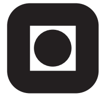

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# Effects of melatonin and cortisol on vitellogenin and estrogen receptor α mRNA expression in 17β-estradiol stimulated Atlantic salmon (*Salmo salar*) hepatocytes

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*Trondheim, May 2013*

Guri Eriksrød Skauen

# Abstract

Knowledge regarding the influence of external and internal factors on teleost fecundity may contribute to increased production and efficiency in aquaculture.

Teleost plasma cortisol levels may be elevated in response to stress, potentially affecting reproduction through a negative impact on vitellogenesis. Melatonin (MEL), on the other hand, is shown to have protective effects in organisms, and large amounts of MEL has been observed in the gastrointestinal tract (GIT) in various species, hence finding its way to the liver.

The subject of this thesis was to document the MEL concentration in Atlantic salmon (*Salmo salar*) vena portae, and compare this to MEL in the circulating blood. Further, the effect of MEL and cortisol on 17$\beta$-estradiol (E2) stimulated vitellogenin (Vtg) mRNA expression in hepatocytes from Atlantic salmon, was investigated *in vitro*.

The presence of MEL in vena portae was documented, seemingly independent of light conditions and pineal MEL levels.

A decrease in Vtg mRNA expression was observed in hepatocytes exposed to cortisol after preincubation with E2 and MEL. This effect was not seen in hepatocytes exposed to cortisol after preincubation with E2 alone.

# Sammendrag

Kunnskap om effekter av eksterne og interne faktorer på reproduksjonsevnen hos teleoster kan bidra til økt produksjon og effektivitet innen oppdrettsnæringen.

Stress kan medføre forhøyede kortisolnivåer i plasma hos teleoster, og dette kan potensielt påvirke reproduksjon gjennom effekter på vitellogenesen. Melatonin (MEL) er derimot vist å ha beskyttende effekter i organsimer, og det er observert store mengder MEL i mage-tarmkanalen i ulike arter, som dermed finner veien til leveren.

Temaet for denne avhandlingen var å dokumentere MEL-konsentrasjonen i vena portae hos atlantisk laks (*Salmo salar*), og sammenlikne denne konsentrasjonen med MEL i det sirkulerende blodsystemet. Videre ble effekten av MEL og kortisol på ekspresjon av vitellogenin mRNA stimulert med 17$\beta$-estradiol (E2) undersøkt *in vitro*.

Tilstedeværelse av MEL i vena portae ble dokumentert, tilsynelatende uavhengig av lysforhold og pineale MEL-nivåer.

Det ble observert en reduksjon i uttrykk av Vtg mRNA i hepatocytter eksponert for kortisol etter preinkubering med E2 og MEL. Denne effekten kunne ikke sees i hepatocytter utsatt for kortisol etter preinkubering med E2 alene.

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# Abbreviations

ACTH adrenocorticotropic hormone

ANOVA analysis of variance

CA catecholamine

CatD cathepsin D

cDNA complimentary DNA

CP crossing point

CPM counts per minute

CYP cytochrome P450

dsDNA double-stranded DNA

E2 17$\beta$-estradiol

ER estrogen receptor

ER$\alpha$ estrogen receptor $\alpha$

ERE estrogen responsive element

EROD ethoxyresorufin-O-deethylase

ETC electron transport chain

FSH follicle stimulating hormone

GABA gamma-aminobutyric acid

GCR glucocorticosteroid receptor

GH growth hormone

GIT gastrointestinal tract

GnIH gonadotropin-inhibitory hormone

GnRH gonadotropin releasing hormone

GnRHR gonadotropin releasing hormone receptor

GtH gonadotropins

HPA hypothalamic-pituitary-adrenal

HPI hypothalamic-pituitary-interrenal

Hsp heat shock protein

Kiss1 kisspeptin

LH luteinizing hormone

LLTP large lipid transfer protein

MEL melatonin

mRNA messenger RNA

mtDNA mitochondrial DNA

NLT nucleus lateral tuberalis

NPO nucleus preopticus

NPY neuropeptide Y

NTNU Norwegian University of Science and Technology

PCR polymerase chain reaction

| | |
|---|---|
| PPD | proximal pars distalis |
| PRL | prolactin |
| qPCR | quantitative real-time polymerase chain reaction |
| RIA | radioimmunoassay |
| SCN | suprachiasmatic nuclei |
| SEM | standard error of mean |
| ssDNA | single-stranded DNA |
| Trp | tryptophan |
| TSH | thyrotropin |
| Vtg | vitellogenin |

# 1. Introduction

The subject of this thesis is the effects of melatonin (MEL) on vitellogenesis in cortisol exposed hepatocytes from Atlantic salmon (*Salmo salar*), investigated by measuring the gene expression of vitellogenin (Vtg) and estrogen receptor $\alpha$ (ER$\alpha$). Cortisol has been demonstrated to have a negative effect on reproduction. MEL, on the other hand, is shown to have a variety of protective effects in organisms. Large amounts of MEL has been observed in the gastrointestinal tract (GIT), hence finding its way to the liver. This has raised the question of the possible role MEL may possess in this area. As a sidestep to the topic, the MEL level in vena portae of Atlantic salmon has been examined and compared to the concentration in the circulating blood. In the following presentation, the theory behind the choice of study is reviewed, leading to the objectives of this thesis.

## 1.1 Background and Motivation

In 2011, Atlantic salmon accounted for 93 percent of the fish farmed in Norway. Totally one million ton was produced, with a primary value of 4.7 billion US dollars (Statistisk Sentralbyrå [2012]). This fast growing industry is constantly looking for ways to improve value along the production chain. Thus, the handling of maturing females has been focused in order to eliminate stress-related negative impacts on their egg quality, which in turn could affect the development and growth of fries. To obtain a high production volume of salmon, a high fry survival is necessary. This is largely dependent on the nutritional condition for the fries, which receive their building materials and energy from the egg. Consequently,

factors that may affect the egg quality are constantly reviewed (Campbell et al. [1994]).

The use of hatcheries, where eggs from salmon are fertilized, hatched, raised to smolts, and then released to the ocean, is increasing (Eagle et al. [2004]). Hatcheries are used for many purposes, including restoring or rehabilitation of stocks, supplementation for wild stock production, supporting fisheries, new habitats colonization and for research (Ritter [1997]). The majority of the hatcheries, however, are seeking to increase the fish production for commercial use (Araki and Schmid [2010]). To ensure a high production, egg quality is of high relevance. According to Bobe and Labbé [2010], this quality may be defined as the capability of the egg to be fertilized and to develop into a normal embryo. Many factors can affect the gamete quality, including nutrition, salinity, photoperiod, temperature, stress and pollutants (Bobe and Labbé [2010]).

### 1.1.1 Atlantic salmon

Atlantic salmon is an anadromous fish and has two life phases: the first period of its life it lives in the river, before undergoing physiological changes and traveling out to the ocean as so called smolt. In the ocean, it feed and grows, and when it is mature it returns to the river where it was born to spawn. The spawning occurs in freshwater in the late autumn. The eggs are covered with stones and gravels, and hatch in spring as fries. For the first period of their lives, the fries get nutrition from a yolk sac (Klemetsen et al. [2003], Aas et al. [2011]).

### 1.1.2 Teleost reproduction

Atlantic salmon is part of the teleost fish family. Teleost fish are generally oviparous that release eggs to the extraneous water with ensuing fertilization, and the developing embryo is receiving optimal nutrients from the egg yolk. The reproduction of teleost fish occurs in cycles involving development of gametes, maturation and spawning, that are managed by interactions between complex endocrine systems modulated by external and internal pathways (Coward et al. [2002]). This overall endocrine control of regulation involves several anatomical structures, including the hypothalamus, pituitary gland, gonad and liver, shown in Figure 1.1.

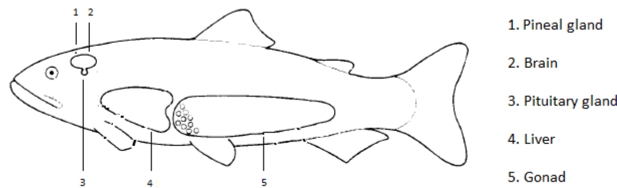



Figure 1.1: Anatomical structures involved in control of reproduction in teleost (modified from Sumpter and Jobling [1995]).

An overview of the regulation of reproduction is shown in Figure 1.2, showing the most important structures, signaling molecules and products. A series of linked endogenous and exogenous signals synchronize the development of the ovarian and the release of the oocyte when it is matured. The brain is receiving information regarding external factors such as photoperiod, temperature, water quality and nutrient supply as well as a variety of social factors. This input is translated into neural impulses affecting the endocrine pathways of the hypothalamus-pituitary-gonadal (HPG) axis. Hypothalamic gonadotropin releasing hormone (GnRH), pituitary gonadotropins (GtHs), sex steroids, progestogens and prostaglandins are important hormones participating in the response. Several growth and paracrine factors are also known to be involved in this respect (Coward et al. [2002]).

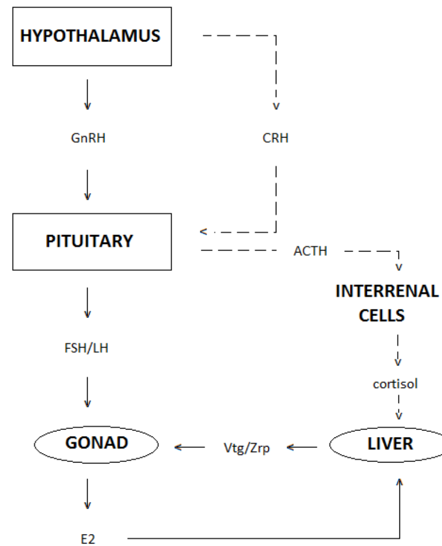



Figure 1.2: Overview of the most important components involved in teleost reproduction.

## Hypothalamus

The hypothalamus plays an important role in the reproduction of teleost. It releases GnRH, a decapeptide regulating the production and secretion of GtH from the pituitary, although it also has the ability to act as a neuromodulator (Amano et al. [1997]). According to Lethimonier and coworkers (2004), eight GnRH variants are found in teleost fish, and it seems like a single species express two or three different GnRH forms. GnRH has a primarily control of the reproductive function, acting on the pituitary (also called hypophysis), starting a cascade resulting in the initiation of sexual maturation (Gopurappilly et al. [2012], Foran and Bass [1999]).

## Pituitary

The released GnRH binds to GnRH receptors (GnRHR) expressed in gonadotroph cell membranes in the pituitary, resulting in synthesis and re-

lease of GtHs (Gopurappilly et al. [2012]). According to Zohar and coworkers (2010), multiple other brain factors influence the function of the pituitary and thus the secretion of GtHs, including dopamine, neuropeptid Y (NPY), gamma-aminobutyric acid (GABA), gonadotropin-inhibitory hormone (GnIH), kisspeptin (Kiss1) and steroid feedback (Zohar et al. [2010], Gopurappilly et al. [2012]).

The main difference between tetrapods and fish in this context, is how the hormones that control the pituitary activity find their way to their target cells. In fish, the short stalk connecting the hypothalamus and the pituitary is comprised of neurosecretory fibers, axons, extending from the hypothalamic neurons to the pituitary. Two constituents, named the adenohypophysis and neurohypophysis, are forming the pituitary gland. The adenohypophysis has different cells secreting pituitary hormones, while the neurohypophysis contains peptide secreting fibers nearby the pituitary cells. The secretion of vasopressin and oxytocin is conducted by the neurohypophysis. The adenohypophysis in fish is subdivided into pars distalis and pars intermedia, where the pars distalis is organized into rostral and proximal pars distalis. The rostral pars distalis generally have corticotrophs (adrenocorticotropic hormone (ACTH) cells) and prolactin (PRL) cells, and some have thyrotrophs (TSH cells). In the proximal pars distalis (PPD), gonadotrophs, growth hormone (GH) cells and TSH cells are present. Plasticity and proliferation may occur, though, at particular stages (Zohar et al. [2010]).

