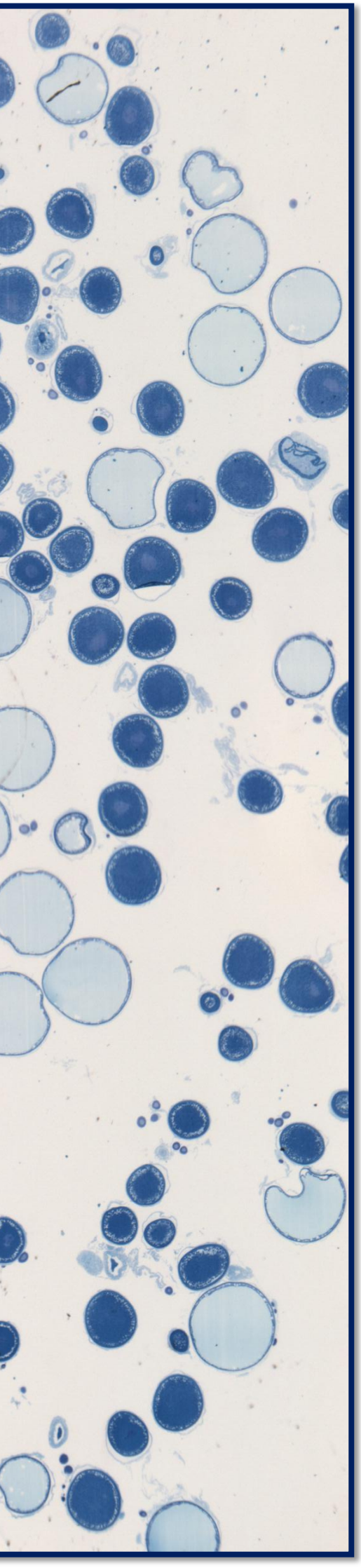


Testosterone in feedback control of final maturation in female Atlantic cod

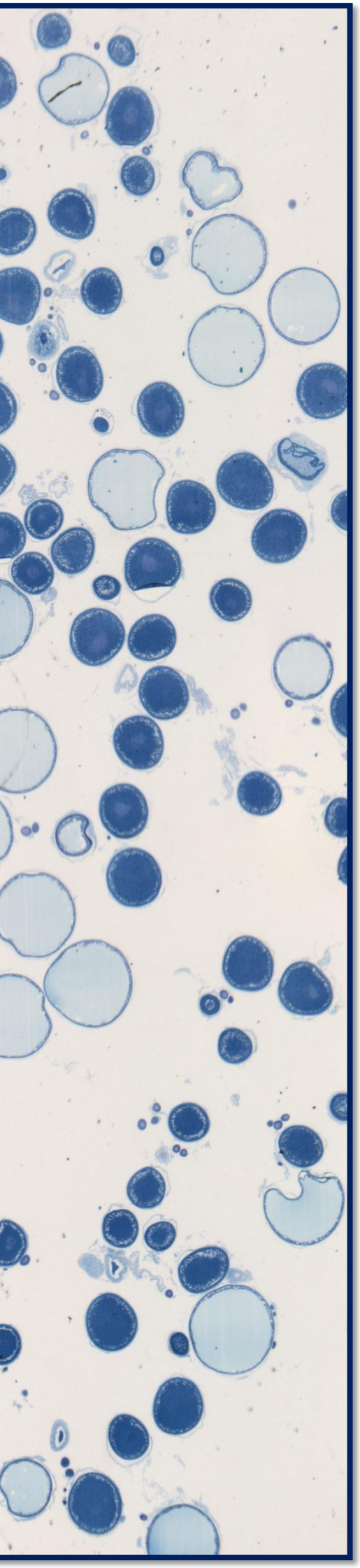
– the effect of testosterone supplementation

Rita de Azeredo

Master's Thesis in Nutrition of Aquatic Organisms in Aquaculture

Department of Biology
University of Bergen
June, 2012

Front page illustration: Atlantic cod gonad sample with vitellogenic, hyaline oocytes and follicular walls



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NIFES
NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH

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Bergen, June 2012

Rita Azeredo

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List of Abbreviations

11-KT	11 - ketotestosterone
17,20β,21-P	17,20β,21-trihydroxy-4-pregnen-3-one
17,20β-P	17,20β-dihydroxy-4-pregnen-3-one
AChE	acetylcholinesterase
ACN	Acetonitrile
ARA	Arachidonic acid
B0	Maximum-binding ELISA wells
$CHCl_3$	Chloroform
COX	Cyclooxygenase
cPGES	Cytosolic PGES
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
E2	Estradiol-17β
EDTA	ethylenediaminetetraacetic acid
EIA	ELISA assay buffer
ELISA	Enzyme-linked immunosorbent assay
EP	PGE_2 Receptor
EPA	Eicosapentaenoic acid
FP	$PGF_{2\alpha}$ Receptor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GVBD	Germinal Vesicle Breakdown
GVM	Germinal Vesicle Migration
HPLC-IT-MS	High Performance Liquid Chromatography Ion-Trap Mass Spectrometry
LC-IT-MS	Liquid Chromatography Ion-Trap Mass Spectrometry
IgG	Immunoglobulin G
IMR	Institute of Marine Research
LH	Luteinizing hormone
LOX	Lipooxygenase
MIH	Maturation-inducing hormone
mPGES	Membrane-bound PGES
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NIFES	National Institute of Nutrition and Seafood Research
NSB	Non-specific binding ELISA wells

OATP	Organic anion transporter polypetide
PBS	phosphate buffered saline
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGES	PGE_2 Synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFS	$PGF_{2\alpha}$ Synthase
PGHS	Prostaglandin endoperoxide synthase
PGT	Prostaglandin transporter
PLA	Phospholipase
PLA_2	Phospholipase A_2
PUFA	Polyunsaturated fatty acid
RF	Response factor
RIA	Radioimmuno assay
T	Testosterone
VTG	Vitellogenin

Abstract

Atlantic cod is a species of very high fecundity and it spawns rather easily and spontaneously in captivity. However husbandry routines, diets, females/males imbalances and stress are believed to be the causes of high mortalities following spawning failure of female cod that become egg-bound. The mechanism behind these events is still not well understood and every possible approach is to be explored. One of these approaches and one of the goals of this thesis is to look deeper at the role of testosterone in cod oocyte growth and maturation by observing the effect of an extra-dose on estradiol-17β levels and oocyte size and stages. Since prostaglandins are known to play an important part in other fish species' oocyte maturation and ovulation, their role in cod ovarian cycle modulation as well as their response to high testosterone levels has been studied.

Three groups of fish were considered: a testosterone group of fish, implanted with testosterone-releasing tubes (n=13), a sham group, in which fish were implanted with the same but empty tube (n=15) and a control group, in which fish were not subjected to any treatment (n=42). The experiment lasted for 23 days. From gonad samples, oocytes were embedded in resin, sectioned and stained in 2 % toluidine blue and 1 % borax, for stages classification and diameter measurements. Blood steroids, testosterone and estradiol, were analysed by Enzyme-linked Immunosorbent Assay and prostaglandins PGE_2, PGE_3, and PGF_2 were extracted, separated and quantified by Liquid Chromatography Ion-Trap Mass Spectrometry.

A very high variance was observed both in steroid and prostaglandin levels. After the experiment, the group treated with testosterone had higher testosterone levels in plasma than both the sham and control group. Higher levels of testosterone did not affect estradiol-17β suggesting that the extra-testosterone was not used as an estradiol-17β precursor. Testosterone did not affect prostaglandin levels and steroids were not correlated with prostaglandins suggesting that ovarian prostaglandins are not regulated by steroids. The three prostaglandins were positively correlated in the control group. $PGF_{2\alpha}$ concentrations were the highest in the three groups while PGE_2 and PGE_3 exhibited very low and similar levels corresponding to a ratio very close to 1. Positive correlation between PGE_2/PGE_3 ratio and the steroids suggests a regulatory role of prostaglandins in steroidogenesis.

1 INTRODUCTION

1.1 Atlantic cod over time

Atlantic cod, *Gadus morhua*, is widely distributed in the Northwest and Northeast Atlantic where, long ago, stocks have been vastly explored. To these stocks are associated high social and economic values. Unfortunately, over-exploitation especially in the East Coast of Newfoundland brought the collapse of the Grand Banks stocks in 1992 and therefore drastic measures were taken and fisheries were prohibited. Nowadays, these stocks are somewhat stable although not fully recovered yet, and captures are far lower than they were before (SOFIA 2010, FAO)

There are also important stocks around Norway such as the Norwegian Arctic stock that spends most of the time in the Barents Sea. Declining fisheries and the consequent high market prices for cod were the stimulus needed to promote the interest in producing cod intensively. Until recently (2010), cod farming had been characterized by the fastest-growing rates (Figures 1.1 and 1.2) but it has been encountering many production and biological obstacles and, in Norway, which is the world's largest producer, amounts of cod reared in captivity have been dramatically decreasing in the last two years (Kristin Hamre, personal communication).

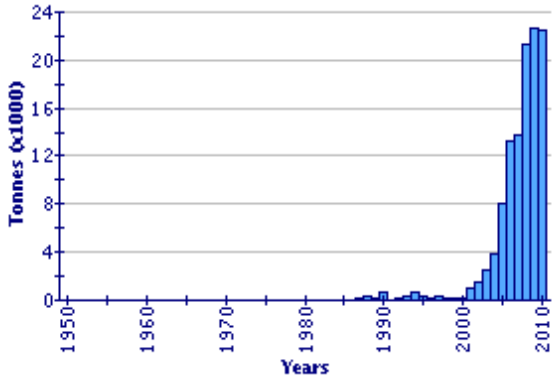

Figure 1.1 Global Aquaculture production for *Gadus morhua* (FAO Fishery Statistic)

Species	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Atlantic cod	169	1 019	1 450	2 565	3 809	8 121	13 228	13 682	21 381	22 729	22 558

Figure 1.2 Norwegian Atlantic cod aquaculture production (t) in the last decade (© FAO - Fisheries and Aquaculture Information and Statistics Service - 24/04/2012)

Cod farming started a long time ago, back to the 1880's, when the Norwegian sea-captain G. M. Darnnevig made some efforts to restore a local stock by releasing yolk-sac larvae in the sea (© FAO, 2012). After unfortunate years, some downs and ups in the success of this activity, advanced juvenile production technology, larger hatcheries along with the advances in photoperiod manipulation of the broodstock were the impulse needed for a more solid establishment of the production and higher growing rates.

Furthermore cod reunites several positive characteristics regarding a farmed species: satisfactory feed utilization and good growth rates as well as short egg and larval stages. However, there are still some challenges that need to be addressed such as differences in egg quality (Salze *et al.*, 2005), dependence on wild broodstock to produce larvae and juveniles (Rosenlund and Halldorsson, 2007), deformities and low growth rates in hatchery stages (Hamre, 2006) and high production costs (Paisley *et al.*, 2010).

1.2 "Why do Atlantic cod females become egg-bound? Studies on possible causes and mechanisms" - Project description

Cod is known to have very high fecundity rates and they spawn rather easily and spontaneously in captivity. Despite that, frequent high mortalities following spawning failure of female cod have been reported in fish farms from every part of Norway (Kristin Hamre, personal communication)

The Norwegian Research Council funded, between 2004 and 2007, a project relevant for this topic: "Optimised nutrition and egg quality in Atlantic cod". In order to investigate the impact of arachidonic acid (20:4n-6, ARA) on reproductive physiology and fecundity, different diet levels were tested in a group of female cod previously transferred from outside netpens to land-based tanks. It was observed that an increase in ARA from 1 to 2% led to higher egg-bound female numbers although the fecundity decreased. When increased to 4%, both fecundity and mortality were reduced. Moreover, higher relative mortalities due to egg-bound situations were reported in fish transferred to inland tanks than in fish kept in the netpens (Hamre *et al.*, 2008).

Therefore, husbandry routines and confinement stress, diet, female/male imbalance may altogether contribute for this situation, but the exact mechanism is not known. It is still

unclear whether there is an inhibition at the final maturation stage, ovulation or even at the oviposition, hence in which exact point in the brain-pituitary-gonad axis does it happen.

Accordingly, the approaches needed in order to outline this problem will be numerous. Luteinizing hormone (LH) assays, feeding and spawning trials and endocrine manipulation will integrate the different approaches suggested in the plan of the project "Why do Atlantic cod females become egg-bound? Studies on possible causes and mechanisms" lead by researchers from the Institute of Marine Research (IMR), the National Institute for Nutrition and Seafood Research (NIFES), Skretting Aquaculture Research Center and the National Marine Fisheries Service, USA.

The relevance of this project is based on the fact that the high mortalities associated to spawning are a serious welfare problem in cod on-growing facilities and broodstock management. It is highly costly to the farmer since it ends up in failed investments and negative yields.

The present master-thesis will be an integrated part of this project where the relation between cod ovarian cycle, steroids (testosterone and estradiol) and prostaglandins will be studied.

1.3 Atlantic cod - General Reproductive Biology

As a demersal, cold-water species, Atlantic cod inhabits, in a general way, the coastal-bottom-waters of the North, Northwest and Northeast Atlantic but its distribution relies mainly on food availability rather than temperature. It can be found as deep as 600 m but it usually occupies shallower continental-shelves. Although it is considered a demersal fish, cod shows more a pelagic character during earlier life stages, spawning or while it is feeding (ICES, FishMap, *Gadus morhua*).

Cod may perform great migrations aiming to reach spawning and feeding grounds. For instance, Arctic-Norwegian cod spends most of its life-time in the Barents Sea. For the spawning season, however, it moves towards the Norwegian coastal waters (*in* © FAO, 2012).

Cod has separate sexes and wild individuals get sexually mature between their second and fourth years. Cod species shows one of the higher fecundity rates and, when in captivity, its fecundity can be even higher than its wild counterparts'. The weight of the total mass of

eggs may sometimes reach more than 100% of their own weight (≈1.5 l/Kg body weight against 0.8l/Kg in wild fish). Cod shows external fertilization and it is a seasonal batch-spawner which means that once in a year, from February throughout the end of April, cod releases batches of eggs with a 2-3-days interval. In captivity, the number of batches is usually up to a maximum of 20 (Kjesbu *et al*., 1996).

The reproductive performance depends on the available energy resources. In an iteroparous spawner, a fish which has several reproductive cycles over its lifetime, these resources are the extra energy that is left after the basic metabolic needs have been fulfilled (Skjaeraasen *et al*., 2009). Being a determinate spawner, the exact amount of eggs a female will be able to release in the spawning season is determinate some months before, during the recruitment period. Therefore, the fecundity of each female basically depends on its energy storages during oocyte recruitment.

Oocyte recruitment occurs during early vitellogenesis and it takes around two months so that it is complete by November (Skjaeraasen *et al*., 2009).

Kjesbu *et al.*(1996) concluded, by looking at time and oocyte diameter relations, that although the interval between batches in cod is around 2-3 days, the time needed for a batch of eggs to be produced is twice that.

1.4 Oocyte Development and Endocrine Regulation

1.4.1 Oocyte Development

The process of oogenesis is assumed to be very similar in every teleost and the several steps will be briefly reviewed here.

The very beginning of oogenesis starts with the proliferation of primordial germ cells that turn into mitotic oogonia. These cells enter, then, the first meiosis and by the time the primary growth starts the oocyte is arrested at prophase of the first meiosis. There are already a few squamous cells surrounding the oocyte that constitute the first follicular cells. Primary growth is characterized by intense RNA synthesis, which leads to the formation of numerous nucleoli around the germinal vesicle, and by the accumulation of cytoplasmatic organelles in clusters that are described as the Balbiani bodies, better described in teleosts by Senthilkumaran *et al.*(2002). It is also during this primary growth that an acellular vitelline

envelope is developed, being frequently referred to as zona radiata, the future egg shell. Towards the end of this phase, the oocyte is completely surrounded by and intimately connected to the granulosa cell layer and the outer theca cell layer. Together, the oocyte and the two cellular layers constitute the ovarian follicle.

The cortical alveolus stage is the next phase. These vesicles contain glycoproteins that are synthesized by the oocyte itself and that will be released into the perivitelline space right after fertilization (Marteinsdottir *et al*., 2000), contributing therefore to the hardening of the egg shell and the avoidance of polyspermy. Towards the end of this stage, the ooplasma is filled by these vesicles and, by the time vitellogenesis starts, they migrate to the periphery of the oocyte. Lipid inclusions may also be seen during the cortical alveolus stage although it is difficult to discern between them and the cortical alveolus. However it is possible that these lipids are already the result of some light vitellogenin (VTG) uptake by the oocyte since vitellogenesis is the very next phase. Besides VTG, vitellogenesis involves the uptake of other lipids. These lipids are transported by other macromolecules such as very low density lipoproteins (Wiegand, 1996).

Teleost oocytes display a great increase in their volume during vitellogenesis and most of the time this growth corresponds to a 50- or 100- fold increase in the gonadosomatic index (Tyler and Sumpter, 1996). The origin of this size and weight gain is the intense uptake of VTG which is a phospholipoglycoprotein produced in the liver and which 79% of its structure are proteins (Jalabert, 2005). Once inside the oocyte, VTG is broken down by cathepsin D into lipovitellins and phosvitins which will represent the energy source for the future coming embryo.

Finally, maturational processes begin to occur in a last phase called final maturation. These transformations will end up in an egg that is prepared to be ovulated, and eventually, fertilized. The nucleus, also known as germinal vesicle, moves towards the animal pole until it finally breaks down with the resumption of the first meiosis, which is arrested in prophase. Consequently, the oocyte enters the second meiosis that is also arrested, but in metaphase II. In Atlantic cod, like in all teleost fish with pelagic eggs, oocytes also undergo an exceptional size increase due to water uptake. This increase may account for 88% of the final egg size and the egg shows now a clear, transparent ooplasma.

Once fully mature, the egg is ready to be ovulated. This particular process is further described below, in the prostaglandins section. All these stages and events occur not independently from one another but in a deeply connected and coordinated way. What determinates their triggering is a complex hormonal system.

1.4.2 Endocrine Regulation

Independently of the reproductive pattern that characterizes a fish, i.e. if it is a synchronous, asynchronous or group synchronous species, external factors such as photoperiod and temperature, are the first indicators to both female and male fish, of the approaching reproduction season. Their impact in the hypothalamus indicates that it is time for preparation for the spawning season and therefore, for the production of gametes (Bromage *et al*., 2001, Davie *et al*., 2007). Environmental light and water temperature are strong inducers of gonadotropin-releasing hormone (GnRH) production in this organ (Bromage *et al*., 2001). In Atlantic cod, this happens therefore in spring, when day length is increasing. Once in the pituitary, this messenger leads to the production of two gonadotropins which concentrations depend on the gametogenesis phase. These gonadotropins firstly referred to as GTH-I and GTH-II corresponded to the mammalian follicle-stimulating hormone (FSH) and LH, respectively (Suzuki *et al*., 1988). As such, fish FSH shows high levels during oocyte growth and vitellogenesis whereas LH rises during maturation and ovulation.

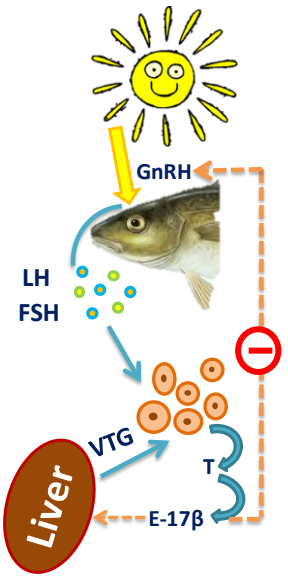

Figure 1.3 Hypothalamus-pituitary-gonad axis – endocrine control of the reproductive cycle.

However these are but intermediates in the roles they play. Once in the ovaries and during oocyte growth, FSH binds to follicular cell receptors, specifically the thecal cell layer, promoting the activation of the adenylate cyclase-cAMP system. This response leads to the synthesis of testosterone (T) that will afterwards diffuse to the granulosa cell layer where the enzyme responsible for the conversion of the precursor T into estradiol-17β (E2) lies. High vascular levels of E2 induce hepatic production and secretion of VTG which is incorporated by the oocyte. All the details described above are illustrated in Figure 1.3.

Maturation-inducing hormone (MIH), similarly to E2, is synthesized in the granulosa cell layer and two different MIH have already been described in teleost fishes: 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) (Patino and Sullivan, 2002). In cod, low plasma levels of 17,20β-P have been reported (Kjesbu *et al*., 1996) and cod ovaries produce predominantly 17,20β,21-P when incubated *in vitro* (Tveiten *et al*., 2010). Taken together, this suggests that 17,20β,21-P acts as the MIH in Atlantic cod. At the end of vitellogenesis, and now under the LH-stimulus, a steroidogenesis-shift occurs so that the vitellogenesis main hormone, E2, is replaced by the MIH. This steroidogenic shift has been shown to happen in the ovarian follicles of medaka, *Oryzias latipes*, (Kobayashi *et al*., 1996).

Estradiol-17β and Testosterone

T and E2 are two important sex steroids that play many physiological roles in the organism. They are, additionally, of utmost importance in regulating reproductive processes and they have been widely described throughout the different species, both mammalian and fish. In teleost females, these hormones along with others are mainly produced in the gonads, specifically in the follicular cells of the ovarian follicle. These cells are tightly coordinated for steroid biosynthesis.