The regulation of gametogenesis in teleost is largely dependent on the pituitary-gonadal axis, where the GtHs are the key hormones. Follicle stimulating hormone (FSH) are in control of the gonadal growth, while luteinizing hormone (LH) is controlling the completing maturation and ovulation (Tyler et al. [1997]). Mediation of the steps of gametogenesis is carried out by steroid hormones, synthesized as a consequence of GtH action, implying an indirectly impact of GtH. Both FSH and LH are steroidogenic and is comprised of $\alpha$ and $\beta$ subunits. During vitellogenesis and spermatogenesis, the plasma concentration of FSH is elevated, while the LH level is low. At the time of spawning there is a decrease in the FSH level and an incline in the plasma concentration of LH (Nagahama [1994]).

### Gonads

In the ovary of teleost, several components are present, with oogonia, oocytes and surrounding follicle cells, in addition to nerve and vascular tissue, as well as supporting tissue. Oocyte growth requires a considerable increase in the follicle cells, which creates a follicle layer, called the granulosa cell layer. The supporting tissue will form the thecal layer, the outer layer of the follicular envelope. This provides a follicle, consisting of one outer thecal cell layer and an inner granulosa layer, separated by a membrane (Nagahama [1994]), shown in Figure 1.3.

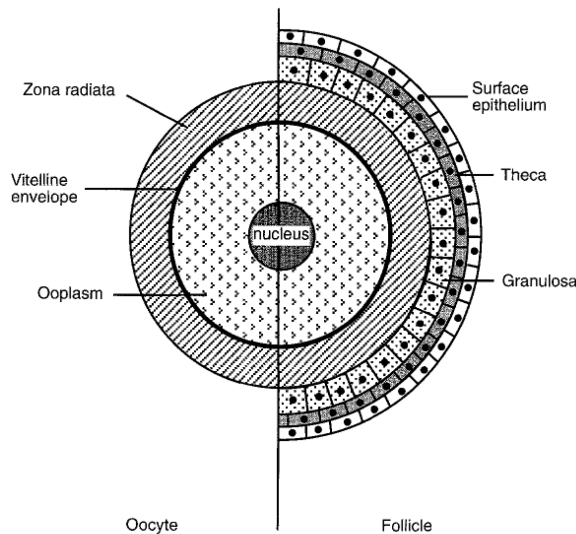



Figure 1.3: The structure of the follicle and the oocyte, and the difference between the two (from Tyler and Sumpter [1996]).

Development of the ovarian is separated into stages based on a variety of criteria. According to Coward and coworkers (2012), the main phases of development are subdivided into oogonial proliferation, oogenesis, primary growth and folliculogenesis, cortical alveolus stage, vitellogenesis, maturation and ovulation. Wallace and Selman [1981] explains four main stages in the teleost oocyte growth. The primary growth are independent of GtH, and in this period a Balbiani body and a number of nucleoli are

created, and then distributed in the ooplasm. The next step depends on the GtH, where the cortical alveoli procursors yolk vesicles are formed. Then, Vtg is obtained from the blood and placed into yolk granules, that at some point will fuse to provide a fluid phase within a layer of cytoplasm. The oocyte growth is accomplished with maturation, the final step (Wallace and Selman [1981]). The end product of the oocyte growth and differentiation is the egg (Lubzens et al. [2010]).

GtH-induced estrogen produced by the ovaries is released to the vascular system, stimulating the synthesis of Vtg in the liver (Nagahama [1994]).

### Liver

Vtg, a glycolipophospho-protein, is derived from the liver, where the vitellogenesis takes place (Coward et al. [2002]). The primary functional cells in the liver are the hepatocytes, which has a variety of features involving secretory, metabolic and endocrine capabilities. These cells form three-dimensional structures with the thickness of one cell, called hepatic laminae (Tortora and Derrickson [2008]). According to Suriawinata and Thung [2011], hepatocytes are polygonal epithelial cells having one or more round nuclei situated. The hepatocytes are arranged in order to provide each cell with direct blood contact. Blood derives from two sources, from the aorta via the hepatic artery providing oxygen and transporting metabolites to the liver, and from the digestive tract by the hepatic portal system (Sherwood and Cengage Learning (Firm) [2010]). According to Ostrander [2000] the fish liver shares several characteristics with the liver of other animals. It is located in the anterior part of the peritoneal cavity, filtering blood through a network of small blood vessels called sinusoids, created by hepatocytes.

The Vtgs belong to the superfamily of the large lipid transfer protein (LLTP), and there are, according to Lubzens and coworkers (2010), a minimum of three Vtgs exist in teleost, named VtgA, VtgB and VtgC. The uptake is executed through receptor-mediated endocytosis by a specific receptor attached to the oocyte membrane (Coward et al. [2002]). The synthesis of Vtg occurs in the liver under influence of 17$\beta$-estradiol (E2) (Tata [1979], Nagahama [1994]), as shown in Figure 1.4. Vtg is then transported through the bloodstream, ending up in the ovary, where it serves

as yolk protein precursor (Tata [1979]). The Vtg is cleaved by the cathepsin D (CatD), forming phosvitin and lipovitellin (Finn and Kristoffersen [2007], Tata [1979]). Phosvitin has a high percent of the amino acid serine, in comparison to other amino acids. It also contains a comparatively high amount of sugar and a single methionine. The lipovitellin contains a higher amount of lipid, including cholesterol, and has a rather large quantity of methionine and a significant lower proportion of the amino acids are serine (Tata [1979]).

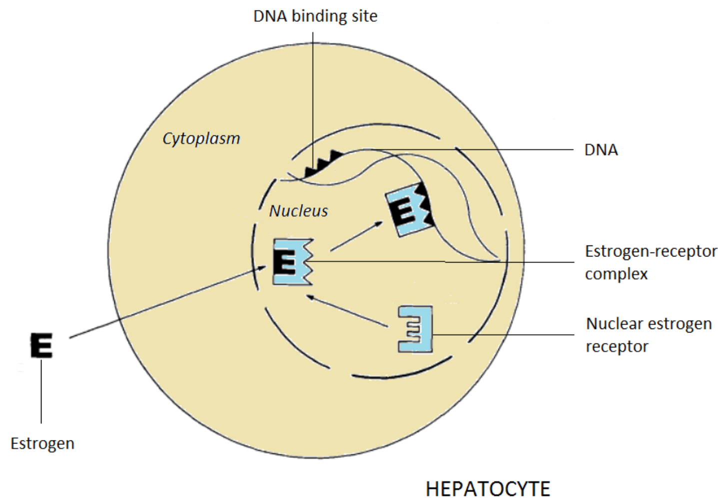



Figure 1.4: Estrogen influence on vitellogenin (Vtg) synthesis (simplified). Estrogen enters the hepatocyte and binds to nuclear estrogen receptor. The estrogen-receptor complex binds to specific DNA sites, activating the expression of the Vtg gene (modified from Hoar and Randall [1988]).

The *in vitro* uptake and incorporation of Vtg in ovarian follicles has been found to be stimulated by FSH, and thyroxin and insulin has been shown to increase this uptake in rainbow trout. A receptor-mediated adenylate cyclase-cAMP system is involved in the stimulation of the thecal cell layer to produce testosterone, by the action of GtH and involvement of several intracellular signaling molecules. The testosterone diffuses into the granulosa cell layer, where the synthesis of E2 from testosterone occurs

(Nagahama [1994]). The arrival of Vtg to the oocyte surface is achieved by crossing from the capillaries of the theca layers to the granulosa layer, via pore canals of zona radiata (Lubzens et al. [2010]).

### 1.1.3 Stress response

The stress system receives and integrates a diversity of signals coming from various pathways, activating the system and triggering responses that are normally adaptive and limited in duration (Charmandari et al. [2005]). In teleost fish, the stress response involves the release of cortisol from interrenal cells and catecholamines (CA) from chromaffin cells. The control of cortisol secretion in fish is due to the hypothalamic-pituitary-interrenal (HPI) axis, with the anterior pituitary gland releasing ACTH, the primary stimulator (Mommsen et al. [1999]). Stimulation of ACTH release is exerted by neuropeptides released from neurosecretory neurons in the hypothalamic nucleus preopticus (NPO) and nucleus lateral tuberalis (NLT) (Bernier and Peter [2001]). Additionally, to increase the transport of oxygen and to obtain energy, the hypothalamic-sympathetic-chromaffin cell axis is activated, resulting the release of the CAs epinephrine and norepinephrin, which in turn impacts the heart and circulating system (Bonga [1997]).

Subjected to stress, the cortisol plasma concentration in teleosts may increases substantially. Cortisol is a glucocorticoid steroid capable of acting both in a genomic and a non-genomic way, providing the possibility of a slow or rapid response, respectively. Their actions rely on glucocorticosteroid receptors (GCRs) for binding to plasma proteins, allowing them to travel, before passing through cell membranes and function as transcription factors (Mommsen et al. [1999]). This steroid receptor signaling pathway, consisting of a ligand and its receptor, along with co-activators and co-repressors, is a complex system controlling a variety of processes on a cellular and physiological level (Bury and Sturm [2007]).

#### Reproduction in relation to stress

Reproduction is critical to survival of the species, and may be especially sensitive to stress exposure. This sensitivity improves the chances of reproducing in an environment that is as safe and suitable as possible. Animals

exposed to stressful handling may have a reduced or absent reproductive capacity (Lovejoy and Barsyte [2011]). In aquaculture, effects of stress has received much attention, and it has to a large extent been focused on research concerning factors involved in fry survival (Campbell et al. [1994]).

Results by Campbell and coworkers (1994) showed a significant reduction in the plasma Vtg levels in female rainbow trout subjected to confinement stress for a period of two weeks, compared to the controls. The plasma cortisol levels in the stress exposed groups were also significantly elevated. According to the study of Carragher and coworkers (1989), a prolonged increase in plasma cortisol levels may reduce the concentration of plasma vitellogenin in female brown trout. The correlation between cortisol treatment and the expression of Vtg mRNA in hepatocytes from Atlantic salmon was investigated in Tran [2010], and according to the work presented, a decrease in the amount of Vtg mRNA in cells treated with 100 nM cortisol or higher was observed, indicating that moderate cortisol concentrations may at least temporary, have a negative effect on Vtg production (Tran [2010]).

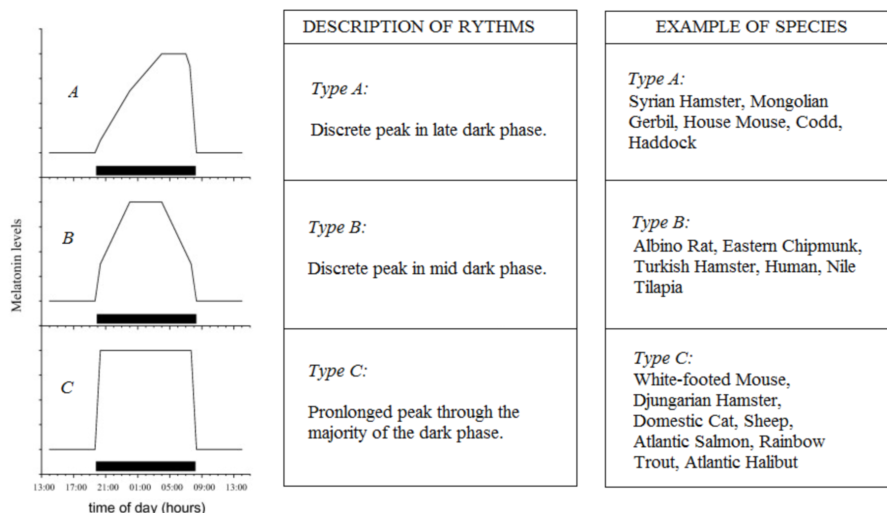
Other studies have been conducted on hepatocytes from Atlantic salmon, with respect to the vitellogenesis. In the work of Kvalsvik [2010], hepatocytes from Atlantic salmon was exposed to melatonin (MEL), and the effect on Vtg mRNA expression was examined. The study showed no significant effect of MEL regarding these aspects in estrogen induced Vtg production in primary hepatocyte cultures from Atlantic salmon. This indicated a negligible or absent effect of MEL in terms of the production of Vtg in hepatocytes.

### 1.1.4 Pineal melatonin

MEL (*N*-acetyl-*5*-methoxytryptamine) is secreted by the pineal gland in accordance with both daily and seasonal rhythms, normally synthesized at a maximum during the dark phase of the day. This is controlled by the circadian oscillator situated in the suprachiasmatic nuclei of the hypothalamus (SCN) (Simonneaux and Ribelayga [2003]). The main function is to communicate the daily light/dark cycle to the body, thus implying the night length and seasonal changes (Cardinali and Pévet [1998]).

According to Falcón and coworkers (2010), the correlation between

light and MEL production is a dose-dependent process. The light response differs between species, as shown in Figure 1.5, and while some react in an on/off manner, like the Atlantic salmon, the rainbow trout and the sheep, others have a more prolonged reaction, as the cod and the house mouse, or a more smooth reaction, which is the case for humans.




| DESCRIPTION OF RYTHMS | EXAMPLE OF SPECIES |
|---|---|
| *Type A:* Discrete peak in late dark phase. | *Type A:* Syrian Hamster, Mongolian Gerbil, House Mouse, Codd, Haddock |
| *Type B:* Discrete peak in mid dark phase. | *Type B:* Albino Rat, Eastern Chipmunk, Turkish Hamster, Human, Nile Tilapia |
| *Type C:* Pronlonged peak through the majority of the dark phase. | *Type C:* White-footed Mouse, Djungarian Hamster, Domestic Cat, Sheep, Atlantic Salmon, Rainbow Trout, Atlantic Halibut |

Figure 1.5: Melatonin profiles in different species of vertebrates. Three types are recognized. Atlantic salmon belongs to type C, having a prolonged peak through the majority of the dark phase (modified from Falcón et al. [2010]).

Other external factors may have an influence on the synthesis of MEL. Temperature has been found to affect the pineal organ and regulate the release of MEL. Internal factors, such as neurotransmittors and hormones, plays a role in the modulation of MEL formation, but knowledge in this area is currently limited. It is believed that high concentration of MEL has an inhibiting action on its own synthesis. Norepinephrine, adenosine, GABA, sexual steroids and glucocorticoids are also thought to play a role in regulation and feed-back mechanisms. Norepinephrine is an important mediator in this regard in mammals, participating in the light signal trans-

mission from the eye to the pineal gland, but this seems to take place in only some of the fish species (Falcón et al. [2010]).

According to Cardinali and Pévet [1998] it is documented that MEL acts through specific membrane receptor sites, and its lipophilic characteristic may allow interactions with intracellular molecules and receptors in the nucleus. MEL is now known to be generated in other organs, were it appears to have local roles, including tissue factor and antioxidant actions (Reiter et al. [2007]).

### Protective effects of melatonin

Free radicals have the ability to make considerable damage to macromolecules in the cell, and a various different devastating actions such as lipid peroxidation and DNA damage may occur due to these highly reactive oxygen agents (Reiter [1993], Susa et al. [1997]). The antioxidant properties of MEL has been known for more than a decade, and in this concern a large number of studies has been conducted (Reiter et al. [2007]). The exceptionally high diffusibility of MEL enables it to enter any cell and cell compartments, and is thereby important for these effects (Reiter [1993]). An electron-rich aromatic indole ring in the MEL structure is responsible for the electron donor property of MEL, and enables the hormone to reduce and repair electrophilic radicals (Reiter et al. [2007]).

According to Pieri and coworkers (1994), the activity of MEL as a peroxyl radical scavenger was twice as high as that of vitamin E. In their study, Wang and coworkers (2004) concluded that MEL, due to functioning as a free radical scavenger, could have a significant protective effect on liver damage. In an other study on hepatocytes, Padilllo and coworkers (2004) found that MEL administration reduced all of the considered parameters related to oxidative stress and cell death of the hepatocytes. According to Susa and coworkers (1997), MEL treatment of primary hepatocyte cultures from rat caused a decline in lipid peroxidation, DNA breaks and cytotoxicity. Additionally, MEL has been shown to reduce mitochondrial DNA (mtDNA) damage, improve electron transport chain (ETC) activity, lower the damage of proteins in mitochondria and preventing apoptosis of normal cells (León et al. [2005]). Other actions of MEL has also been reported. Poon and coworkers (2004) studied MEL receptors in isolated mouse hepatocytes and their results indicated that

MEL may be acting on the liver directly, increasing the plasma glucose concentration.

### 1.1.5 Melatonin in the gastrointestinal tract

According to Bubenik [2002], the GIT is a rich source of extrapineal MEL in vertebrates, and the MEL levels in these tissues may be more than ten to a hundred times higher than in the peripheral blood, and the amount of MEL in the liver may exceed that of the pineal gland with 400 times.

There are a number of theories and suggestions explaining the potential reasons for MEL in the GIT (Messner et al. [2001]). In their study on MEL distribution, Messner and coworkers (2001) determined the presence of MEL in human gut, bile, liver and portal blood, and found that the concentrations in the liver were higher than in the peripheral venous blood, as well as it was detected a slightly higher MEL concentration in the portal blood compared to the peripheral. They conclude that their findings, along with reports implying modulatory effects on functions in the gut and liver, indicates that MEL may act as a mediator in intestinal-hepatic communication.

### 1.1.6 Expression of genes

Gene expression refers to the decoding of the genetic information located in DNA into proteins. This process begins with the transcription of the target gene into a complementary RNA molecule, called messenger RNA (mRNA). The mRNA carries the information out of the nucleus and to the ribosomes for translation, eventually resulting in protein synthesis. Transcription in eukaryotes takes place in the nucleus, and depends on the direction of RNA polymerase. For the RNA polymerase to gain access to the DNA, the chromatin needs to be uncoiled, and control and initiation of the transcription is conducted through promoters and enhancers, along with transcription factors (Klug et al. [2009]).

#### Gene Expression of the vitellogenin gene

Recently, several Vtg genes has been identified, and the more evolved species within teleosts are probably expressing three distinct Vtg proteins,

named VtgAa, VtgAb and VtgC (Hiramatsu et al. [2013]). The Vtgs in teleosts are encoded by a multigene family, believed to have been created by gene duplications (Wang et al. [2010], Wahli [1988]). Expression of the Vtg gene is significantly up-regulated by estrogen (Flouriot et al. [1996]). The action of estrogen occurs via membrane-associated receptors or the soluble intracellular estrogen receptors (ERs) (Fu et al. [2006], Kloas et al. [2000]). It is assumed that estrogen diffuses into the nucleus of cells, where it binds to the ER, forming a transcription factor, which activates target genes containing estrogen response elements (EREs), leading to the Vtg gene activation (Fu et al. [2006]), Wang et al. [2010]. In the study of Hawkins and coworkers (2000), three ERs are described, termed ER$\alpha$, ER$\beta$ and ER$\gamma$. The complex of ER and its ligand is capable of modulating transcription of the target genes directly or indirectly. Directly, it binds to specific enhancer sequences on the DNA, meaning the ERE, which is normally located upstream from the genes. An indirectly approach takes place through interaction with transcription-associated nuclear proteins (Kloas et al. [2000]).

### Gene expression and stress

In response to stressors of both biological and biotic character, a wide range of physiological and chemical changes may occur. These can be observed at the level of the organism, such as an incline in the amount of circulating stress hormones, or at a cellular level, usually involving heat shock proteins (hsp) (Basu et al. [2001]). These proteins are an evolutionary conserved family of cellular proteins found in all organisms studied. Three main families of hsp has been identified, which in non-stressed cells possess important functions concerning metabolism of proteins, involving folding, translocation, assembly and denaturation (Basu et al. [2002], Basu et al. [2001]). Every hsp is not induced by stress, but all stresses that are known are capable of inducing hsp expression (Feder [1999]).

The cellular stress response is initiated by glucocorticosteroids, such as cortisol, binding to a complex composed of a glucocorticoid receptor (GCR) and a hps. The hps is released as a consequence of the ligand binding, and the resulting complex of receptor and ligand is relocated from the cytoplasma to the nucleus, where it acts as a transcription factor, capable of activating or repressing target genes (Momoda et al. [2007]).

## 1.2 Contribution and Scope of this Thesis

Due to the possible transfer of MEL from the GIT to the liver, combined with the reported negative effects of cortisol on hepatocyte vitellogenesis, it is possible that the MEL molecule could modify the cortisol impact on Vtg production.

The aim of this study was to investigate whether MEL may modify the possible negative effects of stress on fish reproduction. Accordingly, the following objectives were formulated:

1. Document the melatonin concentration in vena portae of Atlantic salmon, and investigate its possible variation with melatonin in the general circulation.

2. Investigate how the 17$\beta$-estradiol stimulated Atlantic salmon hepatocyte vitellogenin mRNA expression is affected by cortisol and melatonin exposure *in vitro*.

# 2. Materials and Methods

## 2.1 Experiment 1: Melatonin and Cortisol in Blood Plasma from Atlantic Salmon

This section will cover the background and principle for the use of radioimmunoassay (RIA) to measure melatonin (MEL) concentration in the perifere blood system and the vena portae.

As described in the introduction, it has been shown that MEL is present both in the peripheral blood system, released from the pineal gland, and in the gastrointestinal tract (GIT), which is connected to the liver by vena portae. Experiment 1 was performed to document the presence of MEL and cortisol in vena portae and the circulating blood from Atlantic salmon after handling and anesthetization prior to the blood sampling, and in response to light and darkness.

### 2.1.1 Model Animals

Atlantic salmon with a mean weight and length of 1367 (±152) g and 49.5 (±1.5) cm (Appendix A), respectively, were obtained in October 2012 from Lerøy Seafood Group MIDNOR, outside of Trondheim, and kept at SeaLab, a facility of the Norwegian University of Science and Technology (NTNU). They were distributed into two 400 L flow tanks, where one of the tanks was covered so the fish experienced darkness for three hours, while the other tank was placed in the light.

### 2.1.2 Blood sampling

Blood samples were obtained from six fish in each of the experimental groups, 24 hours after transport from the fish farm. The fish was anesthetized before the experiment, one at the time, using 50 mg/L MS-222 (Tricaine Methane Sulphonate 100% w/w, PHARMAQ Ltd) in salt water. The length and weight was measured after blood sampling. Samples were taken from both vena portae and the peripheral blood system in each fish. For the collecting of blood, heparinized syringes (16.5 IE, LEO Pharma AS) were used. Peripheral blood was sampled from the caudal vein of the fish, before the fish abdomen was opened carefully. The vena portae was found and blood samples was taken by placing the needle with the opening away from the liver, toward the GIT. The head of the fish from the darkness exposed group was covered when sampling the blood, to prevent light exposure. Blood was centrifuged at 3000 rpm for 5 minutes (Minispin®, Eppendorf), and the plasma was transferred to new test tubes, before covered with aluminum foil and placed in a -20°C freezer.

### 2.1.3 Radioimmunoassay

RIA is utilizing that a radio-active antigen and the antigen in the sample will compete for antibody binding sites. According to the kit protocol from Labor Diagnostika Nord GmbH & Co, the amount of $^{125}$I-labelled antigen bound to the antibody is inversely proportional to the concentration of the analyte in the sample. By comparing the radioactivity in the samples with a reference curve made from known standards, the concentration can be calculated.

The plasma MEL concentration was determined by RIA, using $^{125}$I RIA kit (Melatonin Research RIA Kit, Labor Diagnostika Nord GmbH & Co, KG, Germany). This kit provides a direct quantitative determination of MEL in biological fluids. The procedure was performed according to the kit protocol. Some of the results exceeded the standard curve, and was thus diluted 1:2 by using the standard zero solution, before run again. Due to this, a standard curve had to be calculated by the author in order to get the final results (Appendix B).

Using a cortisol RIA kit (Coat-A-Count, Siemens Healthcare Diagnostics Inc., USA), the plasma cortisol concentration was also investigated. This was conducted according to the kit protocol.