On the basis of steroid biosynthesis there is a complex web of signalling and feedbacks that regulate all the events during gametogenesis. The hypothalamus-pituitary-gonad axis is therefore extremely relevant as a vehicle of communication that enables those events to occur in an opportune and coordinated manner.

Although it has been shown that E2 may be synthesized from estrone, another estrogen, in species such as the protogynous wrasse, *Pseudolabrus sieboldi* (Ohta *et al*., 2001) and in the red seabream, *Pagrus major* (Ohta *et al*., 2002), most of the teleosts have T as a substrate in this biosynthetic pathway. Shortly, the preliminary steps happen in the outer thecal layer where cholesterol, the common precursor to sex steroids, is firstly metabolized to pregnenolone. Subsequently, two different pathways may be followed leading to the production of T or 17-Hydroxyprogesterone, the precursors of E2 and 17,20β-P, respectively. These steroids are synthesized in the neighbour granulosa cell under the control of the gonadotropins FSH and LH, according to the timing of the oocyte development. Figure 1.4

shows the reactions in the steroids biosynthesis. The exact roles of FSH and LH in E2 synthesis are still not well comprehended, but it is well established that FSH has a key regulatory role in E2 production by regulating P-450 aromatase activity, which was first established in the brown trout, *Salmo trutta* (Montserrat *et al*., 2004).

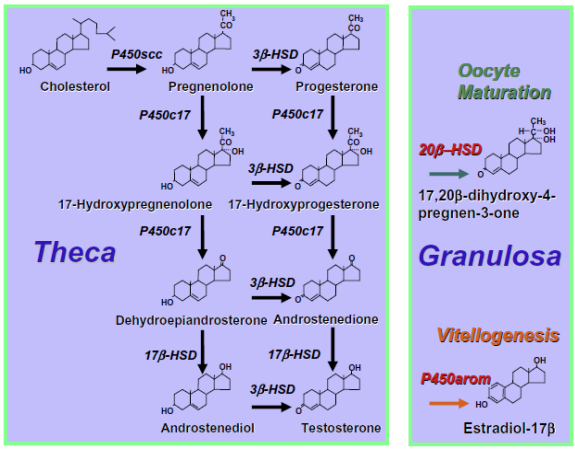

Figure 1.4 Theca and granulosa layers interaction in the biosynthesis of estradiol (Lubzens *et al*., 2010)

E2 is the main inducing-hormone in the synthetic processes of the zona radiata proteins and VTG, both in the liver. The link that binds E2 to vitellogenesis has been deeply studied in many different fish species (Methven *et al.*, 1992, King and Pankhurst, 2003). It is well known that this is the main steroid responsible for the regulation of the liver production and oocyte uptake of VTG, the latter being supported by FSH (Tyler *et al*., 1991). Therefore, at the stage of early vitellogenesis or the yolk granule stage, E2 synthesis is enhanced by FSH and its plasma levels rise. E2 high levels were positively correlated to increasing gonadosomatic index in the amago salmon (Kagawa *et al*., 1983) and peaks in this steroid concentration were measured during conspicuous VTG production (Ng and Idler, 1983, Ohta *et al*., 2001). E2 was higher in cod also, during the spawning season (Dahle *et al*., 2003). Fluctuating levels during the spawning intervals (higher in the beginning and in the end of the spawning intervals) and a general decrease towards the end of the season (Figure 1.5) have been reported in cod (Kjesbu *et al*., 1996), in the goldfish, *Carassius auratus*

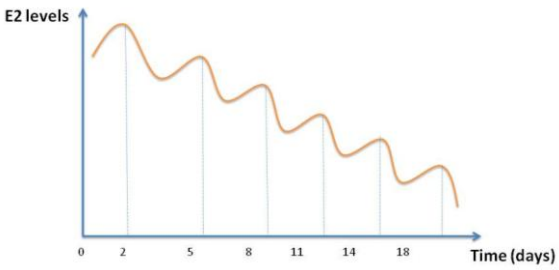

Figure 1.5 Illustration of E2 fluctuations during the spawning season (personal drawing).

(Kobayashi *et al*., 1988) and in the Japanese sardine, *Sardinops melanostictus* (Matsuyama *et al*., 1994). The lower E2 levels are expected since the number of ovarian follicles, i.e. the number of E2-producing structures, is decreasing.

11-ketotestosterone (11-KT) is the most important androgen in males while T is mostly an anabolic agent. Although these are typical male steroids responsible for the stimulation of spermatogenesis and for the secondary sexual male characters, androgens are also present, although at lower levels, in female plasma (Borg, 1994, Young *et al*., 1984). In females, T is basically the main E2 precursor during oocyte growth and vitellogenesis and it plays a key role in feedback control over gonadotropin production in hypothalamus and pituitary (Antonopoulou *et al.*, 1999). However, this happens after aromatization of T to E2.

Kortner (2009) observed that both androgens seem to be present in previtellogenic female Atlantic cod plasma in significant amounts. Particularly the presence of low levels of 11-KT, which is a non-aromatizable androgen, suggests further importance besides that as E2 precursor. In the same study, female cod supplemented with 11-KT displayed higher numbers of more developed oocytes. Similarly, *in vivo* and *in vitro* studies in the shortfinned eel, *Anguilla australis*, reported increased diameter in perinucleolar oocytes when treated with 11-KT (Rohr *et al*., 2001). In female Atlantic cod plasma, T was also detected during the spawning season in spite of its being much lower than E2 (Kjesbu *et al*., 1996).

Moreover, nuclear androgen receptors were detected in the brain tissue of both Atlantic croaker, *Micropogonias undulates*, and kelp bass, *Paralabrax clathratus* (Sperry and Thomas, 1999a, Sperry and Thomas, 1999b). This supports the previous observations that androgens, for themselves and not after being aromatized, must have other physiological roles in fish females. In conclusion, all these studies suggest that androgens have a positive, enhancing effect on the previtellogenic oocyte growth.

Plasma T levels increased during vitellogenesis, reaching a peak before maturation and these high levels were maintained in mature and ovulated fish species (Kagawa *et al*., 1983). In the same study, high T levels persisted even after E2 levels had lowered. These high levels may however be explained by decreased aromatase activity.

Mode of action

Steroids, differently from lipids, are not stored in the cell where they are produced. Rather, they immediately diffuse through the cell membrane and circulate in the blood stream, either bound to albumin or to the sex hormone-binding globulin (Hammond, 2011). Their availability is therefore dependent on their free fraction in the plasma. Gonads are, besides the main synthesis localization, the main target organ for sex steroids and once they are produced, they quickly reach the target cells – the oocytes. Their mode of action is mainly by binding to nuclear receptors.

There are two kinds of receptor-mediated effects: the classical activation of nuclear receptors through which steroids modulate gene expression and the activation of cell-membrane receptors. Once the nuclear receptor is bound to the ligand it will change its conformation and regulate transcription by a co-factor-mediated communication with the transcriptional complex.

Nongenomic effects resulting from the activation of cell-membrane receptors are usually much faster responses (in the order of seconds) and involve signalling pathways with second messengers which can be, among others, diacylglycerol, Ca^{2+}, or cAMP. These receptor/effector-cascades may be triggered not only by the binding of the steroid to its specific receptor, but also by the interactions between the steroid and a modified, classical receptor or some membrane- lipids and proteins. Steroids may still act as co-agonists (Sperry and Thomas, 1999a).

1.5 Eicosanoids

1.5.1 Background

ARA is, although overshadowed by a high number of studies on eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), one of the most studied polyunsaturated fatty acids (PUFA) in marine teleosts. Above all, this is due to its importance as the main precursor of eicosanoids, better discussed further in the text.

ARA belongs to the omega-6 series (n-6) of fatty acids and it consists of a twenty carbon chain with four double bonds (20:4n-6). It is present on the phospholipids bilayer and it has an important structural and integrity role there by providing fluidity and controlling the permeability of the cell. Along with other PUFA, ARA is one of the main components of the

membrane phosphoglycerids being the most common fatty acid associated to phosphatidylinositol. Both EPA and ARA are eicosanoids precursors and hence, they compete for cyclooxygenase (COX) and lipooxygenase (LOX) which are the enzymes involved in the eicosanoids pathway. During the assemblage of the membrane phospholipids EPA incorporation is compromised if ARA levels are too high (Sorbera *et al*., 1998) suggesting that biosynthesis of different eicosanoids is largely dependent on the amount of ARA.

The majority of the marine fish have revealed either lack or very low activity of the enzymes responsible for the desaturation/elongation pathway that produces ARA, DHA and EPA such as C_{18-20} elongase and Δ5-desaturase (Diaz and Arm, 2003) and therefore, these are essential fatty acids that have to be supplied to fish in the fish feed.

ARA has many functions and it is of most importance during fish early stages of life. While feeding sea bream larvae with four different levels of ARA Bessonart *et al.* (1999) observed higher survival rates at the highest concentration levels. A similar approach revealed better survival chances when fish larvae were fed Artemia containing ARA than those fed rotifers deficient in this fatty acid (Koven *et al*., 2001). On the other, it was shown that fish fed high dietary ARA become malpigmented (Estevez *et al.*, 1999, Villalta *et al.*, 2005).

Despite the high n-3/n-6 ratios observed in the marine fish tissues, studies have shown that ARA is considered to be, besides essential, preferred as a precursor of eicosanoids in marine fish (Henderson *et al*., 1985, Anderson *et al*., 1981).

Eicosanoids are oxygenated fatty acids with a twenty-carbon long chain. Their precursors are fatty acids containing at least 3 double bonds. Among these precursors, and perhaps the most important, are dihomo-γ-linolenic acid (DGLA), EPA and ARA. Eicosanoids may be divided into three groups according to the first intervenient enzyme acting on the precursors:

- **Prostanoids** (prostaglandins and thromboxanes), synthesized by cyclo-oxygenases;
- **Leukotrienes and mono-, di- or thri-hydroxy acids**, synthesized by lipoxygenases;
- **Epoxides**, synthesized by cytochrome P-450 epoxygenases.

Their functions are mostly carried out in a paracrine pattern, i.e. they mediate reactions situated either in the cell they were produced or in a nearby cell, and their biosynthesis is inhibited by aspirin and other analgesics and anti-inflammatory drugs (Vane, 1971).

The name prostaglandins comes from the fact that they were first described in the sheep prostate gland (Bergstrom and Sjovall, 1960). Prostaglandins are classified as series 1, 2 and 3 depending on their fatty acid precursors, namely DGLA, ARA or EPA, respectively. The short form for prostaglandin is PG while the ensuing letter indicates either position of the double bond or the constituents of the 5-membered ring. The final subscripted number indicates the number of double bonds on the side chains. For example, PGE_2 and PGE_3 have the same substituent in their ring structure and 2 and 3 double bonds on the side chain, respectively.

Prostaglandins are only produced whenever the cell is stimulated, i.e. whenever they are needed. Therefore they are never stored in the cell. This activation may be mediated by growth factors, cytokines and others.

The very first step of the eicosanoid biosynthesis pathway is the withdrawal of the precursor fatty acid, e.g. ARA, from the cell membrane. A group of acyl-hydrolases called phospholipases is responsible for the release of an unesterified form of ARA. Phospholipase A_2 (PLA_2) is a cytosolic enzyme which is dependent on Ca^{2+} signalling and responsible for the hydrolysis of the *sn*-2 position in the membrane phospholipids that holds ARA (Burke and Dennis, 2009). Diacylglycerol is removed from ARA by the action of a diglyceride lipase. These reactions are limiting the rate at which ARA is recruited, hence, the eicosanoids production. The amount of free ARA is therefore controlled by hydrolysis and reesterification reactions mediated by acyltransferases (Irvine, 1982). There are three main pathways for the production of eicosanoids and they start to differ in the very first enzymatic reaction. ARA is either substrate of COX, or LOX or even cytochrome -P-450 epoxygenase. Prostaglandins are eicosanoids derived from the COX pathway, while leukotrienes are also eicosanoids derived from the LOX pathway.

The term COX pathway is a general way of designating a two-step operating enzyme that transforms ARA into prostaglandin H_2 (PGH_2). COX is also known as the prostaglandin endoperoxide synthase, PGHS (Ricciotti and FitzGerald, 2011). Figure 1.6 describes the COX pathway. In a first step it adds two oxygen molecules to ARA – cyclization - and secondly it

acts as a peroxidase that reduces the fatty acid into what is then PGH_2. COX, or PGHS, has already been described in various studies involving vertebrates such as mammals, birds and both teleosts and cartilaginous fish. In mammals, there are currently two, well-known isoforms of this enzyme commonly named COX-1 (PGHS-1) and COX-2 (PGHS-2). The COX-1 gene is constitutively expressed while the COX-2 gene is an inducible one.

This means that, differently from COX-1 that is permanently present in the nuclear and endoplasmic membranes (Morita *et al*., 1995), COX-2 genes will just be expressed whenever there is a stimulus. Ishikawa and Herschman (2007) found that both zebrafish and rainbow trout have a third contributor, a second inducible and functional COX-2 gene (COX-2b) which, so far, has not been described in other vertebrates.

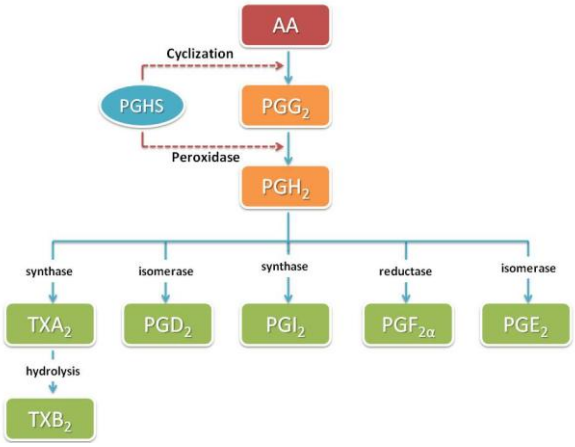

Figure 1.6 General eicosanoid production

1.5.2 Prostaglandin Biosynthesis

Every prostaglandin follows a different production mechanism associated to a specific enzyme. In this review, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2), both from the 2-series, and prostaglandin E_3 (PGE_3) from the 3-series will be discussed.

- **$PGF_{2\alpha}$**

Prostaglandin $F_{2\alpha}$ belongs to the prostaglandin series 2 and has been detected in many different organs. It participates in various biological processes such as contraction of muscles (Cao *et al*., 2002), water excretion (Stier *et al*., 1987) and ovulation (Dozier *et al*., 2008).

This prostaglandin has four stereoisomers: 9α-, 11α-$PGF_{2\alpha}$ (commonly designated as $PGF_{2\alpha}$), 9α-, 11β-$PGF_{2\alpha}$, 9-β, 11α-$PGF_{2\alpha}$ and 9β-, 11β-$PGF_{2\alpha}$ (Watanabe, 2002).

$PGF_{2\alpha}$, synthesis is mediated by PGF synthetase (PGFS) which was first purified and described from bovine lung by (Watanabe *et al*., 1985). This enzyme has a double role since it can produce $PGF_{2\alpha}$ both from PGH_2 by PGH_2 9, 11-endoperoxide reductase, and PGD_2 by PGD_2 11- ketoreductase (Watanabe *et al*., 1986) (Figure 1.7). From PGH_2 9, 11-endoperoxide reductase results 9α-, 11α-$PGF_{2\alpha}$ and from PGD_2 11- ketoreductase results 9α-, 11β-$PGF_{2\alpha}$. Using different rat organ homogenates, Leslie and Levine (1973) observed that $PGF_{2\alpha}$ could also be the result of PGE_2 conversion, being this reaction mediated by PGE-9-ketoreductase with nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) as a cofactor. This enzyme is also able to perform the reverse conversion. No matter what pathway $PGF_{2\alpha}$ comes from, all the enzymes belong to the aldo-keto reductase family.

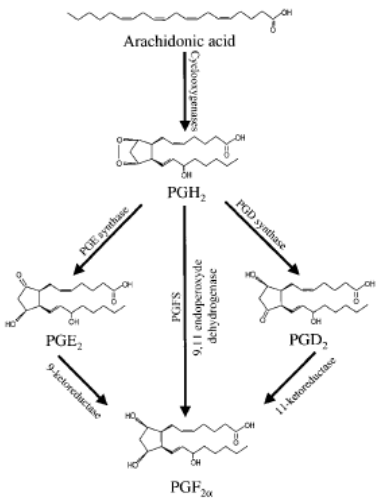

Figure 1.7 $PGF_{2\alpha}$ synthesis pathways (Madore *et al*., 2003)

Furthermore, another enzyme from this family has shown to have PGFS activity in the bovine endometrium (Madore *et al*., 2003). This is an aldose reductase named 20α-hydroxysteroid dehydrogenase (20α-HSD).

- **PGE_2**

Along with $PGF_{2\alpha}$, PGE_2 is a primary prostaglandin and it has several functions. It promotes both anti-inflammatory and pro-inflammatory processes (Ricciotti and FitzGerald, 2011, Ogawa *et al.*, 2009) and it also plays a role in the organism homeostasis (Smith, 1989).

There are three PGE_2 synthases (PGES), two inducible, membrane-bound PGES (mPGES) and one cytosolic (cPGES). Both types belong to a family of enzymes known as Glutathione S Transferase family and both need glutathione as a cofactor for their activity. Ogino *et al*. (1977) first isolated mPGES, whereas Tanioka *et al.* (2000) found a cytosolic version of this enzyme which expression is constitutive and independent from outer stimuli. cPGES seems to couple better with COX-1 during immediate PGE_2 production, to promote cell homeostasis for instance. On the other hand, mPGES works in association with COX-2, under

an induced production that has been settled as a response to pro-inflammatory stimuli. However, this is not a fixed condition. Coupling between these enzymes is under the effect of many variables which may change these patterns (Vazquez-Tello *et al*., 2004).

Figure 1.8 gives a simple description of how these enzyme couples interact to produce PGE_2 whenever it is required and the cell is stimulated by Ca^{2+} signalling (e.g. inflammation). Case a) shows how PGE_2 is produced in a constitutive way. Cytosolic PLA (cPLA), cPGES and COX-1 are permanently present. Constitutively, cPLA moves from its cytosolic location to the nuclear membrane where it cleaves ARA out from the membrane phospholipid bilayer. COX-1 mediates the reaction then, transforming ARA into PGH_2. Finally it is cPGES that transforms PGH_2 into PGE_2 that subsequently exits the cell by passive diffusion or facilitated transport. Regarding scenario b), representing the inducible system of PGE_2 production, it is only when Ca^{2+} signal is present, i.e. when the cell is stimulated, that COX-2 and mPGES genes are expressed. Afterwards the process is similar to a).

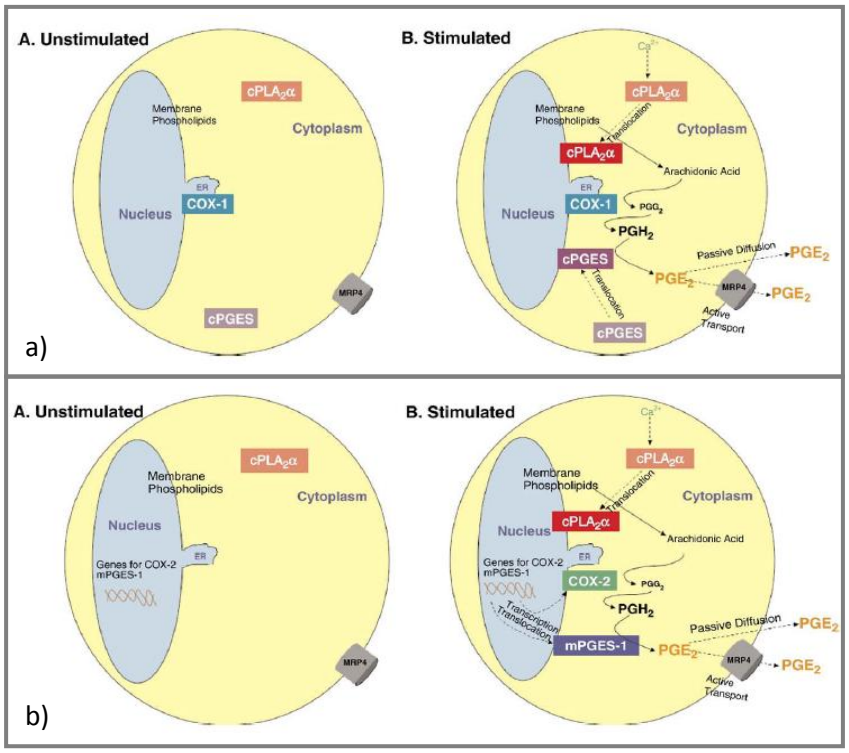

Figure 1.8 PGE_2 synthesis pathways: a) Coordinate production of PGE_2 by COX-1 and cPGES; b) Coordinate production of PGE_2 by COX-2 and mPGES (Park *et al*., 2006)

- **PGE_3**

Little is known about this prostaglandin in fish. Some but not all the 3-series eicosanoids have lower biological activity than series 2 eicosanoids and their precursor, EPA, is not as strong a substrate as ARA is for COX. This series 3 prostaglandin is not very different in structure from its ARA-derived counterpart. PGE_3 has been reported to modulate tumour angiogenesis (Szymczak *et al*., 2008) and the integrity of endothelial cells (Del Turco *et al*., 2007), both in human tissues. It is also associated to human platelets aggregation (Iyú, 2012). Since EPA metabolites activate the same receptors as PGE_2 (Wada *et al.*, 2007), PGE_3 acts also by modulating PGE_2 activity.