## 2.2 Experiment 2: Expression of Vitellogenin and Estrogen Receptor in Hypatocyte Cultures from Atlantic Salmon

To investigate the possible role of melatonin MEL in vitellogenesis, hepatocytes was obtained from Atlantic salmon, treated with 17$\beta$-estradiol (E2), cortisol and MEL, and the effect on gene expression was studied.

### 2.2.1 Model animal

An immature, male Atlantic salmon (*Salmo salar*), 32 cm long and weighing 396.1 kg, was used as model organism. The fish was obtained from Lerøy Seafood Group MIDNOR, outside of Trondheim in Norway, in March 2012. It was kept for one week in advance of the experiment at Sealab research facility (Brattøra in Trondheim), in a 400 L flow through fish tank under controlled light simulating the natural photoperiod, and fed *ad libitum* with standard pelleted feed.

### 2.2.2 Liver perfusion, counting of hepatocytes and plating of cells

The purpose of liver perfusion is to isolate hepatocytes for cultivation. The method was perfomed by the two-step collagenase perfusion described by Berry and Friend [1969], modified by Andersson et al. [1983]. This involves two steps. First, a buffered salt solution containing EGTA is used, which removes the $Ca^{2+}$ causing cell-cell connections to break, and rinses out the blood cells from the liver. In the second step, collagenase is added to the perfusion buffer, enabling the dissolving of intercellular collagen (Segner [1998]). The cells are then plated in a monolayer where they reconnect, and are ready for experimental treatment.

In advance of the perfusion, all glass equipment were autoclaved for 20 minutes at 120°C, and the remaining equipments were sterile. The perfusion solution (PS) I, II and growth medium was prepared beforehand, filtrated in this order, avoiding $Ca^{2+}$ contamination, using a 0.22 $\mu$m Millipore filter (Millipore AS, Oslo, Norway). The filtration was conducted to remove microorganisms and undesirable precipitations. The content of

the PS I and II is shown in 2.1.

Table 2.1: The content of perfusion solution (PS) I and II.

| | PS I (g/L) | PS II (g/L) |
|---|---|---|
| NaCl | 7.14 | 7.14 |
| KCl | 0.36 | 0.36 |
| $MgSO_4$ | 0.15 | 0.15 |
| $Na_2HPO_4$ | 1.6 | 1.6 |
| $NaH_2HPO_4$ | 0.4 | 0.4 |
| $NaHCO_3$ | 0.31 | 0.31 |
| EGTA | 20000.00 | - |
| $CaCl_2x2H_2O$ | - | 0.22 |
| Collagenase | - | 20000.00 |

The fish was anesthetized right before the perfusion, using 50 mg/L MS-222 (Tricaine Methane Sulphonate 100% w/w, PHARMAQ Ltd) in salt water. The anesthetized salmon was dissected by using scissors and scalpels. After carefully opening the fish abdomen, the liver was detected and made accessible. Vena portae was found and a needle connected to the perfusion apparatus was inserted approximately 0.2 - 0.3 cm into the vein. First, the PS I was used for approximately 20 minutes, washing out the blood from the liver. After the color of the liver turned pale and the liver size was increased, the PS II was used for 15 minutes. When the liver had a soft appearance it was taken out of the fish an into a petri dish. The liver was dissolved as much as possible in BSA (0.1%) and the hepatocytes were filtrated through a 150 $\mu$m plankton filter. The cells where centrifuged (Hettich MikroRR, Bäch) for 5 minutes at 50 g, and pellets containing the cells were washed three times with serum-free medium before resuspended in culture medium. The applied medium was DMEM supplemented with 2.5% FBS, 0.3 g/L glutamine (Gibco-Invitrogen, Carlsbad) and 500x antimycotic/antibiotic (neomycin, streptomycin and penicillin, Sigma Aldrich), stored at 10°C and adjusted to pH 7.6 by adding 5M NaOH before use.

The amount of hepatocytes was counted by using a Bürker counting chamber. The cell solution was mixed with tryphanblue solution 1:2 for visualization of potential dead cells, and the required cell survival of >90% was observed. The average number of cells was calculated and the volume needed for plating the cells was estimated. The cell concentration was calculated by Equation 2.1. Test experiments in advance had shown that 6 million cells in each well was desirable.

$$Y = N \cdot \left(1.6 \cdot 10^5\right) \cdot 2 \quad (2.1)$$

where Y = cells/mL, N = average cells/square, $1.6 \cdot 10^5$ = the chamber volume, and 2 = the dilution factor.

For the plating of hepatocytes, thirteen 35 mm Primeria culture plates, each with six wells, were used (Sarstedt, Germany), and the hepatocytes was plated with 3 mL DMEM medium to form a cell monolayer, with a density of $6 \cdot 10^6$ cells.

The hepatocytes were cultured at 10°C in a sterile incubator, without neither $O_2$ nor $CO_2$ additionally supplied. The cells were incubated for 48 hours, allowing for recovery of the hepatocytes from the perfusion-related stress and the restoration of the enzyme activity levels. Hepatocytes has been shown capable of repairing damage due to treatment within few hours after plating, and are able to keep their metabolic activity for 3 to 8 days (Ferraris et al. [2002]).

### 2.2.3 Exposure of hepatocytes

Exposure was performed on eight plates, each with a different exposure, making six replicates of each treatment. One of the plates was used as a control, with only medium added. Only E2 (10 nM) was added to another plate. In three other plates, E2 and cortisol in different amounts, 10 nM, 100 nM and 1000 nM, were added. In three more plates the same three treatments and additionally 400 nM MEL was added. Ethanol was used for dissolving the chemicals. To prevent degradation of MEL by light, aluminum foil was used for cover the MEL containing solutions.

The cells were preincubated with E2 and MEL for 4 hours before the cortisol was applied. The different treatments and the time schedule are shown in Table 2.2 and Table 2.3.

Table 2.2: Time schedule and exposure of plates for preincubation with melatonin (MEL) and 17$\beta$-estradiol (E2). For the groups treated with MEL, 400 nM MEL was used.

| Plate | Exposure time | Treatment | E2 (10 nM) | Melatonin (400 nM) | Cortisol (10 nM) | Cortisol (100 nM) | Cortisol (1000 nM) |
|---|---|---|---|---|---|---|---|
| A | 7:00 a.m. | Control | | | | | |
| B | 7:07 a.m. | E2 | x | | | | |
| C | 7:14 a.m. | E2 | x | | | | |
| D | 7:21 a.m. | E2 | x | | | | |
| E | 7:28 a.m. | E2 | x | | | | |
| F | 7:35 a.m. | E2 + melatonin | x | x | | | |
| G | 7:42 a.m. | E2 + melatonin | x | x | | | |
| H | 7.49 a.m. | E2 + melatonin | x | x | | | |

Table 2.3: Time schedule and exposure of plates to melatonin (MEL), 17$\beta$-estradiol (E2) and cortisol. After 4 hours of preincubation with MEL and E2, cortisol was added in some of the groups. For the groups treated with MEL, 400 nM MEL was used.

| Plate | Exposure time | Treatment | E2 (100 nM) | Melatonin (400 nM) | Cortisol (10 nM) | Cortisol (100 nM) | Cortisol (1000 nM) |
|---|---|---|---|---|---|---|---|
| A | 11:00 a.m. | Control | | | | | |
| B | 11:07 a.m. | E2 | x | | | | |
| C | 11:14 a.m. | E2 + 10 nM cortisol | x | | x | | |
| D | 11:21 a.m. | E2 + 100 nM cortisol | x | | | x | |
| E | 11:28 a.m. | E2 + 1000 nM cortisol | x | | | | x |
| F | 11:35 a.m. | E2 + melatonin | x | x | x | | |
| G | 11:42 a.m. | E2 + melatonin | x | x | | x | |
| H | 11.49 a.m. | E2 + melatonin | x | x | | | x |

Before exposure of the chemicals, an automatic pipette was used to carefully remove the existing medium in all wells, including the controls. The proper treatment solution for each group was added to the associated well with 3 mL medium. This was repeated with the second treatment after preincubation.

### 2.2.4 Harvesting hepatocytes

Harvesting of the treated hepatocytes was performed 24 hours after the first exposure. The medium was carefully removed and 500 $\mu$L Trizol (Gibco-Invitrogen, Carlsbad) was added to each well. Using a cell scraper, the cells was detached from the plates, and the Trizol containing the hepatocytes was transferred to eppendorf tubes. The samples were put on liquid nitrogen rapidly and then placed in a -80°C freezer.

### 2.2.5 Isolation of ribonucleic acid

Total RNA from the hepatocytes was isolated with TRIzol® reagent from Invitrogen (TRIzol® Plus RNA Purification Kit). Six to eight samples were thawed at a time, cell lysis was performed in 0.5 mL Trizol by vigorous pipetting, incubated for 5 minutes in room temperature (RT), and added 0.2 mL chloroform. All tubes were vigorously shaken for 15 seconds, incubated for 2 minutes at RT and centrifuged at 12000 g (Hettich Mikro22R, Bäch). After centrifugation, the water phase containing the RNA was removed to another tube and 0.5 mL isopropylalcohol was added. After 10 minutes of incubation in RT, these tubes were centrifuged at 12000 g for 10 minutes with a temperature of 8°C. The supernatant was removed, and pellets were washed with 75% ethanol, thereafter vortexed before centrifuged at 7500 g for 5 minutes with a temparature of 8°C. The pellets were air dried for 30 minutes, dissolved in 50 $\mu$L DEPC treated (RNase free) water and the tubes were incubated at 60°C for 10 minutes. 10 $\mu$L of each of the RNA solutions was diluted to 50 $\mu$L in order to prevent degradation of the original samples, before all samples were transferred to a -80°C freezer.

### 2.2.6 Synthesis of complementary deoxyribonucleic acid

The RNA quantity and purity was measured by using NanoDrop 1000 (Thermo Scientific) at 260 nm, and the samples were put back in a -80°C freezer.

Complementary DNA (cDNA) was synthesized by using the iScript™ cDNA Synthesis Kit (Bio-Rad) with reverse transcriptase (RT).

The RNA samples were thawed on ice. A reaction mix was made

according to the protocol of the iScript™ cDNA Synthesis Kit (Bio-Rad), containing 4 $\mu$L iScript buffer, 1 $\mu$L iScript reverse transcriptase, and a solution of nuclease-free water containing 1 $\mu$g RNA with a total volume of 15 $\mu$L. Randomly chosen samples without reverse transcriptase were used as controls. Synthesis of cDNA was performed by using a thermocycler (MJ Research PTC200 Thermo Cycler), set at 42°C for 30 minutes and then 85°C for 5 minutes. The synthesized cDNA was put in a -20°C freezer.

### 2.2.7 Quantification of mRNA

Currently, various methods are applied for quantification of mRNA expression, including Nothern blotting, cDNA arrays, *in situ* hybridization and quantitative real-time polymerase chain reaction (qPCR) (Giulietti et al. [2001]). Quantitative variations in gene expression between different samples is to a large extend determined by using qPCR (Taylor et al. [2010]). According to Giuletti and coworkers (2001), qPCR is accurate, sensitive, enabling a high throughput and analysis of small volume samples.

Polymerase chain reaction (PCR) is a technique for amplifying a specific DNA sequence *in vitro*. The amount of DNA is doubled after each so called PCR cycle, involving three steps of reactions. First, the sample is heated to 90-95 °C, which results in the dissociation of the double strand into single strands. In the next step, a temperature between 50°C and 70°C enables added primers to bind to the single-stranded DNA (ssDNA), providing an initiating point for synthesis of a new strand, complimentary to the sequence of interest. The temperature is then set to 70-75°C, optimal for a DNA polymerase applied to the mixture, extending the primers and producing a double-stranded DNA (dsDNA). The number of cycles to be repeated is dependent on the amount of DNA needed, and the PCR is runned by the use of automated machines, called thermocyclers (Klug et al. [2009]).