1.5.3 Prostaglandin Transporters and Receptors

As paracrine mediators, prostaglandins have to be transported out of the cell where they were synthesized. They may trespass membranes by simple diffusion but, since they are charged compounds, their diffusion rates are poor. Most of the time this transport is facilitated by a prostaglandin transporter (PGT) that belongs to the organic anion transporter polypeptide (OATP) family. In a study concerning lung and blood clearance a mediated transport system was observed by looking at both the concentration and flux of prostaglandins (Eling and Anderson, 1976) (Figure 1.9).

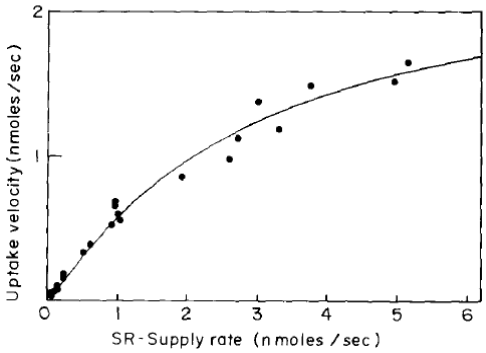

Figure 1.9 Variation of the unidirectional flux of PGE_1 into rat isolated lung with a varying rate of supply of substrate (Eling and Anderson, 1976)

Substrate specificity, different inhibitor sensitivity and PGT mRNA distribution allowed Sala *et al.* (2010) to conclude that this transporter mediates newly synthesized prostaglandins transport out of the cell, transepithelial transport and the prostaglandins clearance from the circulation. Its mRNA is broadly expressed in different tissues from different species such as rat (Sala *et al*., 2010), mouse (Pucci *et al*., 1999) and human (Lu *et al*., 1996, Schuster *et al*., 1997). Evidence has been given that this transporter has high affinity and mediates the uptake of $PGF_{2\alpha}$, PGE_2, PGE_1, and PGD_2 (Itoh *et al*., 1996) and there

are also studies indicating a possible PGT role in the outwards transport of prostaglandins (Chan *et al*., 1998).

It has been suggested that the activation of the $PGF_{2\alpha}$ receptor may down-regulate PGT in a feedback mechanism, decreasing the uptake of prostaglandins by this transporter (Vezza *et al*., 2001). In an attempt to justify this, the authors suggested an amplified effect of local $PGF_{2\alpha}$ concentrations that, otherwise, would be uptaken by PGT and rapidly oxidised inside the cell.

Other members of the OATP family have been shown to transport prostaglandins, along with other compounds, such as the liver-specific transporter (Abe *et al*., 1999). However this and other transports do not have the same affinity and/or specificity that PGT has.

PGH_2, besides being the intermediate precursor of prostanoids is also responsible for triggering signals such as Ca^{2+} and serotonin release or platelets aggregation (Morinelli *et al*., 1987). The latter function makes this compound a strong opponent of its further intracellular product, PGI_2 which inhibits platelets aggregation (Moncada and Vane, 1978). Therefore, the relative extra- and intracellular amounts of PGH_2 are extremely important and so is the system that mediates its translocation, regulating both concentrations. PGT has been shown to transport PGH_2 besides prostaglandins (Chi and Schuster, 2010). It appears that PGT has lower affinity for PGH_2 compared to PGE_2 although the transport rate is higher. However, PGH_2 is more easily diffused through the cell membrane which makes its efflux being mainly by passive diffusion. PGT mediates its import.

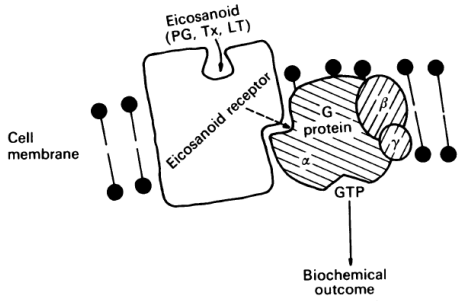

Figure 1.10 Eicosanoid receptors activation (Smith, 1989)

Prostaglandin mode of action is based on their G-protein-coupled receptors activation (Figure 1.10). Every prostaglandin has a receptor with the highest affinity for its ligand but sometimes, some cross-reactivity can be observed between a prostanoid and another prostanoid's receptor (Breyer *et al*., 2001). When activated, most of these receptors either stimulate phospholipases C to produce inositol 1,4,5-trisphosphate and di-acyl-glycerol by cleaving a phospholipid or act by inhibition of adenyl cyclase through inhibitory guanine nucleotide-binding regulatory protein (G_i).

- **PGE_2 receptors (EP)**

Among all the ARA-consequent prostanoids, PGE_2 is the major product and besides playing a role in many processes, PGE_2 sometimes mediates opposing effects (anti- and pro-inflammatory properties, for instance). Maybe because of that, this prostaglandin has different corresponding receptors. These receptors, intuitively named EP_1, EP_2, EP_3 and EP_4, show both the two already described signal pathways, once they are activated (Breyer, 2001) (Figure 1.11). EP mRNAs are not equally expressed throughout the tissues being specifically associated to the various functional effects of PGE_2 (Katsuyama *et al*., 1995). For instance, although EP_2 and EP_4 signal through the same phospholipase C pathway, they are differently distributed. EP_2 appears to be widely distributed in the mouse tissues but it is most abundant in its uterus.

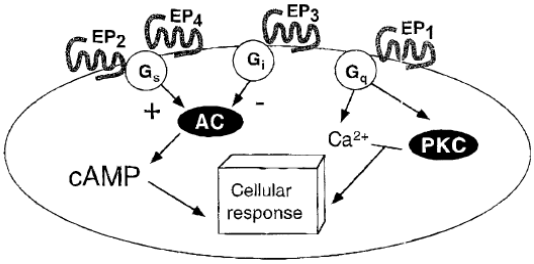

Figure 1.11 PGE_2 receptors activation and their different modes of action (Breyer, 2001)

- **$PGF_{2\alpha}$ receptors (FP)**

FP receptors belong to the group of receptors with a response mechanism based on increased intracellular Ca^{2+}. $PGF_{2\alpha}$ can also bind to EP_1 and EP_2 receptors (Kiriyama *et al*., 1997) and some of its functions are actually mediated by these receptors. Differently from EP, FP receptors do not have subtypes although evidences exist of spliced variants (Pierce *et al*., 1997). They diverge only in 9 aminoacids from the carboxyl-terminal region. This receptor may bind to other agonists rather than $PGF_{2\alpha}$ but the respective affinities are very low.

- **PGE_3 receptors**

With respect to the molecular structure, 3-series prostaglandins are very similar to 2-series prostaglandins. Therefore, PGE_3 usually binds and activate the same receptors that PGE_2 does.

1.5.4 Prostaglandins in Ovulation

Ovulation is the process through which a mature oocyte is released from its ovarian follicle wall. There is a very tight association between the oocyte and the follicle wall provided by the formation of invaginations in the oocyte wall and its consequent microvillus structure. Around these structures and connected to them is the granulosa layer and compounds move in and out through the intermediate pathways. Thus, there must be a regulatory system that mediates the separation of these structures and the rupture of the follicle wall.

Prostaglandin involvement in ovulation

LH, the gonadotropin which regulates ovulation, has response mechanisms mediated by cAMP increased levels, e.g., luteinisation of follicular granulosa cells. It was suggested that prostaglandins could be possible intermediates in other LH stimulated processes such as ovulation since they are associated with signalling mechanisms of adenyl cyclase and cAMP themselves. Therefore, Armstrong and Grinwich (1972), in order to give a more evident proof of the involvement of prostaglandins in ovulation, injected female rats with indomethacin, a potent prostaglandin biosynthesis inhibitor and ovulation was prevented. Prostaglandin production by the ovaries has been mostly explored in mammalian follicles but this is also an emerging topic in teleost fish studies. Stacey and Pandey (1975) applied indomethacin injections directly to the follicles of goldfish to prove that this blockade was happening in the follicle and not via other exterior mechanisms such as LH production. It was demonstrated that upon post-blockade prostaglandin injection (PGE_1, PGE_2 and $PGF_{2\alpha}$) ovulation capacity was restored. Similar findings were reported by Goetz and Theofan (1979) in the yellow perch, *Perca flavescens*, by using indomethacin as an ovulation blocking agent and a post-blockade prostaglandins injection to restore the ovulation capacity.

Regarding prostaglandins from the series 3, Trujillo and Broughton (1995) demonstrated that the consumption of a diet enriched with long chain n-3 PUFA resulted in higher ova release which may indicate a possible role of these prostaglandins in ovulation as well.

Where/which prostaglandins are synthesized?

Increased $PGF_{2\alpha}$ levels in follicle incubates were observed prior to and during ovulation in the brook trout, *Salvelinus fontinalis* (Goetz and Cetta, 1983, Cetta and Goetz, 1982) and yellow perch (Goetz *et al*., 1989a). Assuming that these high levels of prostaglandins were synthesized in the follicle walls, and after being isolated from its surrounding extra-follicular tissue, Goetz *et al.* (1989b) measured prostaglandins produced by the follicles previously treated with ARA. They observed that, although some synthesis could still be measured, PGE_2 levels were not as high as those documented in trout ovaries. Some other compartment in the ovaries would have to compensate this production, then. Furthermore, in the same study, extra-follicular tissue was also tested and high levels of PGE_2 were recorded in these tissue incubates. On the other hand, high levels of $PGF_{2\alpha}$ were being produced in the mature follicle walls during ovulation. This distribution pattern was observed in the goldfish ovaries as well (Goetz, 1991) but in mammals, for instance the rhesus monkey, follicles seem to be synthesizing both PGE_2 and $PGF_{2\alpha}$ in similar amounts (Duffy and Stouffer, 2001, Dozier *et al*., 2008).

Knowing that the extra-follicular tissue of both the brook trout (Goetz *et al*., 1989b) and the yellow perch was able to produce primary prostaglandins, Berndtson *et al.* (1989) wondered if 17,20β-P -induced ovulation would still occur in isolated follicles. They concluded that, even with 17,20β-P stimulus, isolated oocytes did not ovulate. Still, their ability to do so was not compromised, proven by their ovulation following phorbol ester- or calcium ionophore A23187 - treatment.

Studies using yellow perch were performed in order to gain a better understanding of the relationship between prostaglandins and ovulation, and how far the intimate association between extra-follicular tissues and follicles is essential for the ovulation. It was shown that both isolated follicles and extra-follicular tissue can synthesize prostaglandins and that, for ovulation to occur, they must be in close association for at least 24h from the time of incubation (Goetz, 1997). Furthermore, it was also shown that under 17,20β-P control, extra-follicular tissues prostaglandin production

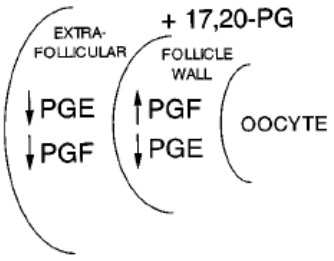

Figure 1.12 Main effect of 17,20β-P on the distinct ovarian compartments regarding prostaglandin synthesis (Goetz, 1997).

decreased (both PGE_2 and $PGF_{2\alpha}$). In contrast, the follicle wall $PGF_{2\alpha}$ synthesis was enhanced and PGE_2's was down regulated (Figure 1.12).

1.5.5 Prostaglandins in Inflammation – parallelism with ovulation

One of the most important reactions which prostaglandins are mediating is the inflammation process. Inflammation is the immune system response against infection or damage of the organism's tissues. During this reaction there are four typical symptoms: redness, heat, oedema and pain. These tissue transformations are driven by an increased localized influx of blood (vasodilatation and increased capillary permeability) and the consequent granulocytes immigration. Many different compounds modulate this response such as histamine, serotonin, bradykinin and prostaglandins. The difference between prostaglandins and other inflammation mediators is that they are not stored in the cell. Instead they are produced in an inducible pattern. Otherwise, their cellular levels are constantly very low. Prostaglandins, especially PGE_2, play an important part in the promotion of those classic symptoms (Ricciotti and FitzGerald, 2011) and hence they are said to have pro-inflammatory properties. However it has been reported some anti-inflammatory activity too, for PGE_2 (Brenneis *et al*., 2011). Binding to their receptors in different cell types is the way prostaglandins act in order to induce the different responses.

Ovulation has been seen as a response of the follicular tissues to an inflammatory reaction induced by the increased level of LH. There are many chemical and physical similarities between the two reactions (ovulation and inflammation): increased blood flow in the ovarian tissue after LH surge, presence of basophils and other types of leukocytes around the Graafian follicle at the time of ovulation (Zachariae *et al*., 1958) as well as proliferating fibroblasts (Espey, 1971). cAMP is also increased in both reactions.

High levels of PGE_2 have been observed in extra-follicular tissues where there are many capillaries. PGE_2 causes vasodilatation and vascular permeability which, during an inflammation, cause the oedema. Hence, it has been suggested that PGE_2 might have a role in the control of the ovarian circulatory system (Goetz, 1991) and this is another clue leading to a possible association between inflammation and ovulation in which prostaglandins act as inflammatory regulators.

2 AIMS OF THE STUDY

Egg-bound cod is a serious and still significant issue in cod farming. Females produce eggs in the traditional spawning season but may not be able to get rid of them by releasing them into the water. Instead, eggs are held in the ovarian lumen and excessive accumulation often leads to death.

There is still so much to be acknowledged about prostaglandins role in fish ovulation dynamics, especially in Atlantic cod, from which we have so little information and so much remains to be unveiled. Most of the prostaglandin studies are concerned with mammalian species and it is already possible to conclude, from the few works that were done in fish, that it is not always advisable to rely on unfounded parallelisms.

The aims of this study were to investigate the following topics:

1. The effect of the T treatment on fish performance
2. The effect of T supplementation on both steroids concentrations after a 23 days treatment.
3. The effect of T on prostaglandin production in cod gonad.
4. Variation of plasma E2, T and gonad prostaglandin concentrations during the ovarian cycle of spawning female cod. By looking at the oocyte development and size distribution, females will be classified in different oocyte development stages.
5. Possible correlations between plasma T, E2 and gonad prostaglandin levels in female Atlantic cod.

3 MATERIALS AND METHODS

3.1 Experimental Design and Samplings

The fish used in this experiment were Atlantic cod, *Gadus morhua*, reared at the IMR, Austevoll Research Station (IMR, 60°05'N 5°15'E) in the GM 30/30 spring production of 2008 using standard hatchery conditions (van der Meeren *et al*., 2007) (Figure 3.1). These fish were fed a commercial diet (Amber Neptun 13mm, Skretting AS, Stavanger, Norway) once a day, until satiation.

Figure 3.1 Tank with fish from the experiment

A total of 192 fish were used for the experiments from which 162 were females and 30 males. Fish were classified in three groups: the T group (n=21, fish with T implants), the sham group (n=21, fish with empty implants) and the control group (n=120, fish without implants). The T group fish were distributed in 3 tanks (seven fish per tank) along with the sham group fish distributed in the same way. 5 males were placed in each one of these tanks. The control group fish were equally distributed in three tanks, 40 fish per tank, along with 10 males per tank.

Out-door round tanks (Ø=3 m, 7000 l) provided with aerated sea-water brought up from a 165 m depth were used. Water temperature was 7.8 °C ± 0.3 °C and the pH between 7.95 and 7.97. The oxygen content in the outlet water was around 75%-80% of the 100% inlet water. Fish were under natural light conditions reduced 70% by tank covering shades.

3.1.1 Experimental set-up

Twenty-one, 1 cm long tubes (diameter ≈1 mm) were prepared with powder T and sealed with silicone in both ends. Also, similar but empty tubes (sham tubes) were provided so that there could be a sub-control group under the exactly same conditions as the T group.

1 ml eppendorf tubes were also labelled for the blood samples and for the resulting plasma samples other eighty-four, 0.5 ml eppendorf tubes were prepared (2 tubes per fish). Syringes for blood were heparinised. A stock solution of Finquel MS222 was prepared in a concentration of 20 mg/L and used as an anaesthetic.

3.1.2 First Sampling

In the 15th of February, 2011, fish (females and males) were firstly anesthetized in a small tank and were afterwards weighed and measured. The initial body weight (mean ± standard deviation, SD) was: T group, 3.164 ± 0.576 kg; sham group, 3.357 ± 0.546 kg). For each female from both the T and the sham groups, a blood sample was taken from the caudal vein as well. T containing tubes were implanted beneath the skin of the abdomen of 21 females. 21 empty tubes were also implanted in the other fish, from the sham group. Fish were marked with a pit tag and finally distributed to the destination tanks.

Blood samples were centrifuged at 12.000 rpm for 2 minutes at 4 ^{o}C, and plasma was collected and divided into two Eppendorf tubes. Plasma samples were stored at -80^{o}C.

3.1.3 Final Sampling

The final sampling lasted for two days, 9th and 10th of March, 2011. Again, fish were weighed and measured and blood samples were taken. Fish were sacrificed and gonads and liver were weighed. Two samples (≈1 g) of gonad were removed and submitted to histology and prostaglandin analyses. Gonad samples for histology were kept in 3.6 % buffered formaldehyde (29.5 mM $NaH_2PO_2 \cdot H_2O$ and 460 mM $Na_2HPO \cdot H2O$). For prostaglandin analyses, gonad samples were distributed in Eppendorf tubes with 20 µl of indomethacin (1 mg/ml in ethanol) and were put in liquid nitrogen at once. They were then stored at -80 ^{o}C.

Due to high mortality, the T group and sham groups were reduced from n=21 to n = 13 and n = 15, respectively.

3.2 Analytical Methods

3.2.1 Histology

Every sample from both T and sham groups was analysed along with 42 samples from the control group. The histological procedure was performed as follows:

3.2.1.1 Dehydration

Gonad samples, fixed in 3.6 % formaldehyde, were washed in 70 % ethanol for 1 hour being left in a shaker at 130 rpm. The ethanol was then changed for a new one and the samples were left to be washed again, this time overnight. The next day, the samples were dehydrated two times, 1 hour each, in 90 % ethanol and one more hour in 96 % ethanol.

3.2.1.2 Embedding

Once the dehydration was finished, samples were placed in histomolds and were infiltrated and polymerized in resin according to the embedding instructions of the Technovit 7100 kit, Heraeus Kulzer.

3.2.1.3 Mounting

The mounting medium, prepared according to the same instructions, was added to the embedded tissues and a wooden block holder, properly labeled, was placed in. Blocks were allowed to harden for at least 1 hour and were afterwards taken out from the histomolds.

3.2.1.4 Sectioning

For each sample, 3 sections of 1 µm width were obtained using a microtome. Once cut out, the sections were put into distilled water (60 ^{o}C) and were picked out by placing the slide underneath them.

3.2.1.5 Staining

Dried slides were stained in 2 % toluidine blue and 1 % borax. This was done by adding a droplet of the solution with the help of a Pasteur pipette until the section was completely covered. After waiting around 35 seconds, the slide was washed with fresh water and left to dry in the hotplate at 60 ^{o}C.

3.2.1.6 Covering glasses

Sections were covered with cover glasses and were kept in the fume hood until the next day.

3.2.2 Determination of oocyte stage and size

Oocytes classification was based on a 6 stages-system (Kjesbu *et al*., 1996) in which the first stage is represented by vitellogenic oocytes and the last by a fully matured hyaline oocytes which are ready to be ovulated. A detailed description is given below:

- **Vitellogenic oocytes**: large, central and round nucleus; conspicuous yolk granules in the cytoplasm, mean diameter ≈ 650 µm
- **Germinal Vesicle Migration I (GVM I)**: eccentric and not so round nucleus and granular yolk, mean diameter ≈ 850 µm
- **Germinal Vesicle Migration II (GVM II)**: proteolytic yolk granules in the vegetal pole and an eccentric nucleus, mean diameter ≈ 875 µm

- **Germinal Vesicle Migration III (GVM III)**: coalescence of yolk granules in the vegetal pole and eccentric, smaller nucleus, mean diameter ≈ 900 µm
- **Germinal Vesicle Breakdown (GVBD)**: no detectable germinal vesicle and an almost complete coalescence of the yolk granules, mean diameter ≈ 975 µm
- **Hyaline oocyte**: complete coalescence of the yolk granules, clear, hydrated ooplasma, mean diameter ≈ 1150µm

Pictures of the slides were taken using a Nikon AZ100 with 0.5 amplification and 3x zoom optic. The camera was a QImaging CAM Fast 1394 connected to the software QCapture V.3.1.2.

Classification was performed with the ImageJ 1.46f software.

3.2.3 Prostaglandin Analysis

3.2.3.1 Homogenization

Prostaglandin analysis was performed in parallel samples to those that were processed for histology. Gonad samples, previously stored at -80°C with indomethacin to prevent the formation of extra amounts of prostaglandins, were firstly homogenized in liquid nitrogen. Samples were maintained frozen and cold with dry ice and, one by one, mixed with liquid nitrogen and processed so that all the tissues from the sample (including oocytes) were crushed to a very fine powder. Afterwards, samples were stored at -80 ^{o}C.

3.2.3.2 Extraction

The extraction procedure involved the addition of a deuterated PGE_2 internal standard (PGE_2-d_4), to the samples. The internal standard (IS) solution was prepared in acetonitrile (ACN) at a concentration of 140 ng/ml and stored at -80°C.

In groups of sixteen at a time, the samples were extracted as follows:

0.2-0.3 g of sample were placed in an initial Eppendorf tube and mixed with 500µl of the ACN solution of IS (140 ng/ml PGE_2-d_4) and 500µl chloroform ($CHCl_3$). The sample was vortex-mixed for 1 minute, centrifuged (3000 rpm) for 2 minutes at room temperature and the organic phase was subsequently collected and transferred to a glass vial (1.5 ml capacity). In the end, the liquid phase, corresponding to the organic phase, was transferred to new glass tubes.

The initial Eppendorf tube containing a flock residue was subjected to the extraction procedure by using subsequent aliquots of 500 µl of pure ACN (without PGE_2-d_4) and $CHCl_3$. After vortex-mixing and centrifugation, the organic phase was added to the initially collected phase and evaporated at low pressure in a vacuum drier system at room temperature in two cycles (30 minutes at 40 bar and 30 minutes at 0 bar).

The dried sample was redissolved in 100 µl of ACN, transferred to a chromatographic glass vial and submitted to liquid chromatography tandem mass spectrometry analysis.

3.2.3.3 Liquid Chromatography Ion-Trap Mass Spectrometry (LC-IT-MS)

- **Principles of the method**

LC-IT-MS is an instrumental technique that allows to physically separate the compounds of a solution and to measure them according to their molecular mass. This is possible due to the association of a mass analyzer such as the ion trap with liquid chormatography. An ion trap is a device capable of ion storage and mass analysis.

The high performance LC-IT-MS (HPLC-IT-MS) system is composed of 4 main elements, shown in Figure 3.2. The sample is loaded into the HPLC column by the autosampler (a). The various components of the sample are eluted along the chromatographic column at different

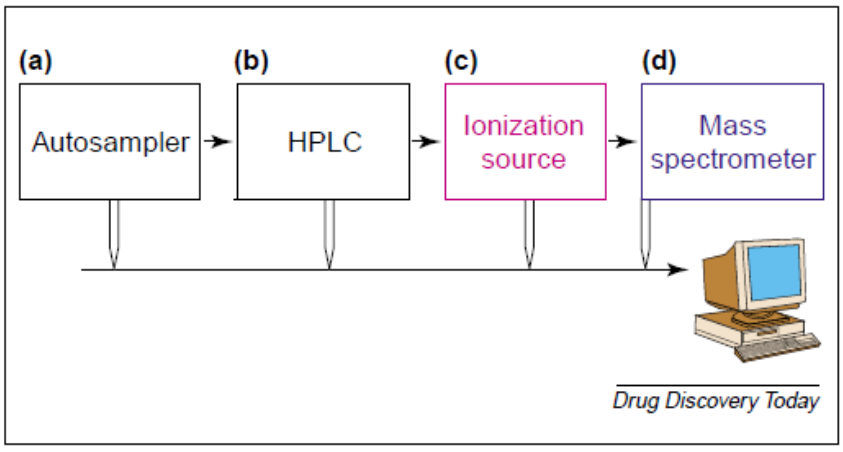

Figure 3.2 Main elements of a LC-MS system (Korfmacher, 2005)

velocities by the mobile phase. The components separation will depend on the chemical nature of the components as well as on the chemical nature of the stationary phase (the column) and also on the physical and chemical interactions between the stationary and

mobile phases. The time a certain compound takes to get out of the column (the retention time) is therefore specific to each component and it allows its identification. The ionization source (c) is the interface between the HLPC (b) and the mass spectrometer (d). This interface is responsible for the ionization of the compounds that starts by its nebulization and further ion evaporation of the droplets. This basically results from the combination of a strong electric field and very high temperatures.

The resulting ions will then pass through a glass capillary to the mass spectrometer where they are fragmented, detected and measured.

- **Choosing this methodology**

Antibody-based assays such as enzyme-linked immunosorbent assay (ELISA) and radioimmuno assay (RIA) are frequently used methods for the estimation of prostaglandins in gonads (Van Der Kraak and Lister, 2008). However, these assays frequently overestimate the levels of prostaglandins due to cross-reactivity with related compounds resulting in reduced selectivity, lack of specificity for complex biological fluids and variability in the quantification of sequential samples. The ability to detect only one compound at the time is also a disadvantage in RIA (Takabatake *et al*., 2002). The inherent disadvantages of ELISA and RIA can be avoided by implementing gas chromatography mass spectrometry or liquid chromatography mass spectrometry (GCMS and LCMS, respectively). A study aiming at comparing ELISA and GC/MS revealed that the estimated concentrations of 8-isoprostaglandin $F_{2\alpha}$ by ELISA were 30-fold greater than those determined by GCMS (Il'yasova *et al*., 2004). Nowadays, it is accepted that GCMS and LCMS outperform antibody-based techniques and they are commonly used in the determination of prostaglandins in a wide variety of samples.

NIFES has developed and validated an IS method for the determination of prostaglandins in cod gonads (Janagap *et al*., 2012). The method exhibited good recovery values, specifically 92-101 % for PGE_2, 92-102 % for $PGF_{2\alpha}$ and 86-99 % for PGE_3. The method is highly selective towards the various analytical species. The optimal concentration of PGE_2-d_4 was 140 ng/ml for evaluating quantitatively the levels of PGE_2, PGE_3 and $PGF_{2\alpha}$ in cod gonads. At this concentration of PGE_2-d_4, response factor (RF) values of 0.932 (for PGE_2 and

PGE_3) and 0.230 (for $PGF_{2\alpha}$) were found for quantifying the various prostaglandins. The quantification of prostaglandins is explained later in the calculations section.

- **Technology specifications**

The LCIT-MS was an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface (ESI), a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector, 25 ml injection volume and 20 min total analysis time. The column used was a Zorbax Eclipse-C8 RP 150 × 4.6 mm, 5 mm (Agilent Technologies. Palo Alto, CA, USA) and it was kept in the column compartment at 40 °C. The solvent system operated in isocratic mode at 0.4 ml/min was ACN with formic acid 0.1 % (v/v) and UV detection at 254 nm. Nitrogen was used as nebulizing and drying gas at 350 °C. The ESI source was operated in negative ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13000 m/z/sec (FWHM/m/z = 0.6-0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC-MSD Trap Software, Version 5.3 from © Agilent Technologies, Inc. 2005. The transitions monitored were m/z 351→333, 315, 271 for PGE_2, m/z 349→331, 313, 269 for PGE_3, m/z 355→337, 319, 275 for PGE_2-d_4, m/z 335 → 317, 273 for PGF_2. Although prostaglandins are eluded at the 8^{th} minute, it was prolonged for 40 minutes to make sure every compound in the solution would be eluted.

All the solutions and equipment used for prostaglandin analysis are fully described in the Appendix II.

3.2.4 Steroid Analysis

ELISA is an assay based on the specificity of an antigen/antibody reaction. The studied analytes were T and E2. In ELISA, these analytes will compete for the antibody against a similar steroid, i.e. T or E2, which concentration is well known. This other steroid is in association with an enzyme which, in this specific situation is the acetylcholinesterase and therefore, we call this analyte the AChE tracer. Both the analytes compete for the binding sites of an analyte-specific rabbit anti-serum. Since the AChE tracer concentration is always the same, it will only depend on the other analyte amount whether it will be more or less successful in binding the anti-serum. By adding the enzyme substrate to the plates with

these compounds it is easy to read the absorbance of its reaction product. The intensity of this absorbance is proportional to the amount of AChE tracer and, hence, inversely proportional to the amount of the analyte (T and E2) (Cuisset *et al.*, 1994, Dahle *et al.*, 2003).

In order to check E2 and T concentrations in fish plasma ELISA was performed to every fish from the T group and the sham group (both before- and after-treatment samples) and to the ones from the control group. The latter group was limited to those fishes histologically analysed. In total, 98 samples were first extracted and afterwards analysed.

Before the main analysis, two tests were done to check on the recovery value of this method. These tests were extraction procedures that included the addition of 5 µl of ^{3}H-labelled testosterone stock solution to the samples, previous to the extraction. Recovery was read in a β-scintillation counter.

3.2.4.1 Extraction

Solutions preparation

A stock solution of potassium phosphate buffer was made with a final concentration of 1M and pH 7.4. This buffer solution was prepared as follows: 66.5 g of dipotassium hydrogen phosphate and 16.075 g of monopotassium dihydrogen phosphate were dissolved in 500 ml MilliQ water in a flask. The pH was controlled with the help of a pH meter and the dissolution was consolidated with a magnetic stirrer.

The 1 M potassium phosphate solution was used to prepare a phosphate buffered saline (PBS) solution at a concentration level of 0.1 M and a pH of 7.4. This was done by dissolving 23,4 g of NaCl (0.40 M) and 370 mg of ethylenediaminetetraacetic acid (EDTA, 1 mM) in MilliQ water, in a total volume of 1000 ml.

Procedure

Samples were taken out of the freezer (-80 ^{o}C) and left at room temperature to thaw.

After samples had been shaken and were completely thawed, 100 µl of plasma were transferred to extraction glass tubes with a pipette. 1 ml of ethyl acetate was then added with a stepette to each sample. After being vortex-mixed for 15 seconds, samples were centrifuged for 2 minutes (1870 rpm, 4 ^{o}C) in the cooling centrifuge. Two well distinguishable phases could be seen: a denser, jelly one in the bottom and a second more liquid phase on

the top, the organic phase. This second phase was transferred with a Pasteur pipette to a new, 6 ml glass tube (Pankhurst and Carragher, 1992).

The procedure was repeated a second time to the initial sample tubes, and after the organic phase had been collected, samples were placed in the Savant vacuum centrifuge to evaporate for 30 minutes.

Once the evaporation was complete, and the samples were perfectly dried, 1 ml of PBS was added to each glass tube. After being vortex-mixed and having acquired an opaque white coloration, the samples were covered with parafilm, placed in the heating block, at 60 ^{o}C for 10 minutes, cooled down, vortex-mixed and finally transferred to labelled 1.5 ml Eppendorf tubes. The samples were stored at -20 ^{o}C until ELISA.

3.2.4.2 ELISA

Solutions preparation

Three buffers were prepared beforehand, one stock solution of phosphate buffer, an ELISA assay buffer (EIA) and a washing buffer.

500 ml of stock solution of phosphate buffer were prepared similarly to the phosphate buffer used for extraction.

23.4 g of NaCl (0.40 M), 370 mg of EDTA (1 mM) and 1 g of bovine serum albumin (BSA) were added to 100 ml of phosphate buffer to prepare EIA assay buffer. MilliQ water was added to dissolve up to a total volume of 1000 ml. The buffers were stirred and stored at 4 ^{o}C.

Finally, for the washing buffer, 10 ml of phosphate buffer (1M) were added to 500 µl of Tween 20, plus MilliQ water making up a total volume of 1000 ml and a final concentration of 0.01 M.

Ellman's reagent was made adding 200 µl of phosphate buffer (1 M) to 20 mg of acetylthiocholin iodide and 21.5 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). MilliQ water was added up to a total volume of 100 ml giving a concentration of 0,002 M. This solution was stored under dark conditions, at room temperature.

Standards assemblage

For the T analysis two glass tubes (6 ml capacity), labeled A and B, were filled with 1500 µl and 1000 µl of EIA buffer, respectively. 500 µl of T standard solution (pure T previously dissolved in ethanol and MilliQ water to a final concentration of 20 ng/ml) were then added to tube A. 1000 µl from tube A were added to tube B.

Each standard concentration was prepared according to Figure 3.3. Final concentrations are listed in Table 4.1.

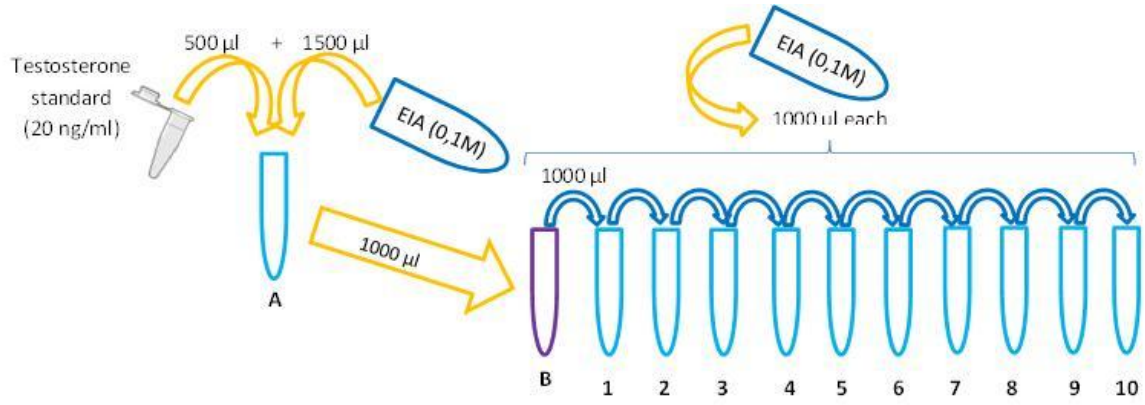

Figure 3.3 Testosterone standards preparation

For the E2 analysis a 6ml tube was added 1500 µl of EIA and 500 µl of E2 standard solution (β-estradiol previously dissolved in ethanol and MilliQ water to a final concentration of 10 ng/ml). This was standard number 1 and the following standards were prepared according to Figure 3.4. Final concentrations are shown in Table 4.2.

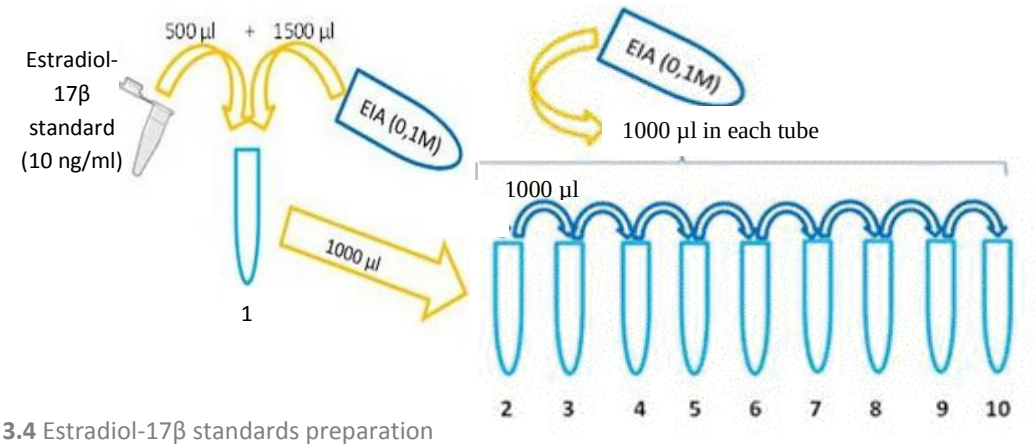

Figure 3.4 Estradiol-17β standards preparation

Table 4.1 ELISA testosterone standard concentrations (ng/ml)

Tube	Concentration (ng/ml)
1	**1.25**
2	**0.625**
3	**0.3125**
4	**0.1562**
5	**0.0781**
6	**0.0391**
7	**0.0195**
8	**0.00977**
9	**0.005**
10	**0.0025**

Table 4.2 ELISA estradiol-17β standard concentrations (ng/ml)

Tube	Concentration (ng/ml)
1	**2.5**
2	**1.25**
3	**0.625**
4	**0.3125**
5	**0.1562**
6	**0.0781**
7	**0.0391**
8	**0.0195**
9	**0.00977**
10	**0.005**

The ELISA solutions were assembled according to previous calculations described later in Appendix I. These solutions were the IS's, T and E2 AChE tracer and EIA anti-serum.

Procedure

Four plates pre-coated with anti-rabbit immunoglobulin G (IgG) were used for each steroid analysis. After properly labelled (I, II, III, IV and date), the plates were washed with 300 µl of the prepared washing buffer with the help of a liquid dispenser.

50 µl of sample were then added to the wells, in duplicates, according to a pre-established plate map (example Figure 3.5) Secondly, standards were transferred having triplicates in the first plate, and duplicates in the three others, also in equal amounts of 50 µl in each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St1	St2	St3	St4	Standards St5	St6	St7	St8	St9	St10	B0 B0	NSB NSB
B	St1	St2	St3	St4	St5	St6	St7	St8	St9	St10	B0	NSB
C	140	141	142	143	Prøver 147	152	153	158	159	160	Tor Dan	Ellman Ell
D	140	141	142	143	147	152	153	158	159	160	Dan	Ell
E	161	164	166	172	177	179	43	44	45	46	48	49
F	161	164	166	172	177	179	43	44	45	46	48	49
G	50	51	52	53	54	55	56	57	58	59	60	Tor Dan5
H	50	51	52	53	54	55	56	57	58	59	60	Dan5

Figure 3.5 ELISA Plate map

Afterwards, the same amount of either T or E2 IS was added to the corresponding wells. Maximum binding to the plate was tested in the wells named B0 and non-specific binding was also assessed in wells labelled NSB (Figure 3.5). EIA buffer was added to these wells, 50 µl and 100 µl, respectively.

Then, 50 µl of AChE tracer and 50 µl of EIA anti-serum were added to every well except to the wells designated to Ellman's reagent. EIA anti-serum was not added to NSB wells. Covered with plastic tape, the plates were shaken for one hour at room temperature and left in the refrigerator overnight.

The following morning the plates were washed three times with 300 µl washing buffer (the second time the washing buffer was left for 10 minutes within the wells). 200 µl of Ellman´s reagent were added to all the wells and, after conveniently covered with plastic tape, the plates were incubated at 20^{o}C for 24 hours or 5 hours, for T or E2 analysis, respectively, before being read in the spectrophotometer at the wavelength of 405 nm.

Whenever the observed binding percentages were lower than 20%, the procedure was repeated with the necessary sample dilutions.

All the solutions and equipment used for steroid analysis are fully described in the appendix (Appendix II).

3.3 Statistical Analysis and calculations

3.3.1 Calculations

Prostaglandins were quantified by using the following equation:

$$[\mathrm{A}] = \frac{[\mathrm{IS}]}{\mathrm{RF}} \times \frac{S_{\mathrm{A}}}{S_{\mathrm{IS}}}$$

where [A] and [IS] represent the concentrations of prostaglandin and internal standard respectively and S_{IS} and S_A represent the recorded signals in ion counts per second (icps) for the internal standard and analyte respectively. RF refers to the response factor.

3.3.2 Statistics

The analyses of the various results were performed by both STATISTICA (StatSoft, Inc. 1984-2011 data analysis software system, version 10.0) and Microsoft ® Office Excel 2007. Most results are presented as mean values with SD. For every test done, the significance level considered was 0.5.

The explored variables were:

- T concentration (ng/ml)
- E2 concentration (ng/ml)
- PGE_2 concentration (ng/g)
- PGE_3 concentration (ng/g)
- PGF_2 concentration (ng/g)
- PGE_2/PGE_3 ratio

Basic dispersion and bar graphs and were made in either Microsoft Excel or STATISTICA. Descriptives and correlation values were obtained using the “Descriptive statistics” and the “Correlation matrices” function, respectively, in STATISTICA . Whenever the experimental data were not normally distributed and/or did not have homogenous variances, the differences between independent groups were tested using the non-parametric Mann - Whitney test.

A non-parametric Wilcoxon Matched Pairs Test was applied to test differences between sampling days and differences within the same group.

4 RESULTS

Fish growth and mortality

T group initial weight was not different from sham group initial weight. After the experiment, weight losses were observed in both the T and the sham groups.

There was no group effect on gonadosomatic and hepatosomatic indexes means (GSI and HSI, respectively) and no differences were found between the T and the sham groups' growth means, as well. A significant difference was found between sham and control groups final weight means.

The highest mortalities were observed in the T group fish, which were significantly different from those in the control group but not from mortalities in the sham group (Table 5.1).

Table 5.1 Initial and final weight, growth, hepatosomatic and gonadosomatic indexes and mortality for the three groups, presented as mean ± standard deviation. The data was analysed by non-parametric Kruskal-Wallis ANOVA to test differences between the three groups and by Mann-Whitney test to find differences between the testosterone and the sham groups. Significant differences are indicated by different superscript letters. Abbreviations: HSI; Hepatosomatic index, GSI; Gonadosomatic index.

Fish Performance According to Groups

	Testosterone Group	Sham Group	Control Group
Initial Weight (g)	3191 ± 721	3369 ± 607	---
Final Weight (g)	2827 ± 708^{ab}	3127 ± 620^{a}	2753 ± 591^{b}
Growth (g)	-364 ± 355	-242 ± 292	---
Growth (%)	-11.29 ± 10.65	-7.09 ± 9.01	---
HSI	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.02
GSI	0.23 ± 0.05	0.25 ± 0.07	0.23 ± 0.05
Mortality (%)	32.96^{a}	20.95^{ab}	9.64^{b}

4.1 Histology and Oocyte Classification

Picture analysis provided an overview over the ovarian maturation status in each female, i.e. whether the fish had just spawned or whether it would be spawning soon. All the

samples contained conspicuous amounts of vitellogenic oocytes and many of them also contained hyaline oocytes in varying amounts. The following Figures 4.1 and 4.2 are two good examples.

It was possible to deduce whether a female had recently spawned or not by the presence of disrupted, empty follicles composed by follicular walls ((c), Figure 4.1).

Furthermore, in most of the samples it was possible to distinguish more than one stage simultaneously. In Figure 4.1, a female from the control group shows at least two different stages: vitellogenic and GVM I oocytes, (a) and (b), respectively. In both stages the cytoplasm is filled with yolk granules, here stained in blue and cortical alveolus gathered at the periphery. Figure 4.2 shows a gonad sample from a female which leading cohort is represented by a high amount of hyaline oocytes. Follicular walls are observed surrounding the oocytes ((c), Figure 4.2).