Through qPCR, the starting amounts of DNA or cDNA can be accurately quantified, by the measurement of the PCR product through detection of fluorescence in each cycle. The fluorescence may be due to dsDNA-binding fluorescent dyes or by sequence-specific probes labeled with fluorescence, such as SYBR Green I. The SYBR Green I attaches to all dsDNA, also potential non-specific PCR products and primer-dimers,

thus requiring high specificity of the PCR (Soheili and Samiei [2005]). To obtain good results, controlled conditions and proper handling methods are important. RNA handling time should be the shortest possible, and high purity and integrity of the RNA is required. To achieve specific amplification with high efficiency, the choice of primer design and target sequence is essential. Reference genes for normalization of the data should also be thoughtfully decided (Taylor et al. [2010]).

The gene expression of Vtg and ER$\alpha$ was quantified by qPCR, using $\beta$-Tubulin as a reference gene (several genes were investigated, $\beta$-Tubulin was the most stable), and running samples without reverse transcriptase as a control. The cDNA was diluted 1:5 by carefully adding 100 $\mu$L of water in each well. Primers (Table 2.4) were diluted 1:20. 10 $\mu$L iTAQ™ SYBR® Green Supermix with ROX (Bio-Rad), 2 $\mu$L autoclaved water, 1 $\mu$L forward primer solution, 1 $\mu$L reverse primer solution and 6 $\mu$L of the proper cDNA solution was added to each required well on a PCR plate. The plate was run in the thermocycler (LightCycler® 480 Real-Time PCR System, Roche) with the cycle parameters shown in Table 2.5.

Table 2.4: Gene sequences of the primer pairs.

| **Target Gene** | **Fw primer** | **Rw primer** |
|---|---|---|
| *Vtg* | 5' - AAGCCACCTCCAATGTCATC | 5' - GGGAGTCTCCCAAGACAA |
| *ER α* | 5' - TCCAGGAGCTGTCTCTCCAT | 5' - GATCTCAGCCCATACCCTCCA |
| *β-Tubulin* | 5' - CCGTGCTTGTCGACTTTGGAG | 5' - CAGCGCCCTCTGTGTAGTGG |

Table 2.5: The cycle parameters of the qPCR run. Step 1, 2 and 3 are repeated in 40 cycles. The temperature of step 3 varies with the target gene.

| Step | Temperature (°C) | Time (sec) |
|---|---|---|
| Activation step | 95 | 180 |
| 1 | 95 | 15 |
| 2 | 60 | 15 |
| 3 | 72 | 15-30 |

## 2.3 Calculation of Results, Validations and Statistical Analysis

This section gives a description of the calculations, validations and the statistical analysis.

### 2.3.1 Experiment 1: Plasma levels of melatonin and cortisol in Atlantic salmon

In this experiment, the concentrations of MEL and cortisol in blood plasma was analyzed by RIA.

#### Calculations of data

The gamma counter outputs counts per minute (CPM). To interpret the results, calculation of the concentration, given in pg/mL for MEL and nmol/L for cortisol, had to be conducted. In order to do this, a standard set of values was used to make a standard curve. For the MEL run, this was performed by creating a MatLab script, generating the standard curve, shown in Figure B.1, and calculating the results. This script created can be found in Appendix B. For cortisol, this was done by the gamma counter, printing the final calculated dose. The dataset used to calculate the mapping was given in the protocol.

#### Validation

For quality assurance of the test, a quality control, or validation, should be performed. For this experiment, the specificity, parallelism, recovery and sensitivity were examined, according to the protocol.

**Specificity** Specificity is evaluated with a non-specific-binding sample, that does not contain added antibodies. If the value is low, the 125I-labelled antigen is specific for the antibody investigated, and the amount of cross-reactions with other elements are low. This value was used to normalize the other data, reducing the effect of background noise.

**Parallelism** Parallelism test is performed using a serial dilution. If the analyte measured is equal to the standard, it implies that non of the other sample components are interfering with the antigen-antibody reaction, and the curve will be parallel to the standard curve. The estimated function based on the parallelism sample values generated a curve that was parallel compared to the standard curve, and can be found in Appendix B.

**Recovery** Recovery of the exogenously added analytes in the plasma is preferable 80-100% of the amount initially added, although acceptable when above 50%. High recovery indicates that none of the other components in the samples are interfering with the reaction. The recovery was found by measuring six spiked plasma samples with a concentration of 200 pg/mL MEL, and the recovery was calculated by Equation 2.2:

$$\% \text{ recovery} = \frac{\text{mean calculated dose in pg/mL} \cdot 100 \text{ \%}}{200 \text{ pg/mL}} \tag{2.2}$$

and found to be 74.7%.

**Sensitivity** Sensitivity gives an indication of the lowest detection limit that can be measured accurately The lowest accurately detectable value of MEL was found to be 14.5 pg/mL.

### 2.3.2 Experiment 2: Expression of vitellogenin and estrogen receptor in hepatocyte cultures from Atlantic salmon

The results in this experiment was obtained by quantitative real time polymerase chain reaction (qPCR), measuring the cDNA in the samples, corresponding to the isolated mRNA level.

#### Calculations of data

The output from qPCR analysis is the crossing point (CP), the point where the fluorescence crosses the threshold fluorescence (Pfaffl [2001]). The CP values are then normalized using a housekeeping gene as a reference. In

this experiment, $\beta$-Tubulin was used. The expression ratio of Vtg and ER$\alpha$ mRNA was calculated by Equation 2.3, presented by Pfaffl [2001].

$$ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}} \tag{2.3}$$

The efficiency (E) was calculated using the software LinRegPCR (Ruijter et al. [2009]).

**Validation**

NanoDrop was used to measure the quantity and purity of the RNA (NanoDrop 1000, Thermo Scientific) in the samples.

To ensure that the RNA samples were not contaminated by DNA, cDNA synthesis was performed on randomly chosen samples without the presence of reverse transcriptase. These samples showed no results in the qPCR run, indicating that DNA contamination during the RNA isolation did not occur.

## 2.4 Statistical analysis

The statistical analysis was conducted using a commercial mathematical software package (MATLAB 8.0, The MathWorks Inc., Natick, MA, 2012b). Statistical significance was set at $p<0.05$. The analysis was conducted by One-Way Analysis of Variance (ANOVA). The scripts written for MATLAB utilized the anova1 function, which performs a one-way ANOVA for comparing the means of two or more groups of data. The function returns the p-value for the null hypothesis that the means of the groups are equal. The written script also provide the ANOVA tables, Appendix C, based on the analysis.

# 3. Results

## 3.1 Experiment 1: Plasma Levels of Melatonin and Cortisol in Atlantic Salmon

Plasma levels of melatonin (MEL) and cortisol, both in the vena portae and in the peripheral blood system (caudal vein), was investigated in Atlantic salmon exposed to light or darkness.

### 3.1.1 Plasma melatonin levels

The mean MEL concentrations in plasma from the caudal vein was 120.7 $\pm$ 43.1 pg/mL in the group exposed to light, and 240.4 $\pm$ 82.1 pg/mL in the group exposed to darkness. In vena portae, mean MEL plasma levels in the group exposed to light was 105.64 $\pm$ 31.1 pg/mL and in the group exposed to darkness a mean level of 90.4 $\pm$ 14.2 pg/mL was measured. An increased plasma MEL concentration in the circulating blood system could be observed in the group experiencing darkness, although this was not statistically significant. No significant difference was observed between the groups exposed to light compared to those exposed to darkness, or between blood plasma obtained from vena portae and the circulating blood (Figure 3.1). ANOVA tables can be found in Appendix C.

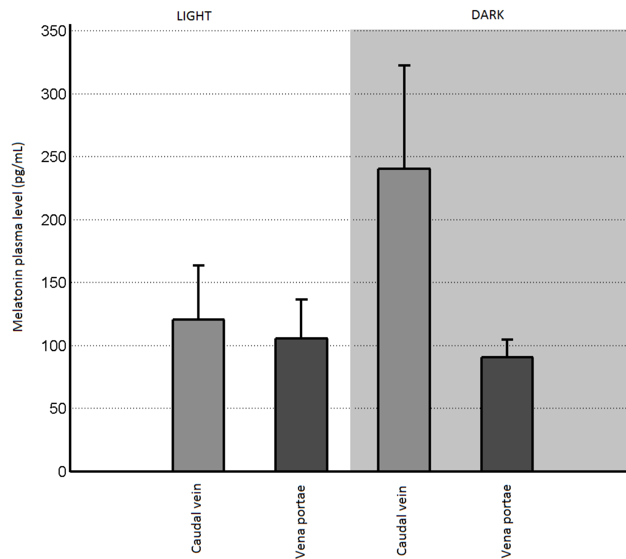



Figure 3.1: Plasma concentration of melatonin (MEL). Sample site is specified at the x axis and plasma concentrations at the y axis. Mean values +standard error of mean (SEM) (n=6) are shown. The treatment (light and dark) is marked in the figure.

In individuals exposed to light, the plasma MEL level did not indicate any evident variation in blood from vena portae and the circulating blood system (Figure 3.2). In individuals exposed to darkness, there was a general tendency of a higher level of MEL in the periphere blood system than in the vena portae.

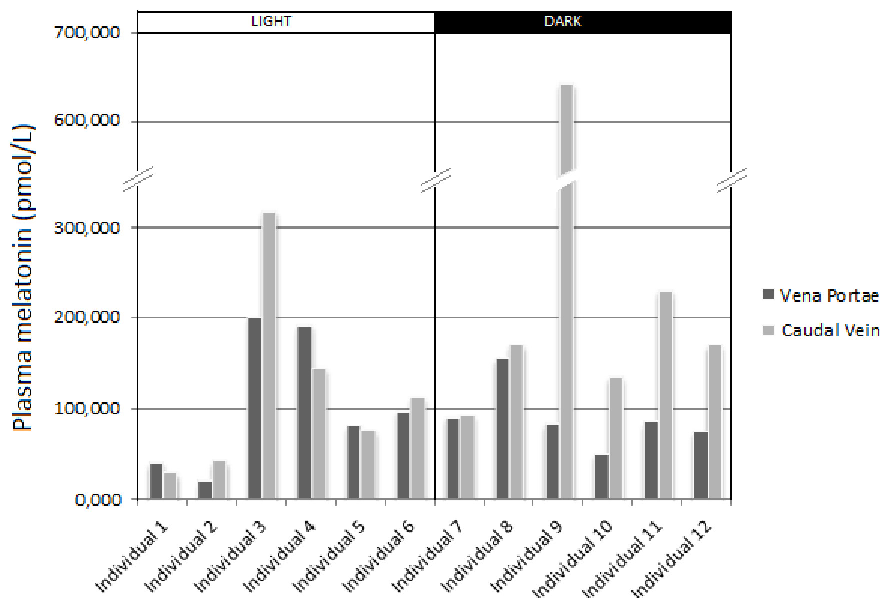



Figure 3.2: Plasma concentration of melatonin in individual fish. The x axis specifies the individual, with plasma concentration on the y axis. The light grey columns are plasma samples from the circulating blood system and the dark grey columns are from the vena portae. The treatment (light and dark) is marked in the figure.

### 3.1.2 Plasma cortisol levels

In vena portae, mean cortisol plasma levels in the group exposed to light was 328.2 ± 49.0 and in the group exposed to darkness a mean level of 299.4 ± 34.1 nmol/L was measured. The mean cortisol concentrations in plasma from the caudal vein was 535.8 ± 43.7 nmol/L in the group exposed to light, and 424.7 ± 68.7 nmol/L in the group exposed to darkness.

Plasma cortisol levels showed a significant difference between the groups (ANOVA: $F = 4.48$, $p = 0.0147$) (Figure 3.3). In the circulating blood plasma an increased cortisol level compared to the blood obtained from

vena portae could be detected. Between the groups exposed to light and darkness, no significant difference could be observed. ANOVA tables can be found in Appendix C.