In both situations, as well as in every other sample, much smaller oocytes in primary oocyte growth were present.

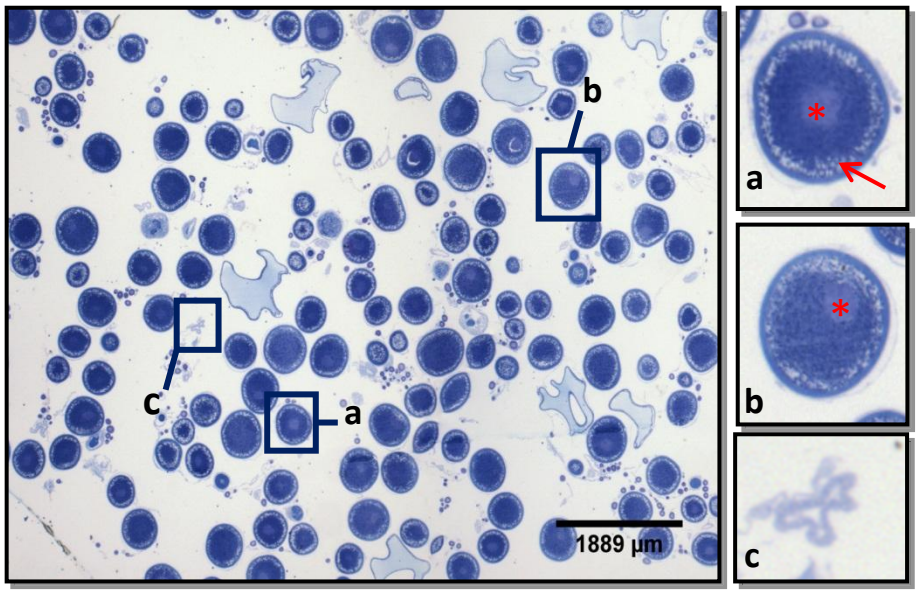

Figure 4.1 Gonad sample from a female belonging to the control group: (a) an apparently vitellogenic oocyte; (b) a germinal migration oocyte; (c) a follicular wall. Red asterisks are indicating the nucleus. Red arrow points to the cortical alveolus.

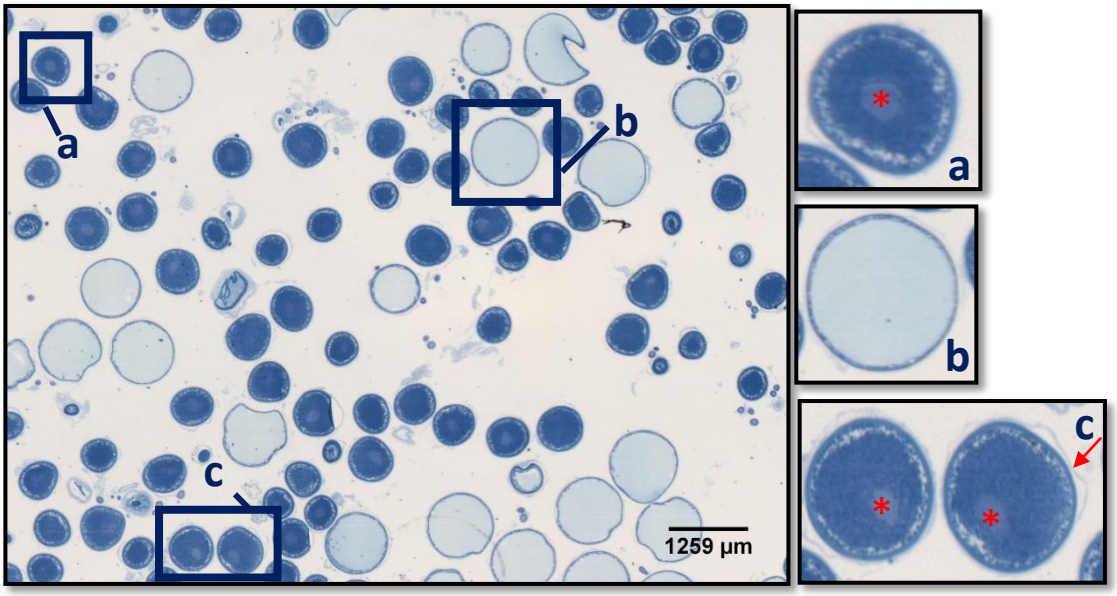

Figure 4.2 Gonad sample from a female belonging to the testosterone group: (a) an apparently vitellogenic oocyte; (b) hyaline oocyte; (c) two germinal vesicle migration oocytes. Red asterisk indicate the nucleus. Red arrow points to the follicular wall around the oocyte.

4.2 Steroid and Prostaglandin Analyses according to the treatment

Neither T nor E2 data were normally distributed and both steroids showed very high SD. In E2 analysis, this value was very close to the mean itself.

4.2.1 T concentration

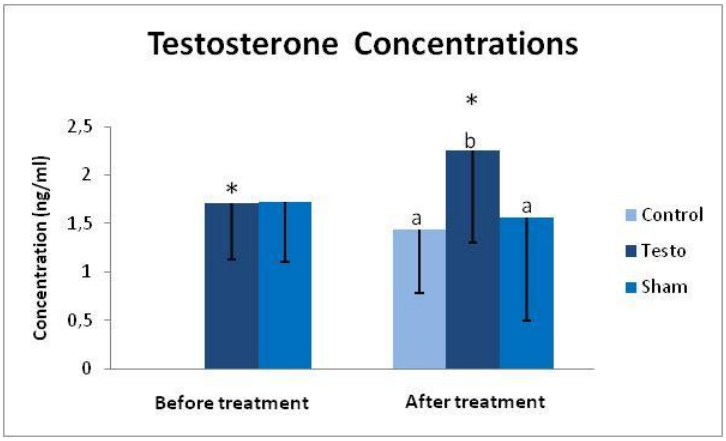

Figure 4.3 Mean testosterone concentration with standard deviation in the three groups, before and after the testosterone treatment. No plasma samples were obtained from the control group, before the experiment. Therefore the comparison between different samplings is not possible for this group. Different letters show significantly different means between groups ($p<0.05$), whereas the asterisk shows significantly different means between the sampling days. Differences between groups were determined by a Mann-Whitney non-parametric test. Differences within groups were tested with a Wilcoxon Matched Pairs non-parametric test.

Figure 4.3 demonstrates T concentrations in fish before and after the treatment. Similar values characterized T and sham groups before the treatment. Plasma samples of the T group taken after the treatment contained significantly higher levels of T compared to plasma samples taken before the treatment ($p<0.05$). The minimum level of T in fish subjected to the extra-dose was 3 times higher than the corresponding value before the treatment, in this group. Such differences were not found in the sham group.

Looking at the three groups after the treatment, significant differences were found between T mean concentrations of the T group and both the sham and the control groups concentrations ($p<0.05$). No differences were found between the sham group and the control group.

4.2.2 E2 concentration

A similar analysis was done to E2 plasma content. Although apparently lower, E2 concentrations after the experiment of fish implanted with T tubes were not significantly different from those of samples taken in the first, initial sampling (Figure 4.4). In the same way, sham group fish analysis did not show any differences before and after the tube implantation.

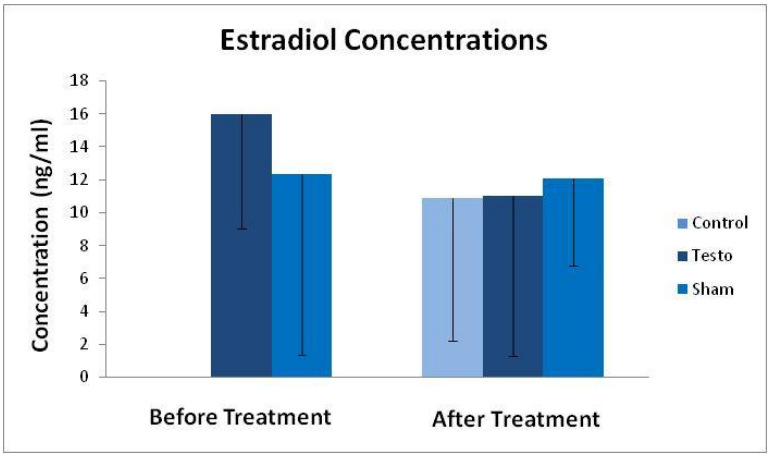

Figure 4.4 Mean estradiol concentrations with standard deviation in the three groups, before and after the testosterone treatment. No plasma samples were obtained from the control group, before the experiment. Therefore the comparison between different samplings is not possible for this group. There were no significant differences, neither between sampling days nor between groups. Differences between groups were tested by a Mann-Whitney non-parametric test. Differences within groups were tested with a Wilcoxon Matched Pairs non-parametric test.

There were no differences between the T group and the sham group before the experiment. After the treatment, E2 levels were similar in the three groups, as well. Furthermore, independently of the time of sampling (before or after treatment) and of the treatment, E2 mean concentrations were approximately 10 fold higher than T mean concentrations.

4.2.3 Prostaglandin concentration

The distributions of the three prostaglandins were not normal and the SD very high. Variances were significantly different between all the three groups.

Although in T and sham groups the three prostaglandins are apparently much lower, mean values of all the prostaglandin concentrations were not significantly different between groups (Figure 4.5). Within each group, however, the mean of $PGF_{2\alpha}$ was significantly higher than the other two prostaglandin means.

On the other hand PGE_2 and PGE_3 shared the same low levels and a mean ratio of 1 equally seen in the three groups: T group (1.00±0.63), sham group (0.98±0.59) and control group (0.96±0.4). Data did not follow a normal distribution and non-parametric tests indicated that the differences between each ratio were not significantly different.

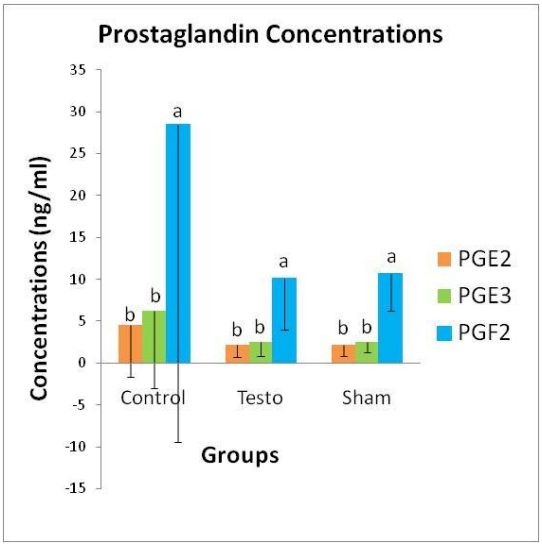

Figure 4.5 Mean prostaglandin concentrations with standard deviation in the three groups, after the treatment. There were no significant differences between groups. Different letters show significant differences between prostaglandins within the same group. Differences between groups were determined by a Mann-Whitney non-parametric test. Differences within groups were tested with a Wilcoxon Matched Pairs non-parametric test.

4.3 Prostaglandin correlation

Plotting the two other prostaglandins against PGE_2 concentration revealed a similar behaviour for the three of them in the control group, i.e. prostaglandins were significantly and positively correlated ($p<0.05$, graph c), Figure 4.6). Correlation matrices have confirmed this by reporting high coefficients of determination (R^2) for these relationships (Table 5.3). Regarding the other two groups (graphs a) and b), Figure 4.6), correlation tests gave no significant results (Tables 5.1 and 5.2, respectively).

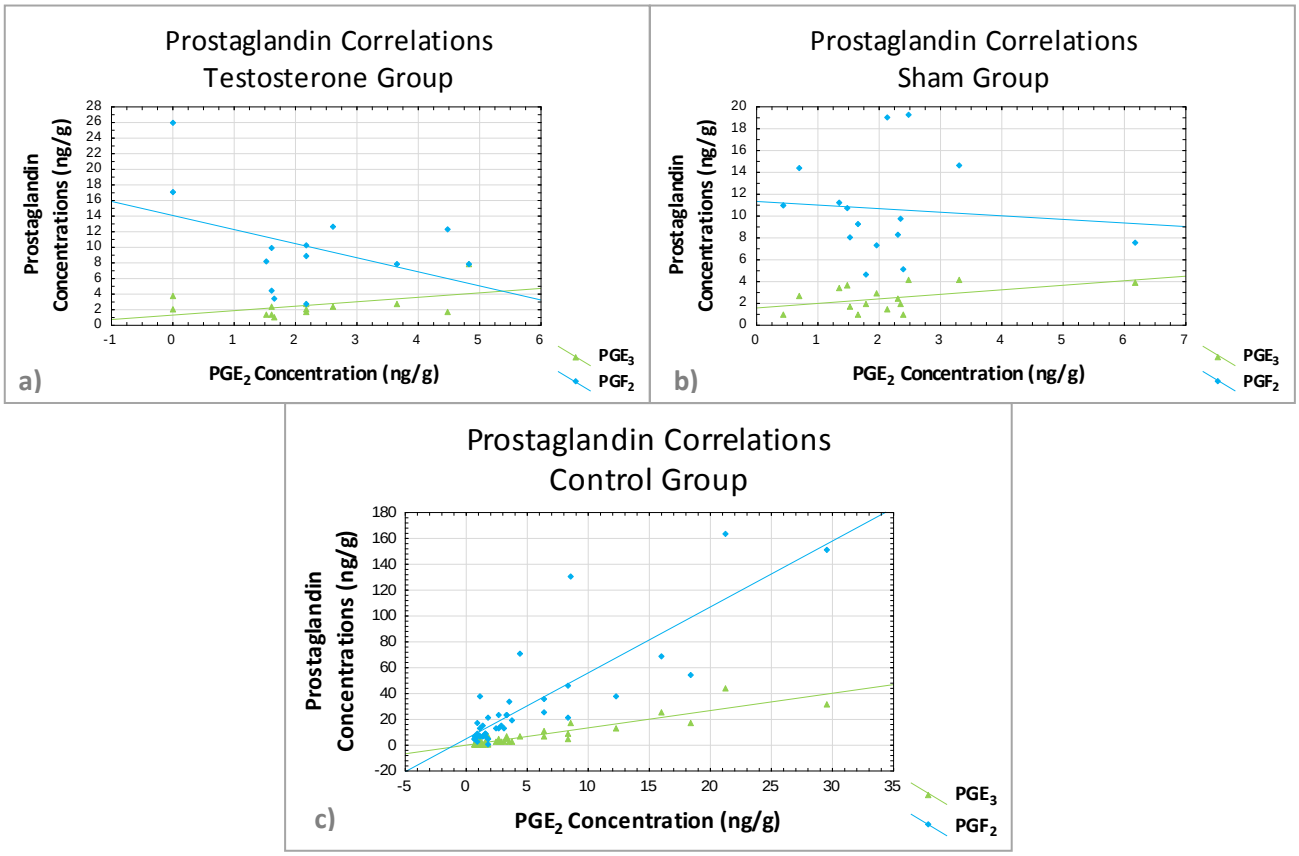

Figure 4.6 Prostaglandin correlation in: **a)** the testosterone group; **b)** the sham group; **c)** the control group. There were no significant correlations in both the testosterone group and the sham group. Positive correlations were found in the control group ($p<0.05$), (PGE_2 – PGF_2, $R^2 = 0.712$), (PGE_2 - PGE_3, $R^2 = 0.836$), (PGE_3 – PGF_2, $R^2 = 0.857$).

4.4 Steroid correlation

Figure 4.7 shows the correlation between T and E2 concentrations in the three groups.

Steroids from fish from the T group did not correlate (graph a), Figure 4.7, Table 5.1). A different scenario is shown in the other groups. Steroids from fish belonging to the sham group were positively correlated (graph b), Figure 4.7). A more gradual and not so marked pattern characterizes steroids correlation in fish from the control group (graph c), Figure 4.7). Correlations for the latter groups were significant ($p<0.05$, Tables 5.2 and 5.3 for sham and control groups, respectively).

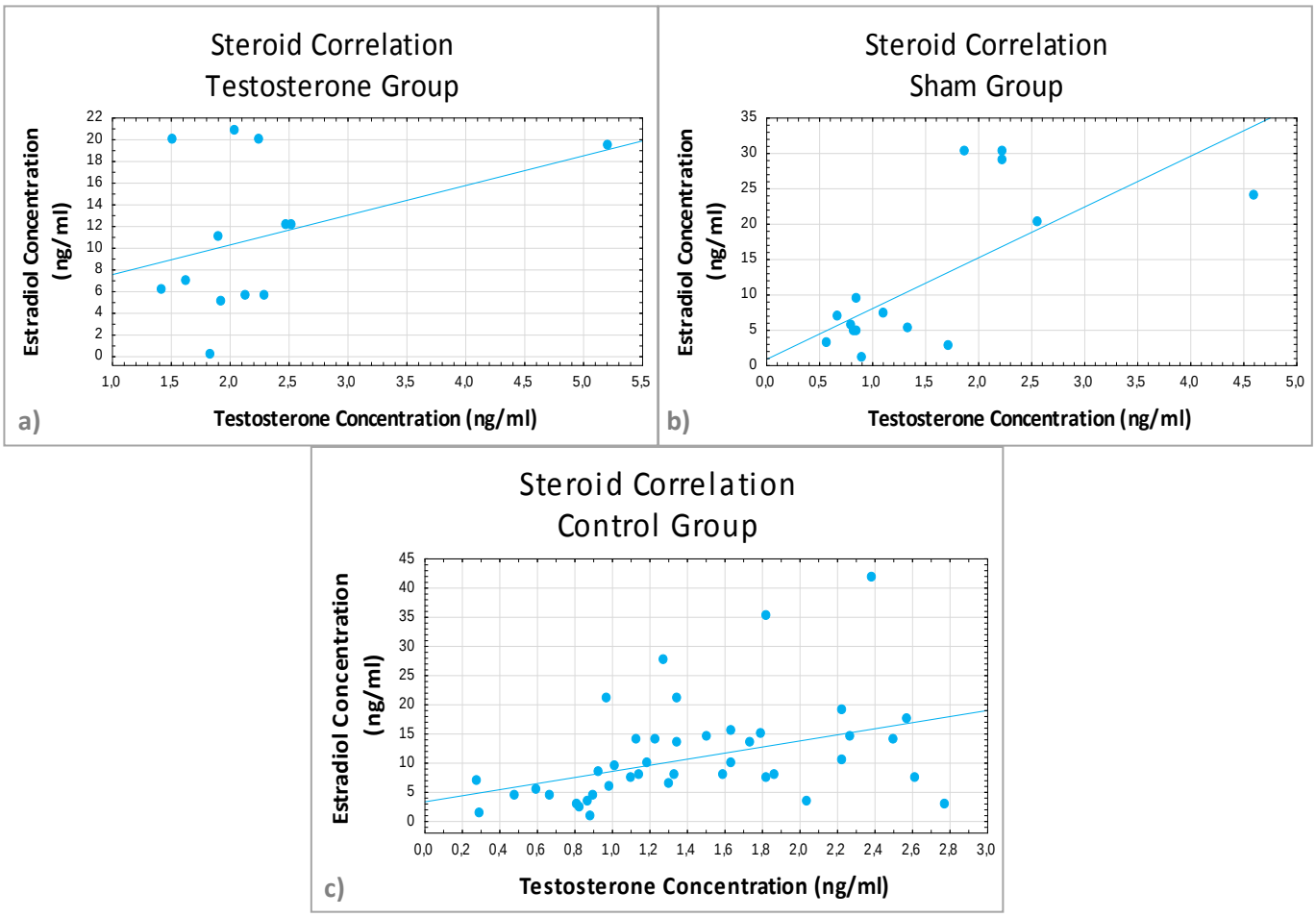

Figure 4.7 Steroid correlation in: **a)** testosterone group; **b)** sham group; **c)** control group. Positive correlations ($p<0.05$) were found between the steroids in both the sham and the control groups, R^2 = 0.484 and R^2 = 0.156, respectively.

4.5 Prostaglandin and steroid correlation

No correlation was observed between the three prostaglandin concentrations and both the T (Figure 4.8) and the E2 levels (Figure 4.9). Correlation values are shown in Tables 5.1, 5.2 and 5.3.