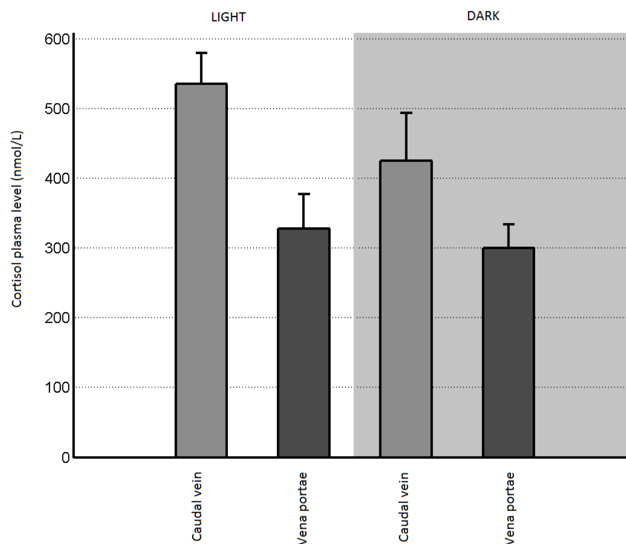



Figure 3.3: Plasma concentration of cortisol. Sample site is specified at the x axis and plasma concentration on the y axis. Mean values +standard error of mean (SEM) (n=6) are shown. The treatment (light and dark) is marked in the figure.

In all individuals, the cortisol level in plasma obtained from the circulating blood system is slightly higher or higher than in plasma obtained from vena portae (Figure 3.4). The cortisol levels in the periphere blood system varied between 300 nmol/L and 720 nmol/L, and in the vena portae the levels ranged from 160 nmol/L to 530 nmol/L.

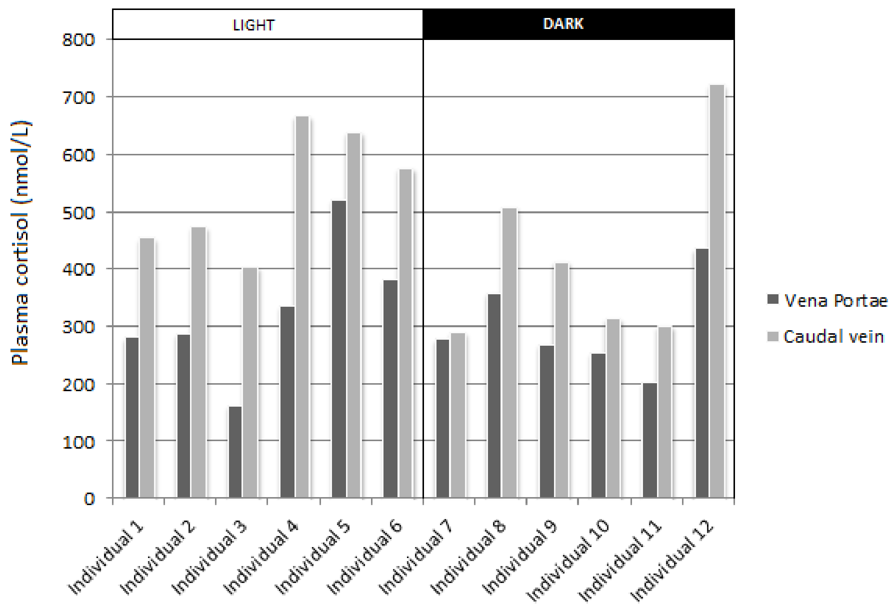



Figure 3.4: Plasma concentration of cortisol in individual fish. The x axis specifies the individual, with plasma concentration on the y axis. The light grey columns are plasma samples from the circulating blood system and the dark grey columns are from the vena portae. The treatment (light and dark) is marked in the figure.

## 3.2 Experiment 2: Expression of Vitellogenin and Estrogen Receptor in Hepatocyte Cultures from Atlantic Salmon

Hepatocyte cultures from a male Atlantic salmon were treated with 17$\beta$-estradiol (E2), cortisol and MEL. The expression of vitellogenin (Vtg) and the estrogen receptor $\alpha$ (ER$\alpha$) mRNA was investigated, and the results are presented below.

### 3.2.1 Vitellogenin expression

The expression of the Vtg mRNA was increased in response to E2 compared to the control (Figure 3.5), with a mean expression ratio of 1.2 $\pm$ 0.3 in the control group and 105.1 $\pm$ 27.9 in the group exposed to E2. There was no significant difference between the three groups treated with different amounts of cortisol (10, 100 and 1000 nM) and E2, with a mean expression ratio of 120 $\pm$ 15.3, 90.9 $\pm$ 7.7 and 98.9 $\pm$ 12.8, respectively. Between the three groups exposed to 10, 100 and 1000 nM cortisol and E2 in combination with MEL, there was a mean expression ratio of 61.6 $\pm$ 5.7, 65.4 $\pm$ 6.9 and 61.7 $\pm$ 14.0, with no significant variations between the values.

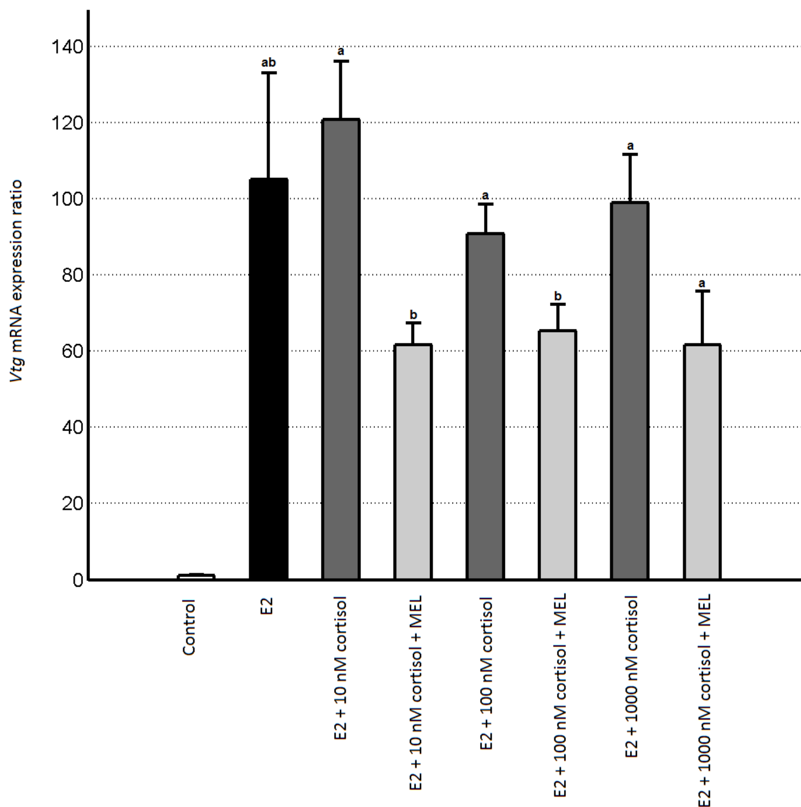



Figure 3.5: *Vtg* mRNA expression ratio. The mean expression ratio +standard error of mean (SEM) (n=6) in the different groups are shown, with treatment at the x axis and expression ratio at the y axis. Statistical significance is indicated with different letters.

There was a significant decrease in the *Vtg* expression ratio in the group treated with 10 nM cortisol and MEL, compared to the group only exposed to 10 nM cortisol (ANOVA: $F = 13.1$, $p = 0.0047$). This was also observed in the groups treated with 100 nM cortisol and MEL, with a decrease in the expression ratio for the cells exposed to MEL (ANOVA: $F = 6.13$,$p = 0.0327$). In the groups treated with 1000 nM cortisol, the decrease was not significant, although a possible decrease could be observed. ANOVA tables can be found in Appendix C.

### 3.2.2 Estrogen receptor $\alpha$ expression

The ER$\alpha$ mRNA expression ratio was elevated in response to the E2 treatment, with a mean expression ratio value of 1.0 $\pm$ 0.1 in the control group and 9.8 $\pm$ 1.5 in the group exposed to E2 (Figure 3.6). Between the extended treated groups, no significant differences could be determined, with mean expression ratio values of 9.9 $\pm$ 1.7, 10.0 $\pm$ 0.7 and 10.3 $\pm$ 1.0 in the groups exposed to E2 and 10, 100 and 1000 nM cortisol, and of 13.0 $\pm$ 2.0 , 9.5 $\pm$ 0.8 and 6.5 $\pm$ 1.4 in the groups treated with E2, MEL and 10, 100 and 1000 nM cortisol, respectively.

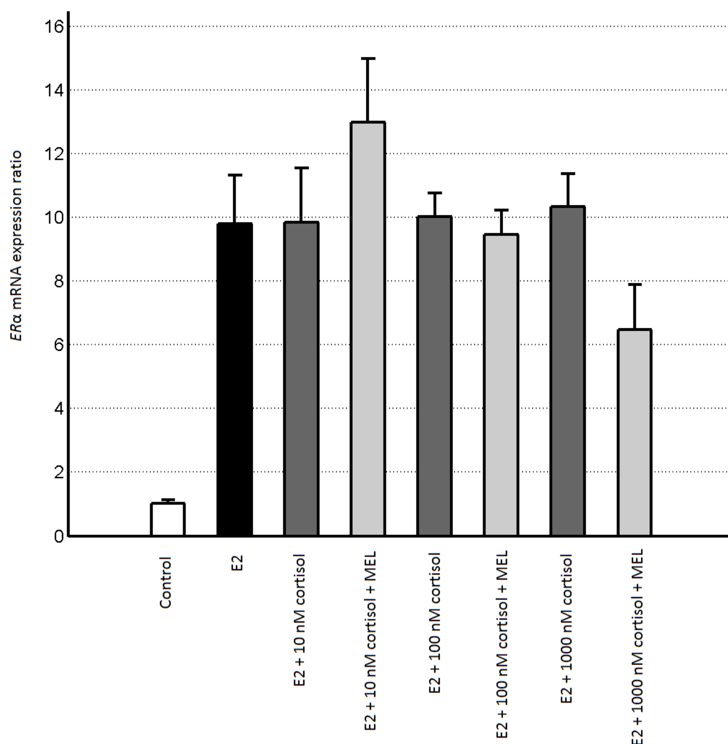



Figure 3.6: Estrogen receptor $\alpha$ (ER$\alpha$) mRNA expression ratio. The mean expression ratio with +standard error of mean (SEM) (n=6) in the different groups are shown, with treatment at the x axis and expression ratio at the y axis.

The expression ratio of the group treated with 10 nM cortisol in combination with MEL may appear to increase slightly, but this is not statistically significant. Between the groups treated with 100 nM cortisol, there was no detectable difference. In the group treated with 1000 nM cortisol and MEL, a small decrease could be detected, but the decrease was not a significant observation (ANOVA: $F = 4.84$, $p = 0.0525$). ANOVA tables can be found in Appendix C.

# 4. Discussion

In response to stress, plasma cortisol levels may be elevated in teleosts, which may reduce fecundity through negative impact on vitellogenesis (Mommsen et al. [1999], Carragher et al. [1989], Tran [2010]). Hence, studies examining the relationship between cortisol and estrogen during the vitellogenic period would be important in order to avoid negative handling routines for maturing salmon. It would also be important to include possible melatonin (MEL) interactions as this hormone is part of the reproductive control (pineal release), and probably also influence the intermediary metabolism (by extrapineal gastrointestinal (GIT) release), through hepatocyte receptors.

## 4.1 Experiment 1: Plasma levels of melatonin and cortisol in Atlantic salmon

The handling and anesthetization of the fish prior to perfusion and retrieval of hepatocytes may have affected the endocrinology of the fish. For technical reasons, this was investigated in a separate experiment, where MEL and cortisol concentrations in blood plasma from Atlantic salmon vena portae was measured by using radioimmunoassay (RIA). MEL and cortisol was chosen due to the high relevancy with respect to Experiment 2. While examining this, the level of MEL and cortisol in vena portae was compared to that of the circulating blood (caudal vein complex), and the potential variations between fish exposed to light and dark were also studied.