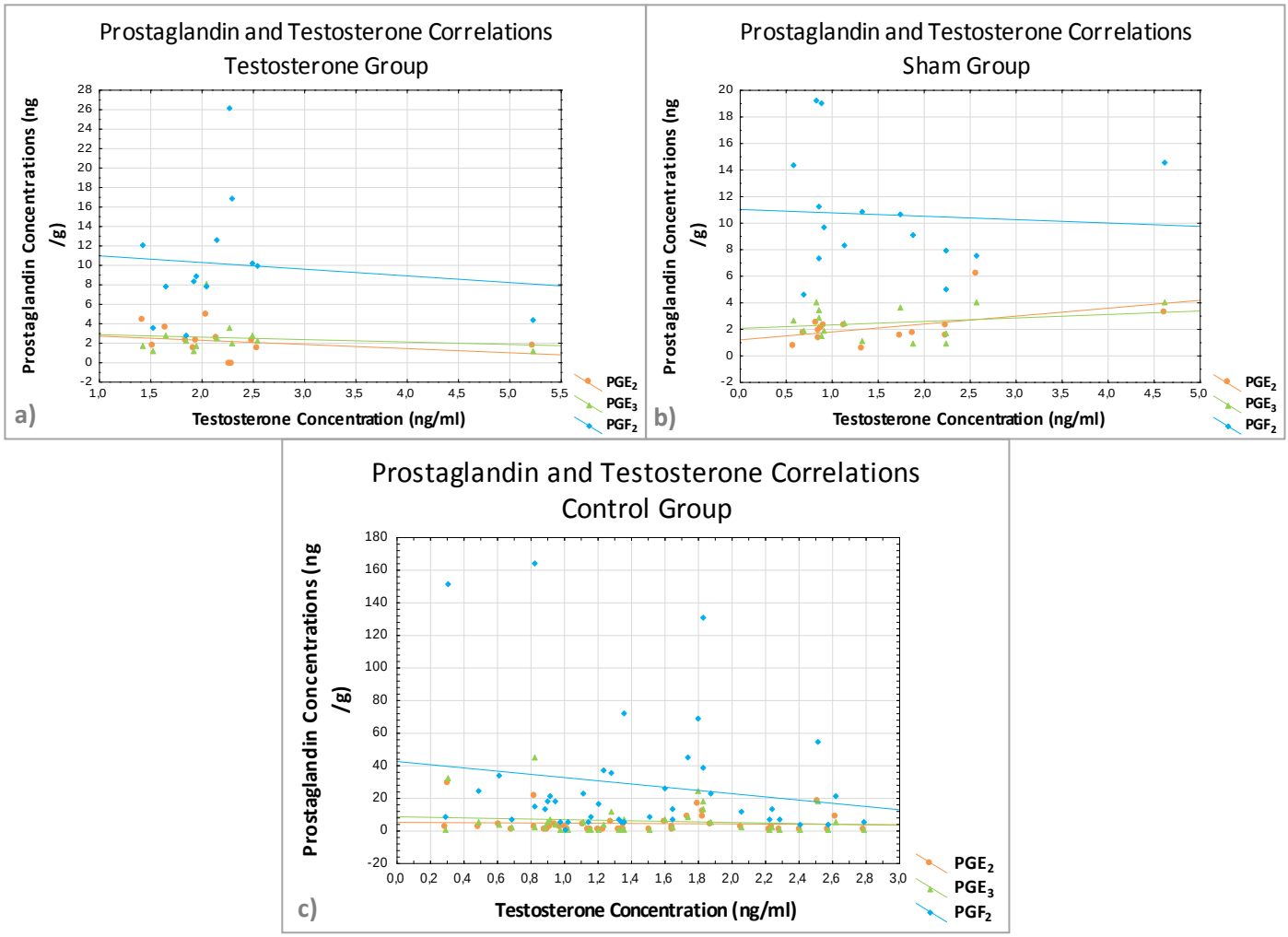

Figure 4.8 Prostaglandin and testosterone correlation in: (a) testosterone group; (b) sham group; (c) control group. No significant correlations were found between prostaglandins and testosterone in any group.

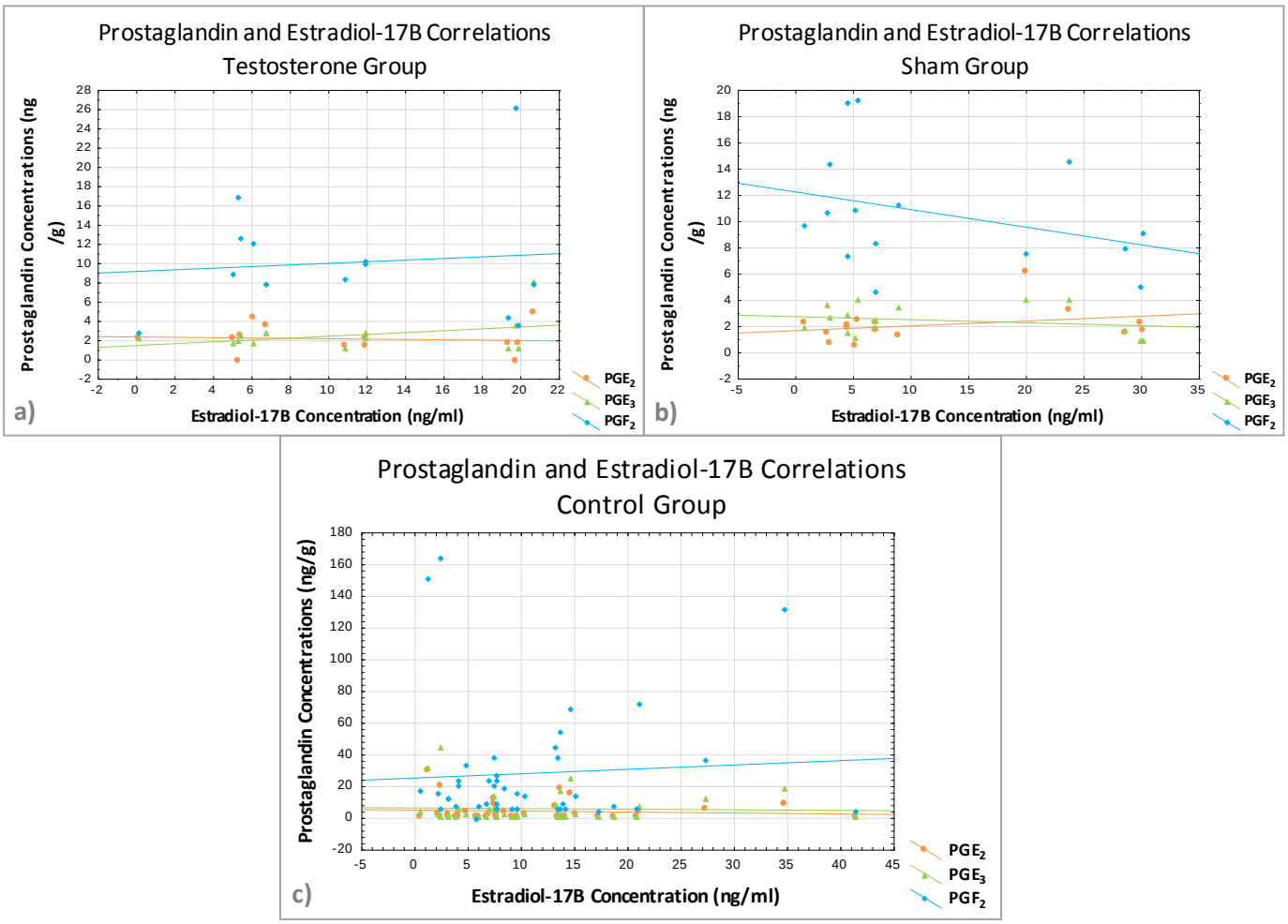

Figure 4.9 Prostaglandin and estradiol-17β correlation in: (a) testosterone group; (b) sham group; (c) control group. No significant correlations were found between prostaglandins and estradiol-17β in any group.

4.6 Steroid and prostaglandin ratio correlation

There were no significant correlations between the steroids and the PGE_2/PGE_3 ratio in the both the T and the control groups (graphs a) and c), Figure 4.10, Tables 5.1 and 5.3, respectively). On the other hand, E2 and the ratio were significantly and positively correlated in the sham group ($p<0.05$, graph b), Figure 4.10, Table 5.2).

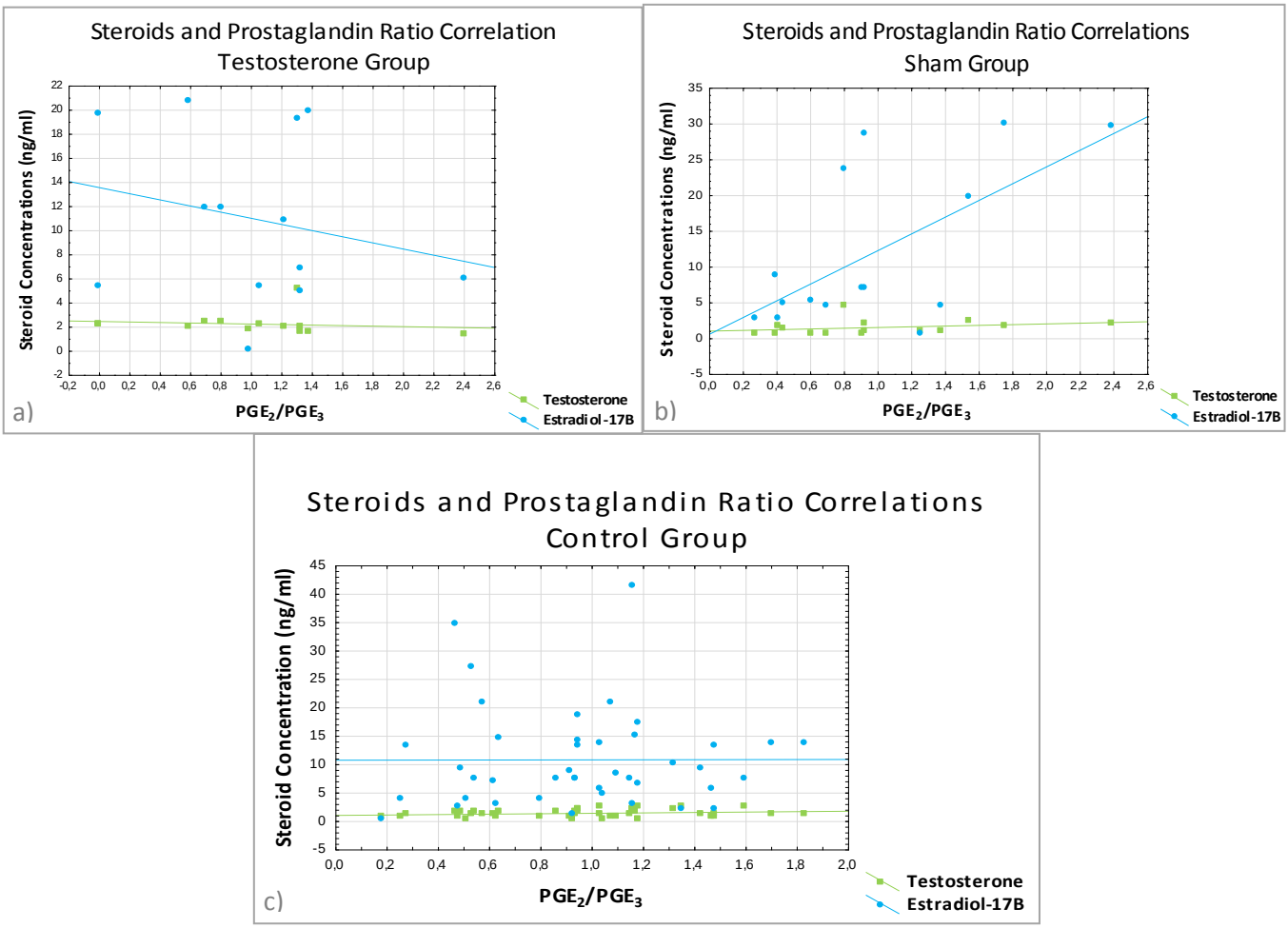

Figure 4.10 Steroid and PGE_2/PGE_3 ratio correlation in: (a) testosterone group; (b) sham group; (c) control group. A positive correlation was found between estradiol-17β and the ratio in the sham group ($p<0.05$), $R^2 = 0.397$.

Table 5.2 Coefficients of determination (R^2) and their statistical significance between all the variables in the testosterone group.

Correlations
Testosterone Group

		Testosterone (ng/ml)	Estradiol (ng/ml)	PGE_2	PGE_3	PGF_2	PGE_2/PGE_3
Testosterone (ng/ml)	R^2		0.1420	0.0787	0.0179	0.0113	0.0196
	p		p=0.204	p=0.353	p=0.663	p=0.729	p=0.648
Estradiol (ng/ml)	R^2	0.1420		0.0076	0.1397	0.0089	0.0532
	p	p=0.204		p=0.777	p=0.208	p=0.759	p=0.448
PGE_2	R^2	0.0787	0.0076		0.2146	0.1803	0.370
	p	p=0.353	p=0.777		p=0.111	p=0.148	p=0.027
PGE_3	R^2	0.0176	0.1397	0.2146		0.0247	0.140
	p	p=0.663	p=0.208	p=0.111		p=0.608	p=0.208
PGF_2	R^2	0.0113	0.0089	0.1803	0.0247		0.2773
	p	p=0.729	p=0.759	p=0.148	p=0.608		p=0.064
PGE_2/PGE_3	R^2	0.0196	0.0532	0.370	0.140	0.2773	
	p	p=0.648	p=0.448	p=0.027	p=0.208	p=0.064	

Each correlation is described by R^2 (the upper value) and the statistical significance.
Significant differences are marked with red colour (p<0. 05).

Table 5.3 Coefficients of determination and their statistical significance between all the variables in the sham group.

Correlations
Sham Group

		Testosterone (ng/ml)	Estradiol (ng/ml)	PGE_2	PGE_3	PGF_2	PGE_2/PGE_3
Testosterone (ng/ml)	R^2		0.4835	0.2273	0.0583	0.0037	0.0782
	p		p=0.004	p=0.072	p=0.386	p=0.829	p=0.313
Estradiol (ng/ml)	R^2	0.4835		0.092	0.0479	0.1099	0.3970
	p	p=0.004		p=0.272	p=0.433	p=0.228	p=0.012
PGE_2	R^2	0.2273	0.092		0.2283	0.0097	0.1962
	p	p=0.072	p=0.272		p=0.072	p=0.727	p=0.098
PGE_3	R^2	0.0583	0.0479	0.2283		0.1052	0.2089
	p	p=0.386	p=0.433	p=0.072		p=0.238	p=0.087
PGF_2	R^2	0.0037	0.1099	0.0097	0.1052		0.1232
	p	p=0.829	p=0.228	p=0.727	p=0.238		p=0.200
PGE_2/PGE_3	R^2	0.0782	0.3970	0.1962	0.2089	0.1232	
	p	p=0.313	p=0.012	p=0.098	p=0.087	p=0.200	

Each correlation is described by the R^2 (the upper value) and the statistical significance.
Significant differences are marked with red colour (p<0. 05).

Table 5.4 Coefficients of determination and their statistical significance between all the variables in the control group.

Correlations

Control Group

		Testosterone (ng/ml)	Estradiol (ng/ml)	PGE_2	PGE_3	PGF_2	PGE_2/PGE_3
Testosterone (ng/ml)	R^2		0.1558	0.0024	0.0151	0.0289	0.0537
	p		p=0.010	p=0.757	p=0.438	p=0.282	p=0.140
Estradiol (ng/ml)	R^2	0.1558		0.0070	0.0013	0.0039	0.0000
	p	p=0.010		p=0.598	p=0.820	p=0.694	p=0.985
PGE_2	R^2	0.0024	0.0070		0.8364	0.7115	0.0275
	p	p=0.757	p=0.598		p=0.000	p=0.000	p=0.294
PGE_3	R^2	0.0151	0.0013	0.8364		0.8572	0.1432
	p	p=0.438	p=0.820	p=0.000		p=0.000	p=0.013
PGF_2	R^2	0.0289	0.0039	0.71150	0.8572		0.1446
	p	p=0.282	p=0.694	p=0.000	p=0.000		p=0.013
PGE_2/PGE_3	R^2	0.0537	0.0000	0.0275	0.1432	0.1446	
	p	p=0.140	p=0.985	p=0.294	p=0.013	p=0.013	

Each correlation is described by the R^2 (the upper value) and the statistical significance.
Significant differences are marked with red colour (p<0. 05).

5 DISCUSSION

5.1 Effect of T on fish performance

The T treatment did not influence either the fish weight loss or the GSI except for a higher final weight in the sham group than in the control group. The unaltered GSI seem to suggest that T treatment does not influence gonad development.

As there was no weight measurement in the control group during the first sampling, it was not possible to calculate the growth in this group.

Moreover, mortality was higher in the T group compared to the control group. Since there was no difference between the two treatment groups means, the cause for the higher mortalities in the T and the sham group was most probably the handling stress.

Therefore, the T treatment does not seem to have a positive effect on fish performance.

5.2 Effect of T on steroids and prostaglandins

5.2.1 Steroid concentrations analysis

T is of utmost relevance in male fish, both during its differentiation as a male and also during reproduction (Miura *et al*., 1991). Besides that, T is involved in adaptation to stress, homeostasis and immune function (Cuesta *et al*., 2007). However, 11-KT is the most important male androgen in teleost fish.

Nevertheless, the androgen tested in this study was T, which is the most abundant androgen in female plasma being, sometimes, even higher than in males (Campbell *et al.*, 1976, Stuartkregor *et al.*, 1981).

T levels increased during vitellogenesis and were still high after ovulation, even though E2 levels decreased in the Amago salmon, *Oncorhynchus rhodurus* (Kagawa *et al*., 1983). The non-aromatizable steroid 11-KT is present in Atlantic cod females plasma and oocyte growth seems to be influenced by its concentration (Kortner *et al.*, 2009). Non-aromatizable androgen injection in the European eel, *Anguilla anguilla*, has been shown to have an indirect effect on hepatic vitellogenesis, stimulating VTG production by binding to E2 receptors when in high concentrations (Peyon *et al*., 1997).

The role of androgens in Atlantic cod females, especially 11-KT, has been given a lot of attention regarding its growth-enhancing effect on previtellogenic oocyte growth and development (Kortner *et al.*, 2009, Kortner *et al.*, 2008, Kortner and Arukwe, 2007).

In this study instead, mature female Atlantic cod were used. An extra-dose of T was supplied to these fish and later, its concentrations were related to E2 plasma content and to prostaglandin levels.

5.2.1.1 Changes in steroid concentrations during the treatment

Plasma steroid analysis from T and sham group fish **before the experiment** indicated that females have significant amounts of circulating T during vitellogenesis. E2 was also very high, reaching concentrations 10 fold higher than T levels.

The T levels reported here were much higher than those observed by Kortner *et al.* (2009), whose studies were focused on Atlantic cod previtellogenic oocytes. One role of T is as a precursor of the main vitellogenesis-inducing steroid, E2. During the spawning season, cod ovaries are extremely active in vitellogenesis (Kjesbu *et al*., 1996). Thus, as an aromatizable androgen, T levels are expected to be higher during vitellogenesis than during the previous primary oocyte growth.

Accordingly, E2 levels from plasma samples taken **before the treatment** were much higher than those measured in the previtellogenic females (Kortner *et al.*, 2009). Such steroid amounts were in accordance with those observed previously in spawning females by Kjesbu *et al.* (1996).

After the treatment, the sham group showed unaltered T levels which were similar to the control group levels. Differently, the fish belonging to the T group showed a significantly higher T circulating level when compared to the same levels 23 days before. This increase shows that T was successfully delivered by the silicone implants. In the study of Kortner *et al.* (2009) fish showed no differences in the plasma T levels after 21 days of a T treatment administered by three separate injections. The fact that such differences were found in the present study may be explained by the chosen delivery method which was continuous throughout the experiment.

Regarding the E2 levels, there were no significant alterations after the treatment, although a slight decrease can be observed in both the T and the sham groups. Methven *et al.* (1992) noticed a gradual decrease in E2 levels throughout the spawning season while

studying the halibut, *Hippoglossus hippoglossus*, which is also a batch spawner. In Atlantic cod this has been described as well (Kjesbu *et al*., 1996). This matter will be discussed later in this section.

5.2.1.2 Differences between the groups

The analysis of the samples taken **before the experiment** indicated that both T and E2 levels were not significantly different between T group and sham group and this was important as a basic premise for the onset of the experiment.

On the other hand, **after the experiment**, T group T levels were significantly higher (around 1.5 times higher) than those of both the sham and control groups. Despite that, the extra T had no effect on E2 levels in the T group: all the three groups presented very similar E2 means after the experiment.

Kortner *et al.* (2009) found an effect on E2 levels after 21 days, after having supplied extra-doses of 11-KT and T to previtellogenic females. In fish given 11-KT the E2 levels increased in parallel to gradually lower T concentrations. This was most likely due to the P-450 aromatase activity, transforming T into E2. In the present study, however, T levels were higher after treatment. Attending the fact that the increased T levels did not affect E2 levels, leads to the conclusion that the extra-dose was apparently not being conducted to the follicles to be used as a substrate for P-450 aromatase.

However, it is very important to have in mind that the control of reproduction involves the entire reproductive axis – the pituitary as well. Kortner's study (2009) did not focus on the pituitary's involvement in the 11-KT-induced oocyte growth. Therefore, the role of 11-KT in the increasing E2 levels is far from being clear.

As it has been said before, female cod ovaries are very active in vitellogenesis. A possible explanation for the fact that T was not used as an E2 precursor would be that female cod have naturally higher levels of both steroids during vitellogenesis than during any other time of the oocytes development (Nagahama, 1994, Dahle *et al.*, 2003). Thus, an additional supply of T is not having any effect on E2 synthesis. There was no intermediate monitoring of the steroid concentrations throughout the experiment. This would imply more fish handling and the subsequent stress pressure on fish which would lead to even higher mortalities. Therefore, it is impossible to know whether the delivered T was just being

accumulated in the blood stream and metabolized afterwards or whether it was being used for another purpose.