### 4.1.1 Plasma melatonin levels

The results from this study documented the presence of MEL in vena portae of Atlantic salmon. This hormone level was not affected by exposing the salmon to darkness, which indicates negligible changes in the GIT melatonin production. The vena portae MEL level was comparable to that seen in the the general circulation under light conditions. During darkness, however, the mean peripheral MEL concentrations were increased by 99%, signifying the increased pineal synthesis. There were no significant difference in MEL concentrations with respect to the circulating blood system between the fish exposed to light or darkness. Individual variations are expected in biological experiments, due to genetic and physiological factors. All individuals are unique, and may respond to treatment differently. Variations in gene expression occur both among populations and individuals, and responsiveness to stressors and cortisol is an individual characteristic (Oleksiak et al. [2002], Øverli et al. [2006]).

In the study of Bubenik and Pang [1997], the MEL level was investigated in GIT tissue from fish, amphibians and a reptile, finding concentrations between 100 pg/g of tissue and 250 pg/g of tissue in carp and trout. This corresponds to the observations in the present study, confirming the presence of MEL in vena portae in Atlantic salmon. As described in the introduction of this thesis, MEL concentrations, both in the GIT and in the liver, have been found to be higher than that in the circulating blood. This was not documented in the present study, and might be due to differences in diet or intestinal activity.

The MEL production in Atlantic salmon has been found to increase rapidly in response to darkness, with elevated levels remaining through the dark period, before a rapid decrease in response to light (Randall et al. [1995]), as illustrated in Figure 1.5. In the study of Randall and coworkers (1995), clear light/dark MEL levels were always observed, although with a higher amplitude during the summer months.

The findings of this study documents that MEL is present in the vena portae, and consequently, that hepatocytes are somewhat experienced to MEL from the GIT system. It can, however, be assumed that this only represent a low background level. Additionally, the results showing no visible variation between MEL in vena portae in groups exposed to light and dark, implies that the vena portae is an exclusive system and there

are no indications of pineal MEL influence. This is consistent with other findings. Environment, food intake and other factors may play a large role in the GIT MEL synthesis, while MEL in in the circulating blood is primarily controlled by the light/dark signal. The study of Bubenik and coworkers (2000) showed that a large quantity of MEL is secreted into the vena portae in Yorkshire pigs. They also found that the GIT MEL secretion to a larger extend is associated with nutritional factors, than to that of photoperiod, and that an incline in the MEL concentration in the vena portae occurs in relation to major food intake.

### 4.1.2 Plasma cortisol levels

The cortisol level results in this study varied between 300 nmol/L and 750 nmol/L in the periphere blood system, and between 160 nmol/L and 530 nmol/L in the vena portae. According to Olsen and coworkers (1992), resting levels of cortisol in Atlantic salmon has been found to be less than 28 nmol/L, and after acute stress, the levels may vary between 190 and 690 nmol/L. The levels may also be affected by physiological processes, such as smoltification, which may elevate the resting levels up to 70 nmol/L. From Figure 3.4, there is a tendency of increased cortisol levels during the sampling (individual 4,5 and 6, and individual 12). This may reflect that these individuals experienced more disturbance due to the out-taking of other fish. Considering this, the fish of the present study may have experienced stress as seen from elevated cortisol levels, prior to the heptocyte harwest.

Cortisol may induce the activation of cytochrome P450 (CYP), which could lead to a decrease in the MEL level (Facciolá et al. [2001]). The presence of cortisol could affect the results, by interfering with the MEL, 17$\beta$-estradiol (E2) or estrogen receptor (ER). However, due to the liver perfusion method and the metabolism by the cells themselves, it is unlikely that significantly levels of cortisol remained in hepatocytes at the time of experimental treatment.

## 4.2 Experiment 2: Expression of vitellogenin and estrogen receptor in hepatocyte cultures from Atlantic salmon

The possible effects of MEL in combination with cortisol on vitellogenesis was investigated *in vitro* in hepatocytes from Atlantic salmon. Fish hepatocyte cultures maintain their metabolic properties, and the use of such cell cultures has the advantage of allowing highly controlled experiments regarding the physiological and environmental conditions (Anderson et al. [1996], Navas and Segner [2006]). *In vivo* studies are time-consuming, more expensive and may provide ethical issues. However, *in vitro* studies have limitations, including the absence of many metabolic steps, providing a simplified neuroendocrine environment. For this reason, the results must be transferred to the *in vivo* system with caution (Pelissero et al. [1993]).

In this study, it was decided to use male fish. Both male and female fish carry the Vtg gene, but the transcription of the gene is estrogen dependent, and thus normally confined to females. However, exposed to natural or synthetic estrogen, male fish are able to produce Vtg (Tyler et al. [1996]). Maturing females may express a high and variable amount of Vtg, while male fish has a low baseline expression, hence is the use of male fish often preferable (Harries et al. [1999]).

### 4.2.1 Vitellogenin expression

The results showed a clear Vtg mRNA expression response to E2 exposure, documenting the desired initiation of vitellogenesis. Exposure with cortisol after preincubation with E2 did not result in any observable change, despite cortisol concentrations ranging from physiological to almost pharmalogical levels. These results deviate from most other studies demonstrating a suppressive effect of cortisol on the hepatocyte vitellogenesis in fish. According to Lethimonier and coworkers (2000), negative effects of stress on vitellogenesis in salmonids are well documented, and linked to increased cortisol levels (Roy et al. [1990], Campbell et al. [1992], Pottinger and Pickering [1990], Contreras-Sánchez et al. [1998], Carragher et al. [1989]). In the study of Tran [2010], a decrease of *Vtg* expression

was observed in Atlantic salmon hepatocytes after cortisol exposure for 12 hours, although for the lower exposure concentrations (10 - 100 nM), this effect could not be detected after 24 hours of exposure. Higher cortisol exposure concentrations did still have a moderate negative effect on Vtg mRNA expression after 24 hours. However, there are also indications of the opposite. The study of Pelissero and coworkers (1993) found no effect on the Vtg level in response to the cortisol treatment, although this was examined by detection of Vtg in the medium.

Clearance of cortisol by the organism is mainly due to uptake and catabolism by the tissue, but also binding proteins and target tissue receptors may be involved. Cortisol has a lipophilic character, and is thought to enter the cell through passive diffusion, although carrier-mediated uptake has been reported. Inside the cell, cortisol is either binding to a receptor or metabolized by enzymes, which may include reductases, oxidoreductases and CYP dependent hydroxylases. The primary organ for degradation of cortisol is the liver (Mommsen et al. [1999]).

Breakdown of cortisol during the incubation time of the hepatocytes may have occured, and could be self-regulated by the cortisol treatment, since cortisol has been demonstrated to increase the level of ethoxyresorufin-O-deethylase (EROD), an important catalyst of CYP activation (Andersson and Förlin [1992]). However, the clearance of cortisol takes time, and even high cortisol levels did not have any effect, and it is therefore unlikely that catabolism of cortisol is the cause of a possibly missing glucocorticoid Vtg mRNA depression. Additionally, transcriptional depression of the *Vtg* mRNA by cortisol is observed in other studies, with a comperative setup and within the same range in relation to exposure time and cortisol concentration.

One difference between the present study and the experiment of Tran [2010] is, however, the 4 hour E2 preincubation of hepatocytes. If the effect of cortisol acts through inhibitory mechanisms prior to the binding of E2 to the ER, or before the binding of the E2-ER complex to the ERE, the E2 treatment in advance of cortisol exposure could have blocked the suppressive impact of cortisol. According to Teitsma and coworkers (1998), cortisol may reduce the hepatic binding of E2, and this could take place either as a consequence of a decreased ER transcription or by destabilization of ER mRNA. If the E2-ER complex has already formed

and bound to the ERE, the cortisol may not be able to cause such a suppressive effect.

The results presented in the study of Lethimonier and coworkers (2000), indicate that the decrease of ER and Vtg expression by cortisol takes place at a transcriptional level, and their studies imply that the effect is not due to indirect effects mediated by other hormones. They found that glucocorticoid receptor (GCR) inhibited the E2-stimulated transcriptional activity of the ER promoter, and argue that the transcriptional interference between GCR and ER is unlikely to occur as a consequence of direct protein-protein interactions, and suggest a possible interaction with other transcription factors or co-activators.

In the present study, a significant decrease in the Vtg mRNA expression was documented in hepatocytes preincubated with both E2 and MEL before the addition of cortisol. This differs from other studies presenting no effects or a possible protective role of MEL. Dietary intake of tryptophan (Trp) has been shown to increase the synthesis and release of MEL by the GIT in rainbow trout (Lepage et al. [2005]. Herrero and coworkers (2007) have proposed that MEL may have a counteracting effect on the glucocorticoid responses induced by stress in mammals, and Trp in the diet has been shown to counteract stress-induced cortisol increase in rainbow trout plasma. In the study of Herrero and coworkers (2007), a MEL supplemented daytime diet increased the plasma concentrations of MEL. Trp did not show the same increase, but both MEL and Trp supplement had a reducing effect in high cortisol levels in European bass. A study performed on Atlantic salmon hepatocytes showed no effect of MEL on the expression of *Vtg* (Kvalsvik [2010]). According to Facciolá and coworkers (2001), MEL is rapidly metabolized by CYP in the human liver, and this may also be the case in the liver of teleosts. Since cortisol has been shown to raise the level of EROD, this could explain a lack of effect of MEL, but does not explain the observed negative effect.

MEL has been shown to have antiestrogenic actions, having the ability to down-regulate the circulating estrogen levels, either by suppressing gonadal synthesis, by down-regulation of enzyme activity involved in estrogen synthesis, or by interacting with ER directly or indirectly (Sánchez-Barceló et al. [2005]). Only the latter might be relevant in the present study, as the *in vitro* system does not provide gonadal involvement. Since

estrogen is metabolized by CYP (Tsuchiya et al. [2005]), and CYP may be induced by cortisol (Andersson and Förlin [1992]), the combined effect of MEL and cortisol, could lead to a significant decrease in the estrogen level, which may result in a decrease in the Vtg mRNA expression. However, in the study of Kvalsvik [2010], no negative effect of MEL was observed, and the study of Mazurais and coworkers (2000) did not find any effect of MEL on the ER in the liver of rainbow trout.

The incubation time and concentrations of E2 and MEL in the present study is within the range of the study of Mazurais and coworkers (2000) and Kvalsvik [2010]. What differs from the experimental setup of Kvalsvik [2010] is the addition of cortisol. In the present study, preincubation of E2 and MEL before the addition of cortisol could be an important factor of the experimental design. The lack of effect observed in the study of Kvalsvik [2010], may indicate that the combination of MEL and cortisol is crucial in this regard. No significant difference was found between the three groups treated with MEL and various cortisol amount. This implies that it is the presence of cortisol, not the level, that may be essential in relation to the possible combined effect with MEL.

Transcription of *Vtg* depends on the nuclear estrogen receptor (ER), enhancing proteins that functions as transcription factors. Its interaction with E2 forms a dimeric complex which recognizes and binds to the estrogen responsive element (ERE) located upstream of, or within the DNA, resulting in the activation or enhanced transcription of the Vtg gene (Babin et al. [2007]), as shown in Figure 4.1.

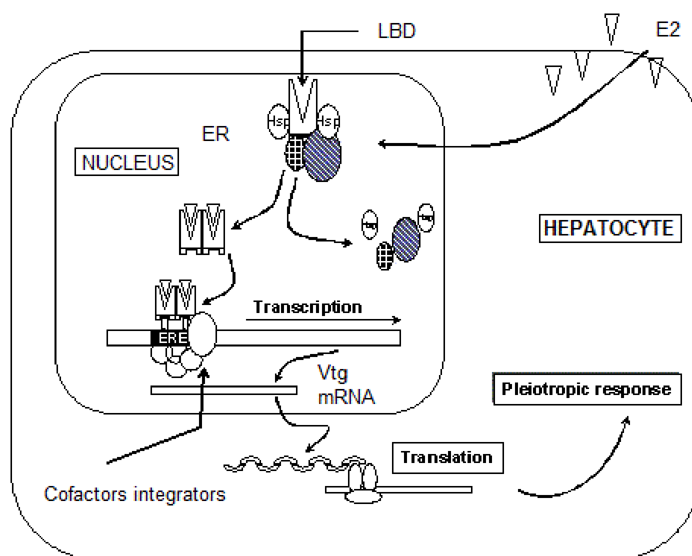



Figure 4.1: Transcription of vitellogenin (Vtg) mRNA. Estrogen (E2) enters the cell and binds to ligand binding domain (LBD) of the estrogen receptor (ER). The E2-ER complex dimerize and binds to estrogen responsive element (ERE) on the DNA, initiating transcription of Vtg mRNA. At the same time, cofactors (co-activators and co-repressors) participates by enhancing or repressing the transcription (modified from UZ Gent, Center for Medical and Urological Andrology [2012]).