5.2.2 Prostaglandin concentration analysis

Many studies have been carried out on prostaglandin involvement in fish ovulation but none have been performed in Atlantic cod. The specific cases of brook trout (Cetta and Goetz, 1982), yellow perch (Goetz *et al*., 1989a), pond loach, *Misgurnus anguillicaudatus*, (Ogata *et al*., 1979) and goldfish (Stacey and Pandey, 1975) have been thoroughly studied. Many of them follow the same pattern of prostaglandin dynamics during maturation and ovulation. Generally, it seems that PGE_2 amounts are the highest until the oocyte maturation. Then, under the MIH control, $PGF_{2\alpha}$ starts to increase while PGE_2 decreases. At the time of ovulation, $PGF_{2\alpha}$ is the most conspicuous prostaglandin (Goetz, 1997, Goetz and Cetta, 1983, Goetz, 1991, Cetta and Goetz, 1982). Similarly to PGE_2's, the production of $PGF_{2\alpha}$ is suppressed in the extra-follicular tissues during ovulation (Goetz, 1997).

On the other hand, a higher ovulation-inducing potential was found in PGE_2, after being administered to mature oocytes which were incubated firstly with MIH and indomethacin (Goetz and Theofan, 1979).

5.2.2.1 Prostaglandin individual levels

The first thing to be noticed was the conspicuous amounts of $PGF_{2\alpha}$ which were, in every fish from all the groups, considerably higher than the other two prostaglandins. Regarding the much lower PGE_2 and PGE_3 concentrations, the three groups considered here were characterized by PGE_2/ PGE_3 means very close to 1.

This big contrast between them has been reported previously by other authors who showed increased $PGF_{2\alpha}$ concentrations and decreased PGE_2 levels at the time of ovulation (Cetta and Goetz, 1982). These levels were measured in the brook trout whole ovary tissue, similarly to the present study.

Another *in vitro* study reported higher PGE_2 levels compared to $PGF_{2\alpha}$ in the control incubates of the yellow perch ovaries (Goetz, 1997). However, under the effect of MIH, $PGF_{2\alpha}$ increased while PGE_2 decreased.

Attending to these findings, it seems that $PGF_{2\alpha}$ is the primary prostaglandin more deeply involved in the maturation and ovulation processes.

It is important to have in mind that the brook trout is a synchronous spawner. On the other hand, cod is a group synchronous batch spawner. This means that trout has an ovary in which oocytes are in the same stage at the same time until they are spawned, once in a year. On the other hand, cod spawns several times during a spawning season and therefore, there are more than one oocyte stage at the same time in the gonad.

Eicosanoids actions will depend on the EPA/ARA ratio in the cell membranes and this, in turn, is influenced by the diet's fatty acid content (Tocher, 2003). Most of the diets that are being used for experiments, which are actually common commercial diets, have considerably high EPA/ARA ratios (Henrotte *et al*., 2010, Salze *et al*., 2005). EPA competes with ARA in the eicosanoids biosynthesis. Some of its 3-series prostaglandins are less biologically active than their 2-series counterparts (Goldman *et al*., 1983). Salze *et al*. (2005) observed a very high EPA/ARA ratio in Atlantic cod eggs in both wild and farmed broodstock. Since EPA levels are apparently higher in the cell membranes and since it competes with ARA as a substrate for COX, low PGE_2/PGE_3 ratios should be expected. However, COX has a preference for ARA and therefore, ARA is usually the main eicosanoid precursor in fish species (Henderson *et al*., 1985, Anderson *et al*., 1981). Furthermore, Janagap (2012) has shown that cod head-kidney cells were producing even more PGE_2 when supplied with EPA instead of decreasing its amount due to competition with ARA for COX. One explanation suggested by the author was that higher amounts of EPA would promote the release of ARA from the cell membrane. More ARA available means more substrate for COX, and therefore, higher PGE_2 levels.

Sorbera *et al.* (2001) have demonstrated in the sea bass, *Dicentrarchus labrax*, that EPA was actually ineffective in inducing oocytes maturation and was even inhibiting the gonadotropin-induced maturation.

The high similarity reported here between PGE_2 and PGE_3 concentrations seem to indicate that COX is using both ARA and EPA as substrates in the same proportion. Based on the previous statements, EPA normal concentration in the cell membrane does not compromise the production of 2-series eicosanoids. Thus, it is possible to speculate that PGE_3 might be produced after some specific unknown participation in maturation and/or ovulation processes. Due to its resemblances with PGE_2 it would be interesting to understand how this prostaglandin's dynamics in this process are.

5.2.2.2 Differences between the groups

This study showed no significant differences in the mean concentrations of any prostaglandin between the three groups indicating that T had no effect on prostaglandin levels even when it was externally supplied. Steroids relation with prostaglandins has been given some attention both in fish and mammals. Most of the time the steroid tested is progesterone but non-progestational steroids such as E2 and T have also been used. These results are in accordance with Goetz *et al*. (1989a) who observed no T or E2 significant effect on PGE_2 and $PGF_{2\alpha}$, in the yellow perch mature oocytes.

In rats, mRNA of the PGE_2 receptors EP_1, EP_2, EP_3 and EP_4 is regulated by E2 and also by progesterone (Blesson *et al*., 2012). On the other hand, T production was enhanced by ARA treatment (Van Der Kraak and Chang, 1990, Mercure and VanDerKraak, 1996). To test whether this action was mediated by ARA metabolites, the authors added different inhibitors of enzymes involved in the ARA metabolism. Enhanced T production was not observed and levels were similar to those from the control group, indicating that ARA-metabolites mediated the increase in T concentration.

However, it is reasonable that no T effect was observed on prostaglandins. The fact that there was no effect of the T supply on both the steroids suggests that there was no effect on oocyte development either. Thus, the same levels of prostaglandins would be expected in both the treated and in the non-treated fish.

In this study, and for the first time in adult female cod, prostaglandins were assayed in the whole gonad tissue (both follicles and extra-follicular tissue). The amounts described here are therefore a sum of the two compartments and no conclusions can be taken from that perspective. Also, the present study is an *in vivo* experiment on female cod which gonads not only show maturing but also vitellogenic oocytes. Most of the other studies were performed *in vitro* and were focused on maturating follicles only. Therefore, with different phases of the cod oocytes development present at the same time, it is not advisable to speculate about the specific roles of each one of the prostaglandins considered here.

5.3 Data distribution

Generally speaking, steroid and prostaglandin concentration distributions were characterized by high variances. In certain cases, SD's were higher than the respective means.

The presence of different cohorts of oocytes at the same time in the cod ovaries could be an explanation for the wide data distribution in the sense that both steroid and prostaglandin levels, as key ovarian regulators, would be different according to the oocyte stage.

A different suggestion is that the variances are the result of another variable effect that was not measured in this study.

Despite being one of the main aims of this study, it was not possible to classify the oocyte stages. Such classification would allow relating steroid and prostaglandin levels to different ovarian phases and hence, better understanding the steroid and prostaglandin dynamics in the cod ovaries. Full description on this topic is given further in the text.

5.4 Correlations

5.4.1 Steroid correlations

Statistics did not indicate any significant correlation between the two steroids in the T group, meaning that extra T-supply did not contribute to strengthen up the correlation with E2. On the other hand significant positive correlations were found between steroids both in the sham and in the control groups. The correlations were not very strong but the relation is expected during vitellogenesis where these steroids play key roles: E2 as the estrogen responsible for the induction of VTG hepatic-production and T as E2 precursor.

The absence of a correlation in the T group suggests that there is a plateau that is reached after having gradually increased T concentration and consequently also increased E2 levels. It seems that P-450 aromatase or other E2 biosynthesis intermediate will no longer respond to T levels after a certain maximum amount. The questions then would be from which basal concentration would this happen and whether such amounts can actually be measured in a non-treated fish.

5.4.2 Correlation between prostaglandins

The three prostaglandins were significantly and positively correlated with each other in the control group.

By analysing previous studies it seems that primary prostaglandins such as PGE_2 and $PGF_{2\alpha}$ have distinct roles. In most cases, during maturation and ovulation there is an increase of one prostaglandin while the other's production seems to be suppressed. However, this is highly depending on the analysed tissue, on the oocyte stages and on the fish species (Goetz, 1991, Goetz, 1997, Goetz and Cetta, 1983).

The present positive correlations between prostaglandins indicate that the fish with the highest PGE_2 levels also has the highest $PGF_{2\alpha}$ levels. However, these results do not invalidate the existence of more complex interactions between prostaglandins. Differently from the other studies, this experiment was performed on the whole gonad of cod and therefore, it is not advisable to make further conclusions.

The simultaneous increases were however not found in the other two groups. Since no T effect was observed on prostaglandin concentrations, the absence of prostaglandin correlations of both the T and sham groups may perhaps be attributed to stress stimuli to which fish from both groups were equally subjected.

Finally, if the three prostaglandins vary in a similar way, it is expected that the PGE_2/PGE_3 ratio and individual prostaglandins positively correlate, as well. This was observed both in the control group and in the T group.

5.4.3 Correlation between steroids and prostaglandins

No correlations were observed between T and prostaglandins in any group. Likewise, varying E2 levels were not significantly correlated with the eicosanoid levels.

However, there may be an effect of prostaglandins on steroidogenesis. E2 showed positive correlation with the PGE_2/PGE_3 ratio in the sham group.

Pre-matured oocytes treated with ARA showed significantly increased T concentration and indomethacin addition was successful in inhibiting increased production of T (Van Der Kraak and Chang, 1990). This suggests that ARA metabolites may be involved in steroidogenesis.

PGE_2/PGE_3 ratio means are the same in the three groups. What differ between the sham and the control groups are both the presence of prostaglandin correlations in the control

group and the strength of the steroids correlation, which is stronger in the sham group. Stress may explain this situation. A hypothesis would be that, under the stress caused by the tube implantation, the different prostaglandin levels, although not significantly different from other groups, may have changed enough to have an effect on E2 levels in these fish. Thus, by affecting E2 levels, this perturbation would strengthen up the correlation between the steroids, which is stronger in the sham group than in the control group.

5.5 Oocyte stages classification and diameter measurements

Due to failure of the methods to classify the ovulation stages, no data are given for this variable.

In the first method, oocyte classification was mainly based on 4 criteria: the nucleus presence/absence, shape (whether it was perfectly round or not), position (central or peripheral) and the yolk granules organization in the cytoplasm. 6 oocyte stages were considered in the classification of each sample. It was the intention to place a female in a certain category, i.e. a specific time of the ovarian cycle, according to the amount of oocytes of each stage that had been found in her gonads.

However, at the same time that this plan was being materialized, the methodology chosen turned out to be somehow inadequate.

The gonad histology process resulted in nice blue stained oocyte sections which would allow obtaining pictures good enough for the classification according to the criteria described above. Despite that, the magnification used in the microscope (x0.5) and the 3x zoom were not strong enough to clearly distinguish stages from one another. Higher magnification would mean more pictures per section and it would be, in the end, too time-consuming. Classification was done, but due to its inaccuracy, the data were not adequate.

After this first attempt, the decision was to obtain oocyte diameters which corresponded to the equatorial plan of the oocyte. Vitellogenic oocytes have a central nucleus. This measurement would be perfectly accurate and reliable for vitellogenic oocytes, as long as the section contained the nucleus in it. Unfortunately, it is not certain that a section belongs to a vitellogenic oocyte just because it contains a central nucleus. A GVM I, II or even III oocyte has its nucleus in a peripheral position, heading towards the animal pole. Still, the resulting oocyte section may have a central nucleus if the sectioning is done as it is

exemplified in Figure 5.1. This would lead to the misconception of measuring a section of a GVM I, II or III and considering it a smaller vitellogenic oocyte.

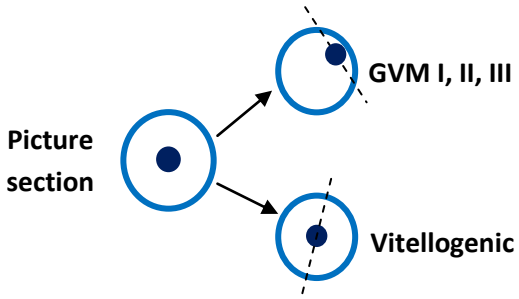

Figure 5.1 Oocyte section possible positions. A section with a central nucleus may correspond to an equatorial plan of a vitellogenic oocyte but it may also belong to a GVM I, II or III oocyte.

An alternative suggestion was to use the fraction of hyaline oocytes as an indicator of the female position in the ovarian cycle, i.e. the higher the amount of hyaline oocytes, the closer a female would be to ovulation. Since no nucleus is visible, hyaline oocytes would easily be identified and counted in each female. However, the amount of these oocytes cannot be used as an indicator of a female placement in the ovarian cycle as it is explained next.

Until the end of vitellogenesis, oocytes have different growth rates (Kjesbu *et al*., 1996). Afterwards, between each batch spawn, a leading cohort matures and ovulates at the same time. Thus, hyaline oocytes evolve at the same rate, i.e. their number does not increase over time, and they last no more than one and a half day inside the ovary, before they are ovulated and spawned. Therefore, the **amount** of hyaline oocytes may perhaps be considered an indicator of fecundity, rather than an indicator of the ovarian cycle stage. Instead, the **presence** of hyaline oocytes in a female gonad only indicates that the female is about to spawn.

A different strategy should have been adopted to accomplish this classification. Measuring the diameter of the whole oocyte, instead of oocyte sections, would have been a reliable and more accurate method.

The determination of the oocyte maturation stages should have been the basis for the female placement in the ovarian cycle association with the steroid and prostaglandin levels. Each female would have been classified according to her oocyte leading cohort stage and it

would be plotted against the corresponding steroid and prostaglandin levels. This may have given a more detailed picture of the prostaglandin dynamics and their involvement in maturation/ovulation.

The time needed for a batch of eggs to be fully developed and ready to be expelled is around 5-6 days (Kjesbu *et al*., 1996). Moreover, the interval between batches in cod, similarly to the halibut, is 2-3 days (Norberg *et al*., 1991) Thus, different cohorts of oocytes must be present in the ovary at the same time. This has been reported in cod more than once (Kjesbu *et al*., 1990, Kjesbu *et al*., 1996) and was also observed here, during pictures analysis. In most pictures it was possible to distinguish at least 2, when not 3 different stages of oocytes.

5.5.1 Female placement in the ovarian cycle – effect on steroids

When the gonad is full of hyaline oocytes, a second batch is already being produced, in an earlier stage. Thus, E2 levels in such a female may be higher than they are expected to be in the presence of hyaline oocytes.

The amounts of E2 and T could not be related to the different oocyte development stages but it is expected that E2 levels vary in a cyclic way as it has been previously shown by Kjesbu *et al*. (1996). E2 is expected to be higher both in the first and the last part of the batch interval.

The same study revealed that both the amount and the size of oocytes in each batch decrease towards the end of the spawning season. As a consequence, E2 concentrations slowly and gradually decrease as the end of the season approaches.

Although not significantly, mean E2 and T levels after the treatment were slightly lower than the corresponding levels before the treatment which is in accordance with the previous statement.

5.5.2 Female placement in the ovarian cycle – effect on prostaglandins

Similarly to steroids, the absence of the oocyte stages variable does not allow to fully comprehend prostaglandin dynamics during the ovarian cycle.

Different prostaglandins have been shown to have different effects on oocyte maturation and ovulation and it also varies between species. Regarding ovulation Kagawa *et al*. (2003) have reported a high stimulatory potential for $PGF_{2\alpha}$ in the Japanese eel. This was

the most powerful prostaglandin compared to other prostaglandins such as PGE_2 and it was observed in maturing oocytes. On the other hand, in the yellow perch, PGE_2 was the most effective in restoring ovulation in oocytes treated with indomethacin (Goetz and Theofan, 1979). Finally, high levels of $PGF_{2\alpha}$ were measured in the goldfish immature follicles while just small amounts of prostaglandins were produced by the mature follicle walls (Goetz, 1991).

The prostaglandin data from cod ovary samples only show that the most conspicuous prostaglandin is PGF_2. It also shows that the females with the higher amounts of one prostaglandin are also the ones with higher levels of the other prostaglandins. Unfortunately, trying to relate these levels to a specific role in the ovarian cycle would be to make unfounded speculations.

6 CONCLUSION

Steroid analysis demonstrated that significant levels of T circulate in plasma from female cod. Despite that, T did not have any effect on any studied variable. However, treatment with tube implants either filled with T or not, significantly increased mortalities, probably due to stress. Considering the initial aims of this study, the analysis of the present results allows to make the following conclusions:

1. Treatment did not affect weight and GSI which indicates that the gonad development is not influenced by higher T levels. However, higher mortalities in both T group and sham group suggest that fish performance is negatively affected by the treatment.
2. The unaffected E2 levels and the absence of a correlation between the two steroids in the fish treated with T indicate that T supplementation did not influence E2 production. Higher levels of T in the T group suggest that T was not used as an E2 precursor in these fish. This seems to indicate that T may also have another different role in reproduction.
3. The absence of T supplementation effect on prostaglandin levels suggests that eicosanoid ovarian levels are not regulated by steroids.
4. It was not possible to relate either the steroids or the prostaglandins to ovarian cycle phases due to inadequate methodology. Therefore, no conclusions can be done either to steroid or prostaglandin variation during the oocyte development. Nevertheless, $PGF_{2\alpha}$ seems to be the most abundant prostaglandin the female Atlantic cod ovaries during the spawning season. PGE_2 and PGE_3 were comparatively much lower and share very similar concentrations. This seems to indicate that COX is mediating both prostaglandins biosynthesis in the same proportion which subsequently suggests an intimate involvement of PGE_3 in oogenesis.

Prostaglandin positive correlations revealed a similar behaviour for the three of them. The absence of prostaglandin correlations in both the T and the sham groups may be associated to a stress stimuli response.

5. Steroid positive correlations in both the sham and the control groups are in accordance with what is expected in a spawning female, which ovaries are very active in vitellogenesis.

The fact that steroids were not correlated with any prostaglandin supports the hypothesis that steroid do not regulate prostaglandin activity. However, E2 was positively correlated with PGE_2/PGE_3 ratio in the sham group. This fact may suggest that prostaglandins are regulating steroidogenesis under stressful environments.

7 FUTURE CONSIDERATIONS

7.1 Method improvements

In order to accurately classify oocytes one should mostly rely on diameter measurements instead of ultra-structural features. Diameter should be obtained from fixed, whole oocytes instead of sectioned oocytes and further classification can then be made according to each oocyte stage mean diameter. Oocyte sections should be used for ultra-structural analyses, only.

This study was based on a T supplement that was provided by an implanted silicone tube which was slowly releasing the steroid throughout the experiment period. Only two samplings were done, one on the day of implantation and the second on the last day of the experiment. Therefore steroid data gives either the starting or the final concentrations.

To better monitor the steroids dynamics and the extra T-dose effect on the steroids and prostaglandin levels, intermediate plasma and gonad biopsies should be done to fish. Thereby, it would be easier to understand at which point the correlation between steroids is disrupted, i.e. at which concentration does the role of T as a precursor cease as well as the point at which T plasma concentrations are permanently high.

7.2 Future studies

Further studies should to be done on prostaglandin involvement in maturation and ovulation of cod oocytes, especially series-3 prostaglandins. *In vitro* studies to individual oocytes will allow detailed prostaglandin analysis. Also, in vivo treatments with prostaglandins and inhibitors should be done in order to better understand their involvement in steroidogenesis and ovulation.

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8 APPENDIX

Appendix I

ELISA Components Assemblage

For ELISA, the T and E2 AChE tracer and EIA antiserum were prepared according to the requested plates number:

T AChE Tracer (20x diluted)

- Total amount of liquid for plates = 4 plates x 5000 µl pr. Plate = 2000 µl + 2000µl = 22000 µl
  - Original T AchE Tracer amount = 22000 µl (total amount of liquid) / 20 (dilution factor) = 1100 µl
  - Solved in EIA = 22000 µl (total amount of liquid) – 1100 µl (Original T AChE Tracer) = 20900 µl EIA

T EIA antiserum (50x diluted)

- Total amount of liquid for plates = 4 plates x 5000 µl pr. Plate = 2000 µl + 2000µl = 22000 µl
  - Original T EIA antiserum amount = 22000 µl (total amount of liquid) / 50 (dilution factor) = 440 µl
  - Solved in EIA = 22000 µl (total amount of liquid) – 440 µl (Original T EIA antiserum) = 21560 µl EIA

T internal standard (10x diluted)

- Total amount of liquid = 1000 µl
  - Original T internal standard amount = 1000 µl (total amount of liquid) / 10 (dilution factor) = 100 µl
  - Solved in EIA = 1000 µl (total amount of liquid) – 100 µl (Original T internal standard) = 900 µl EIA

E2 AChE Tracer (40x diluted)

- Total amount of liquid for plates = 4 plates x 5000 µl pr. Plate = 2000 µl + 2000µl = 22000 µl

- Original E2 AchE Tracer amount = 22000 µl (total amount of liquid) / 40 (dilution factor) = 550 µl
- Solved in EIA = 22000 µl (total amount of liquid) – 550 µl (Original E2 AChE Tracer) = 21450 µl EIA

<u>E2 EIA antiserum (20x diluted)</u>

- Total amount of liquid for plates = 4 plates x 5000 µl pr. Plate = 2000 µl + 2000µl = 22000 µl
  - Original E2 EIA antiserum amount = 22000 µl (total amount of liquid) / 20 (dilution factor) = 1100 µl
  - Solved in EIA = 22000 µl (total amount of liquid) – 1100 µl (Original E2 EIA antiserum) = 20900 µl EIA

<u>E2 internal standard (15x diluted)</u>

- Total amount of liquid = 1000 µl
  - Original E2 internal standard amount = 1000 µl (total amount of liquid) / 15 (dilution factor) = 66.6 µl
  - Solved in EIA = 1000 µl (total amount of liquid) – 66.6 µl (Original E2 internal standard) = 933.4 µl EIA

Appendix II

Origin of Chemicals and equipment

Experiment

Solution	Description
Testosterone (17β-Hydroxy-3-oxo-4-androstene, 17β-Hydroxy-4-androsten-3-one, 4-Androsten-17β-ol-3-one)	Sigma-Aldrich Art.Nr. T1500, St. Louise, USA

Histology

Equipment	Description
Shaker	KS250, IKA Labortechnik,
Microtome	LEICA RM 2155 (Fabr. Nr. 0774/12- 1997).

Solutions	Description
3.6% buffered formaldehyde	Sigma, Art. Nr. HT5012
Borax	Sigma. Art. Nr. HT1002

Prostaglandins Analysis

Equipment	Description
Evaporator	Labconco vacuum drier system, Kansas, MO
Centrifuge	VWR Galaxy 14D
Centrifuge	Eppendorf Centrifuge 5804
Vortex	*VWR international* vortex
Scale	Mettler AT200

Solutions	Description
PGE2-d4 (99%)	Cayman Chemical (Ann Arbor, MI, USA)
Acetonitrile (ACN, 99.8%)	Sigma-Aldrich (St. Louis, MO, USA)
Formic acid (98%)	Sigma-Aldrich (St. Louis, MO, USA)
Chloroform (99.8%)	Merck (Darmstadt, Germany)

ELISA

Solutions	Description
^{3}H-labelled testosterone	American Radiochemicals Inc (ARC; USA)
Dipotassium hydrogen phosphate	Sigma, Art. Nr. P3786
Monopotassium dihydrogen phosphate	Sigma, Art. Nr. 60219
Sodium chloride	Sigma, Art. Nr. S7653
Ethylenediaminetetraacetic acid	Sigma, Art.Nr. e5134, 500g
Ethyl Acetate	Merck, Art.Nr. 1.09623, 2.5l
Acetylthiocholin iodide	Sigma, Art. Nr. A5751
5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)	Fluka, Art.Nr. 43760, 25g
Tween 20	Sigma. Art.Nr. P9416, 100 ml
Bovine serum albumin	
Steroids Internal Standards	Extracted from vitellogenic females' plasma at the Austevoll Research Station
Testosterone (Testosterone standard (20 ng/ml)	Steraloids Inc (USA), Art. Nr. A 6950-000
β-Estradiol (Estradiol standard)	Sigma, Art. Nr. E1132
Testosterone trial- Mouse Anti-Rabbit IgG Coated Plates	Cayman Chemical Item Nr. 400007; Lot 0409285-2;
Estradiol trial - Mouse Anti-Rabbit IgG Coated Plates	Cayman Chemical Item Nr. 400004; Lot
Testosterone AChE tracer	Cayman Chemical Item Nr. 482700; Lot 0419236-2
Testosterone EIA Anti-serum	Cayman Chemical Item Nr. 482702; Lot 21584-141787
Estradiol AChE tracer	Cayman Chemical Item Nr. 482250; Lot 0415676-1
Estradiol EIA Anti-serum	Cayman Chemical Item Nr. 482252; Lot 0411895-2

Equipment	Description
Cooling centrifuge	Beckman, GPKR Centrifuge
Vacuum Centrifuge	Savant Automatic Environmental Speedvac System AES1010 with RC110B Radiant Cover
β - scintillation counter	Packard
MilliQ water system	Milli-Q Integral Water Purification System, Millipore
Shaker	Titramax 1000 (Heidolph)
Liquid dispenser	Thermo Scientific Multidrop 384 (Labsystems)
Spectrophotometer	UVMax microplate reader, Molecular Devices

Appendix III

Data

Fish	group	testo (ng/ml)	testo (before)	Estradiol (ng/ml)	Estradiol (before)	C [PGE2] (ng/g)	C [PGE3] (ng/g)	C [PGF2] (ng/g)	pge2/pge3
45	a	2.53	1.73	11.98	9.32	1.593996	2.311011	9.925519	0.689739531
48	a	1.95	2.14	4.97	17.34	2.178666	1.637748	8.811902	1.33028123
51	a	1.92	1.72	10.84	19.89	1.53539	1.265197	8.282608	1.213557927
52	a	1.52	2.38	19.88	9.81	1.658334	1.198967	3.591148	1.383135458
53	a	1.64	1.17	6.81	13.32	3.667835	2.772982	7.778964	1.322703971
54	a	5.23	2.53	19.33	7.51	1.628863	1.252312	4.505089	1.300684529
55	a	2.49	2.03	11.88	14.65	2.179745	2.688743	10.33436	0.810692952
61	a	2.14	0.98	5.47	12.19	2.614447	2.474671	12.59396	1.056482641
62	a	1.43	0.45	6.05	4.94	4.461053	1.85983	12.18524	2.398634587
63	a	2.3	1.96	5.36	36.88	0	1.983152	16.95996	0
68	a	2.05	1.92	20.72	34.14	4.81539	8.033781	7.747208	0.59939273
70	a	2.26	1.41	19.75	18.35	0	3.63339	26.14598	0
71	a	1.85	1.88	0.09	9.59	2.160716	2.174847	2.713883	0.99350261
43	b	1.12	2.11	7.02	10.97	2.301877	2.496839	8.252661	0.92191619
44	b	0.59	1.42	2.99	9.93	0.699697	2.599639	14.33161	0.269151438
46	b	0.85	1	4.51	14.85	1.973274	2.841522	7.315312	0.694442506
49	b	0.86	0.77	8.99	17.15	1.352463	3.411475	11.18022	0.396445374
50	b	2.25	2.51	28.64	13.43	1.519393	1.652891	7.989309	0.919233697
56	b	0.69	1.34	6.89	8.46	1.787819	1.986428	4.646454	0.900017046
57	b	0.88	1.12	4.54	9.17	2.151638	1.558653	19.02867	1.380446804
58	b	0.91	1.97	0.8	9.39	2.333349	1.852632	9.688719	1.259477975
59	b	2.24	1.68	29.88	15.39	2.380227	0.994543	5.064703	2.393287688
60	b	4.61	3.09	23.75	5.45	3.306542	4.091122	14.56804	0.808223681
64	b	1.34	2.09	5.09	23.41	0.456867	1.05773	10.8908	0.431931644
65	b	0.82	1.31	5.43	19.31	2.481796	4.094434	19.16068	0.606138903
66	b	2.57	2.03	19.87	7.23	6.18237	4.009036	7.524785	1.542108847

67	b	1.88	1.43	30.12	15.7	1.665425	0.950925	9.202751	1.751373671
69	b	1.73	1.96	2.66	4.94	1.483225	3.610641	10.68489	0.410792591
127	c	0.29		6.7		1.663276	1.406592	9.058139	1.182486462
105	c	0.9		0.42		0.883057	5.011894	17.99988	0.176192309
106	c	1.14		13.65		0.746812	0.439621	5.71452	1.698764031
107	c	1.64		9.54		1.108542	2.28179	6.550982	0.485821196
108	c	1.6		7.74		6.373678	7.381905	26.50318	0.863419239
109	c	0.68		3.97		1.496049	1.880288	6.845763	0.795649073
110	c	0.61		4.93		3.58518	3.450202	33.73802	1.039121656
111	c	1.32		5.9		1.414836	1.368823	7.804532	1.033615465
112	c	1.87		7.71		3.412401	6.298812	22.97816	0.541753123
113	c	1.8		14.62		16.00869	25.1922	68.99122	0.635462181
114	c	0.91		4.01		1.689862	6.641618	20.61901	0.254435321
115	c	2.28		14.11		0.782484	0.824829	6.726993	0.948662028
116	c	1		5.77		1.741483	1.187384	0.148088	1.466655204
117	c	1.36		13.44		1.07974	0.730991	5.939156	1.477090191
118	c	0.83		2.09		2.942143	1.986341	14.89509	1.481187607
119	c	2.58		17.35		0.665626	0.565869	4.133432	1.176290934
122	c	1.74		13.18		8.325983	8.779058	45.13993	0.948391366
123	c	1.51		14.01		1.493852	0.818579	9.348218	1.824932402
124	c	1.83		34.75		8.488426	18.0956	131.304	0.469087715
125	c	1.2		9.55		1.370509	0.962165	16.14276	1.424401335
128	c	1.15		7.72		0.873203	0.759735	8.632334	1.149350972
129	c	1.24		13.47		1.187319	4.213314	37.54758	0.281801622
133	c	2.05		3.14		2.467091	2.122829	12.34009	1.162170961
134	c	2.4		41.44		0.982197	0.843328	3.741014	1.164667308
136	c	2.23		18.62		1.290444	1.367723	7.121284	0.943497955
137	c	1.83		7.34		12.36424	13.26092	38.30184	0.932381585
140	c	1.65		15.1		2.676679	2.280897	13.2792	1.173520583
141	c	2.79		2.36		0.712413	0.527301	5.156679	1.351055536
142	c	0.31		1.17		29.53189	31.78782	151.1568	0.929031479
143	c	2.24		10.2		3.014905	2.292401	13.37234	1.315173475
147	c	2.51		13.61		18.30349	17.81023	54.99475	1.027695572
152	c	1.35		21.02		4.364493	7.529595	71.47226	0.579645123
153	c	2.62		7.35		8.413122	5.26473	20.73801	1.598015789
158	c	1.34		7.67		0.598308	0.642488	5.173615	0.931236745
159	c	0.94		8.34		3.756899	3.439642	18.85541	1.092235344
160	c	0.82		2.43		21.24268	44.66742	163.8329	0.475574499
161	c	0.49		4.17		2.664432	5.227591	24.38303	0.509686316
164	c	0.88		3.14		1.06035	1.69445	13.00385	0.625778073
166	c	1.02		9.07		0.865753	0.940942	6.213064	0.920091569
172	c	0.98		20.94		1.721577	1.594732	5.332092	1.07953984
177	c	1.11		7.01		3.366706	5.452398	23.60286	0.617472547
179	c	1.28		27.39		6.299419	11.78385	36.31774	0.534580563

Statistical Treatments

Levene's Tests for Homogeneity of Variances

Tables 1, 2, 3 and 4. Levene's test for homogeneity of variances. Red values indicate significant differences (p<0. 05).

	Levene's Test for Homogeneity of Variances (First_dilution_25.01 in Testoste Effect: group Degrees of freedom for all F's: 2, 67			
	MS Effect	MS Error	F	p
testo	0,37534	0,27950	1,34286	0,26803

Table 1. Levene's test for the testosterone concentration variable.

	Levene's Test for Homogeneity of Variances (Data_30.11.11_treinc Effect: Group Degrees of freedom for all F's: 2, 67			
	MS Effect	MS Error	F	p
Estradiol	69,1166	27,1630	2,54450	0,08607

Table2. Levene's test for the estradiol-17β concentration variable.

	Levene's Test for Homogeneity of Variances (Data_30.11.11_treinc Effect: Group Degrees of freedom for all F's: 2, 67			
	MS Effect	MS Error	F	p
C [PGE2] ng/ml	586,28	77,886	7,52739	0,00112
C [PGE3] ng/ml	1292,44	179,222	7,21140	0,00145
C [PGF2] ng/ml	24195,10	3129,13	7,73222	0,00095

Table 3. Levene's test for the prostaglandin concentrations variables.

	Levene's Test for Homogeneity of Variances (Data_30.11.11_treinc Effect: Group Degrees of freedom for all F's: 2, 67			
	MS Effect	MS Error	F	p
pge2/pge3	0,15549	0,09191	1,69185	0,19195

Table 4. Levene's test for the PGE_2/PGE_3 ratio variable.

Statistical Tests

Tables 5, 6 and 7. Mann-Whitney test for differences between the three groups' testosterone mean concentrations, after treatment (**a** is the testosterone group, **b** is the sham group and **c** is the control group). Red values indicate significant differences (p<0. 05).

	Mann-Whitney U Test (Testosterone data_2versions) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum b	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N b	2*1sided exact p
testo	239,000	167,000	47,0000	2,30326	0,02126	2,30326	0,02126	13	15	0,01948

Table 5. Differences between the testosterone group and the sham group.

	Mann-Whitney U Test (Testosterone data_2versions) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N c	2*1sided exact p
testo	524,000	1016,00	113,000	3,15981	0,00157	3,15998	0,00157	13	42	0,00107

Table 6. Differences between the testosterone group and the control group.

	Mann-Whitney U Test (Testosterone data_2versions) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum b	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N b	Valid N c	2*1sid exact
testo	414,500	1238,50	294,500	-0,36244	0,71702	-0,36247	0,71699	15	42	0,7131

Table 7. Differences between the sham group and the control group.

Tables 8 and 9. Wilcoxon Matched Pairs Test for differences in testosterone mean concentrations between the first and the last sampling days (**a** is the testosterone group, **b** is the sham). Red values indicate significant differences (p<0. 05).

	group=a Wilcoxon Matched Pairs Test (Testosterone data_2version Marked tests are significant at p <,05000			
Pair of Variable	Valid N	T	Z	p-value
testo & testo*	13	14,0000	2,20139	0,02770

Table 8. Testosterone group.

	group=b Wilcoxon Matched Pairs Test (Testosterone data_2versi Marked tests are significant at p <,05000			
Pair of Variable	Valid N	T	Z	p-value
testo & testo*	15	39,0000	1,19272	0,23298

Table 9. Sham group.

Tables 10, 11 and 12. Mann-Whitney test for differences between the three groups' estradiol-17β mean concentrations, after treatment (**a** is the testosterone group, **b** is the sham group and **c** is the control group).

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable Group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum b	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N b	2*1sided exact p
Estradiol	195,000(	211,000(	91,0000(	0,27639:	0,78224'	0,27639:	0,78224'	13	15	0,78564!

Table 10. Differences between the testosterone group and the sham group.

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable Group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N c	2*1sided exact p
Estradiol	377,000(	1163,00(	260,000(	0,24763·	0,80441:	0,24763:	0,80441·	13	42	0,80676!

Table 11. Differences between the testosterone group and the control group.

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable Group Marked tests are significant at p <,05000									
variable	Rank Sum b	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N b	Valid N c	2*1sided exact p
Estradiol	420,000(	1233,00(	300,000(	-0,26276!	0,79272!	-0,26277:	0,79272!	15	42	0,79491·

Table 12. Differences between the sham group and the control group.

Tables 13 and 14. Wilcoxon Matched Pairs Test for differences in mean estradiol-17β concentration between the first and the last sampling days (**a** is the testosterone group, **b** is the sham).

	group=a Wilcoxon Matched Pairs Test (ELISA analysis) Marked tests are significant at p <,05000			
Pair of Variables	Valid N	T	Z	p-value
after_estradiol & before_estradiol	13	25,0000(	1,43265(	0,15195'

Table 13. Testosterone group.

	group=b Wilcoxon Matched Pairs Test (ELISA analysis) Marked tests are significant at p <,05000			
Pair of Variables	Valid N	T	Z	p-value
after_estradiol & before_estradiol	15	59,0000(	0,05679(	0,95470!

Table 14. Sham group.

Tables 15, 16 and 17. Mann-Whitney test for differences between the three groups' prostaglandin mean concentrations, after treatment (**a** is the testosterone group, **b** is the sham group and **c** is the control group).

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum b	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N b	2*1side exact p
C [PGE2] (ng/g)	193,000	213,000	93,0000	0,18426	0,85380	0,18428	0,85378	13	15	0,8561
C [PGE3] (ng/g)	182,000	224,000	91,0000	-0,2763	0,78224	-0,2763	0,78224	13	15	0,7856
C [PGF2] (ng/g)	177,000	229,000	86,0000	-0,5067	0,61235	-0,5067	0,61235	13	15	0,6177

Table 15. Differences between the testosterone group and the sham group.

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N c	2*1sided exact p
C [PGE2] (ng/g)	346,000	1194,00	255,000	-0,3466	0,72882	-0,3466	0,72882	13	42	0,73195
C [PGE3] (ng/g)	349,000	1191,00	258,000	-0,2872	0,77391	-0,2872	0,77391	13	42	0,77659
C [PGF2] (ng/g)	273,000	1267,00	182,000	-1,7928	0,07299	-1,7928	0,07299	13	42	0,07272

Table 16. Differences between the testosterone group and the control group.

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum b	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N b	Valid N c	2*1sided exact p
C [PGE2] (ng/g)	403,000	1250,00	283,000	-0,5708	0,56810	-0,5708	0,56810	15	42	0,57193
C [PGE3] (ng/g)	411,000	1242,00	291,000	-0,4258	0,67020	-0,4258	0,67020	15	42	0,67343
C [PGF2] (ng/g)	356,000	1297,00	236,000	-1,4225	0,15486	-1,4225	0,15486	15	42	0,15639

Table 17. Differences between the sham group and the control group.

Tables 18, 19 and 20. Wilcoxon Matched Pairs Test for differences between mean prostaglandin concentrations within the same group (**a** is the testosterone group, **b** is the sham group and **c** is the control group).

Pair of Variables	group=a Wilcoxon Matched Pairs Test (Data_30.11.11_trein Marked tests are significant at p <,05000 Valid N	T	Z	p-value
C [PGE2] (ng/g) & C [PGE2] (ng/g				
C [PGE2] (ng/g) & C [PGE3] (ng/g	13	41,0000	0,31448	0,75315
C [PGE3] (ng/g) & C [PGE2] (ng/g	13	41,0000	0,31448	0,75315
C [PGE3] (ng/g) & C [PGE3] (ng/g				
C [PGF2] (ng/g) & C [PGE2] (ng/g	13	0,0000	3,17979	0,00147
C [PGF2] (ng/g) & C [PGE3] (ng/g	13	1,0000	3,10991	0,00187

Table 18. Testosterone group.

Pair of Variables	group=b Wilcoxon Matched Pairs Test (Data_30.11.11_trein Marked tests are significant at p <,05000 Valid N	T	Z	p-value
C [PGE2] (ng/g) & C [PGE2] (ng/g				
C [PGE2] (ng/g) & C [PGE3] (ng/g	15	41,0000	1,07912	0,28053
C [PGE3] (ng/g) & C [PGE2] (ng/g	15	41,0000	1,07912	0,28053
C [PGE3] (ng/g) & C [PGE3] (ng/g				
C [PGF2] (ng/g) & C [PGE2] (ng/g	15	0,0000	3,40777	0,00065
C [PGF2] (ng/g) & C [PGE3] (ng/g	15	0,0000	3,40777	0,00065

Table 19. Sham group.

Pair of Variables	group=c Wilcoxon Matched Pairs Test (Data_30.11.11_trein Marked tests are significant at p <,05000 Valid N	T	Z	p-value
C [PGE2] (ng/g) & C [PGE2] (ng/g				
C [PGE2] (ng/g) & C [PGE3] (ng/g	42	315,000	1,70675	0,08786
C [PGE3] (ng/g) & C [PGE2] (ng/g	42	315,000	1,70675	0,08786
C [PGE3] (ng/g) & C [PGE3] (ng/g				
C [PGF2] (ng/g) & C [PGE2] (ng/g	42	1,000	5,63290	0,00000
C [PGF2] (ng/g) & C [PGE3] (ng/g	42	1,000	5,63290	0,00000

Table 20. Control group.

Tables 21, 22 and 23. Mann-Whitney test for differences between the three groups' PGE_2/PGE_3 mean ratios, after treatment (**a** is the testosterone group, **b** is the sham group and **c** is the control group).

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable group Marked tests are significant at p <,05000							
variable	Rank Sum a	Rank Sum b	U	Z	p-value	Z adjusted	p-value	Valid N a
pge2/pge3	197,000	209,000	89,0000	0,36852	0,71248	0,36857	0,71244	13

Table 21. Differences between the testosterone group and the sham group.

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N c	2*1sided exact p
pge2/pge3	386,000	1154,00	251,000	0,42593	0,67015	0,42593	0,67015	13	42	0,67381

Table 22. Differences between the testosterone group and the control group.

	Mann-Whitney U Test (Data_30.11.11_treinos2) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum b	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N b	Valid N c	2*1sided exact p
pge2/pge3	404,000	1249,00	284,000	-0,55272	0,58045	-0,55272	0,58045	15	42	0,58422

Table 23. Differences between the sham group and the control group.

Tables 24, 25 and 26. Mann-Whitney test for differences between the three groups mortalities after treatment (**a** is the testosterone group, **b** is the sham group and **c** is the control group).

	Mann-Whitney U Test (Basic data_Rita) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum b	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N b	2*1sided exact p
Tank Mortality (%)	221,500	184,500	64,5000	1,49712	0,13436	1,53014	0,12598	13	15	0,12998

Table 24. Differences between the testosterone group and the sham

	Mann-Whitney U Test (Basic data_Rita) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum a	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N a	Valid N c	2*1sided exact p
Tank Mortality (%)	565,000	975,000	72,0000	3,97205	0,00007	4,37107	0,00001	13	42	0,00002

Table 25. Differences between the testosterone group and the control

	Mann-Whitney U Test (Basic data_Rita) By variable group Marked tests are significant at p <,05000									
variable	Rank Sum b	Rank Sum c	U	Z	p-value	Z adjusted	p-value	Valid N b	Valid N c	2*1sided exact p
Tank Mortality (%)	456,000	1197,00	294,000	0,37150	0,71026	0,40474	0,68566	15	42	0,71318

Table 26. Differences between the sham group and the control group.