The findings of the present study indicates that the cortisol may act in a different way in cells preincubated with E2 and MEL, than in cells preincubated with E2 alone. As shown in Figure 4.1, there are several possible sites of action for inhibition of the Vtg mRNA expression. Interference by other molecules may affect the cell uptake of E2, or the binding of E2 to the ER. The release of hsp and other associated proteins may be subject to influence, and prevention of binding of the E2-ER complex to the ERE is an other possibility. Cofactors involved in initiation of transcription could also potentially be affected. It is thus several possibilities for MEL to act upon this system, and otherm yet unknown, factors may

also be involved in this regard.

In human mammary adenocarcinoma, MEL has been found to inhibit the activation of E2-induced ER DNA binding, but not the binding of E2 to the ER or the localization of ER in nucleus (García Rato et al. [1999]). The inhibitory capability of MEL is suggested to be caused by a destabilizing effect of a receptor, mediated by MEL, on the binding of E2-ER complex to ERE. Preincubation with MEL could enable this effect, reducing the expression of Vtg mRNA. Still, such a potential inhibitory effect of MEL on the binding of the E2-ER to the DNA did not affect the results of Kvalsvik [2010] and Mazurais and coworkers (2000), and it remains uncertain whether this may take place in the hepoatocytes of teleosts, and if so, what impact the addition of cortisol may have in this regard.

Regulation of gene expression may act in several ways and on different levels. Modulations and effects can also take place on the synthesized protein. In this regard, experiments investigating the final Vtg protein may find different results. The findings of Herrero and coworkers (2007) imply that MEL may have a promising effect on reducing the negative impacts of enhanced cortisol levels, and there might be a possibility of a different effect in relation to the *Vtg* mRNA expression when providing MEL to the living organism.

### 4.2.2 Estrogen receptor $\alpha$ expression

In the present study of the expression of $ER\alpha$, an up-regulation was observed by E2, documenting an initiating effect of E2 on ER$\alpha$ mRNA expression. Between the group treated with E2 and groups treated with E2 and cortisol, no difference could be observed. This deviates from other studies, where cortisol has been found to have a suppressing effect on the ER. The study of Lethimonier and coworkers (2000) found an inhibitory effect of cortisol on vitellogenesis in rainbow trout, both *in vivo* and *in vitro*, and the results indicate that this may be explained by transcriptional interference on the *ER* expression. A decrease in the mRNA levels of *ER* was found in hepatocyte aggregates. As in section 4.2.1, the results could be due to the preincubation with E2 before the cortisol addition, allowing E2 to bind to the ER, and the complex to bind ERE, enabling transcription of E2 dependent genes.

No significant effects on the *ER* expression of cortisol combined with MEL could be documented, although a decrease in the group treated with E2, 1000 nM cortisol and MEL in relation to the group treated with only E2 and 1000 nM cortisol, was seen. The results of this experiment demonstrates no obvious correlation between the effect of cortisol and MEL on the *Vtg* expression and the ER expression, indicating that the possible negative effects on the Vtg mRNA expression by MEL in combination with cortisol may involve other processes, and act through a different signaling pathway than on the expression of ER. The study of Kvalsvik [2010] showed a small, but significant up-regulation in hepatocyte *ER*$\alpha$ expression after 24 hours of incubation with E2 and MEL. In Mazurais and coworkers (2000), no effect of MEL on the ER in hepatocytes was observed. It is therefore uncertain whether MEL has any effect on the ER receptor in the teleost liver.

The preincubation of hepatocytes with E2 and MEL combined could have created the opportunity for MEL to inhibit the binding of the E2-ER complex to the ERE, as suggested by García Rato and coworkers (1999). Thus, the hormone could be acting on this complex in a specific way, perhaps by cofactors specific for the Vtg gene transcription. This would not necessarily affect the expression of the ER, only the expression of the Vtg mRNA.

# Conclusions

1. The presence of melatonin in Atlantic salmon vena portae was documented, being independent of environmental photo conditions. There were no indications of influence from the pineal melatonin levels on this system.

2. After preincubation of Atlantic salmon hepatocytes with 17$\beta$-estradiol and melatonin, a reducing effect on the vitellogenin mRNA expression *in vitro* was only seen when hepatocytes were exposed to cortisol and melatonin in combination.

# Perspectives

### Melatonin from the gastrointestinal tract of Atlantic salmon

Future experiments should consider to study the effect of diet and food intake on the salmon gastrointestinal tract (GIT) melatonin (MEL) production, and delivery through vena portae to the hepatocytes.

### Melatonin and cortisol exposure of hepatocytes from Atlantic salmon

The present study showed that MEL in combination with cortisol may have a negative effect on the expression of vitellogenin (Vtg) mRNA. To the author‘s knowledge, these findings have not been reported in other studies, and needs further investigations.

This experiment was an *in vitro* study, carried out on hepatocyte cultures from Atlantic salmon. It would be interesting to investigate whether the same results could be reproduced *in vivo*.

### Interactions of other hormones on hepatocyte vitellogenesis

The possible role of MEL in hepatocytes of teleosts remains to be resolved, and potential interactions with other molecules are also relevant attentions for further studies. Especially, one should examine if hepatocytes produced hormones, as insulin-like growth factor (IGF), participates in cortisol or MEL mediated impact on the mRNA Vtg activity.

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# Appendix

# A. Weight and Length of Atlantic salmon (Experiment 1)

Table A.1: Weight and length of the fish, measured before blood sampling.

| Individual | Treatment | Weight (g) | Length (cm) |
|---|---|---|---|
| 1 | Light | 1391.4 | 49.5 |
| 2 | Light | 1220.0 | 50.0 |
| 3 | Light | 1070.0 | 45.0 |
| 4 | Light | 1347.0 | 49.0 |
| 5 | Light | 1376.0 | 49.0 |
| 6 | Light | 1620.2 | 51.0 |
| 7 | Darkness | 1352.9 | 50.0 |
| 8 | Darkness | 1375.3 | 49.0 |
| 9 | Darkness | 1544.2 | 51.0 |
| 10 | Darkness | 1187.2 | 48.5 |
| 11 | Darkness | 1357.6 | 49.0 |
| 12 | Darkness | 1565.2 | 52.0 |

# B. Standard curve, MEL, RIA

## B.1 Standard curve for Melatonin RIA

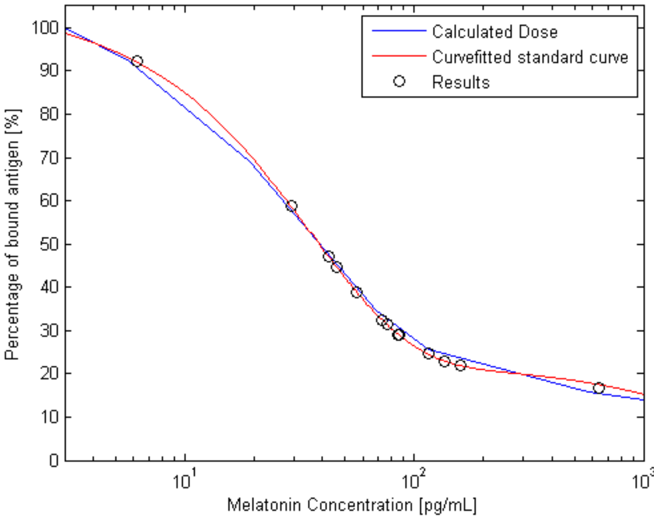


Figure B.1: Generated standard curve for Melatonin RIA. The blue line is the generated standard curve, the red line is the curvefitted standard curve used for the calculations of the results.

## B.2 Script: ExperimentPostprocessing.m

The following script calculate the polynomial of the standard curve, and plot the results of the exponential function. The function become:
$f(x) = a \cdot e^{(b \cdot x)} + c \cdot e^{(d \cdot x)}$

upquote

```
% ExperimentPostprocessing.m
%
%     Generic script for post processing of RIA experiment.
%
%     Author :  Guri Skauen
%     Date   :  24.02.2013
%
%
%     Ref:
%           [1] Melatonin Research RIA, "125 I - Radioimmunoassay
%               reagents for the direct quantitative determination
%               of Melatonin in biological liquids", Labor
%               Diagnostika Nord GmbH & Co, 2005
%
clear all;
close all;
clc;

%% Load data to workspace, and create variables

data.RIA.stdCurve.defDose = [...       % Standard curve data. [pg/↩
    mL CPP]
                    3.0  2191;
                    6.0  2033;
                    20.0  1561;
                    60.0  863;
                    200.0  680.5;
                    600.0  483.5;
                    2000.0  391.5];

data.RIA.stdCurve.calcDose = [...       % Standard curve data. [pg↩
    /mL CPP]
                    3.0  2191;
                    5.70021  2033;
                    19.45272  1561;
                    68.56031  863;
                    115.77033  680.5;
                    572.39712  483.5;
                    2000.0  391.5];

data.RIA.experiment.result.CPM = [...
                                1353;
```

```
                                            1115;
                                            605;
                                            818;
                                            797;
                                            948;
                                            1065;
                                            750;
                                            499;
                                            626;
                                            659;
                                            749;
                                            2032];

NSB = 160;                  % Non spesific binding
B_0 = 2191;                 % Standard A


%% Calculate the needed data

% Construct data for standard curve by creating the variable of ↩
    the
% precentage of CPM of each standard in relation to the CPM of the↩
     zero
% reference.
%
% According to the protocol [1], the equation to solve become:
%               ((B - NSB) / (B_0 - NSB)) * 100
%

% Calculations for calculated dose
data.RIA.stdCurve.calcDosePros = data.RIA.stdCurve.calcDose;
for i = 1:length(data.RIA.stdCurve.calcDosePros(:,1))
    data.RIA.stdCurve.calcDosePros(i,2) = ...
        ((data.RIA.stdCurve.calcDosePros(i,2) - NSB) / (B_0 - NSB)↩
            ) * 100;
end

% Calculations for defined dose
data.RIA.stdCurve.defDosePros = data.RIA.stdCurve.defDose;
for i = 1:length(data.RIA.stdCurve.defDosePros(:,1))
    data.RIA.stdCurve.defDosePros(i,2) = ...
        ((data.RIA.stdCurve.defDosePros(i,2) - NSB) / (B_0 - NSB))↩
             * 100;
end


%% Curvefit the standardcurve
% utilizing the MATLAB curvefit tool, cftool().
%   finction call:
%       cftool(data.RIA.stdCurve.calcDosePros(:,1), ...
%                                     data.RIA.stdCurve.↩
    calcDosePros(:,2))
```

```
%
%       cftool(data.RIA.stdCurve.defDosePros(:,1), ...
%                                          data.RIA.stdCurve.↩
    defDosePros(:,2))
%
%   Using exponential curvefit:
%
%       eqn : f(x) = a*exp(b*x) + c*exp(d*x)            (1)
%       Itterations: 3000
%       Coefficients (with 95% confidence bounds)
%

%%   Result for calculated dose in precent
curvefit.results.calcDosePros.a = 83.2;
curvefit.results.calcDosePros.b = -0.0281;
curvefit.results.calcDosePros.c = 22.24;
curvefit.results.calcDosePros.d = -0.000372;

curvefit.results.calcDosePros.goodness.SSE = 10.81;
curvefit.results.calcDosePros.goodness.Rsquare = 0.9987;
curvefit.results.calcDosePros.goodness.AdjustedRsquare = 0.9973;
curvefit.results.calcDosePros.goodness.RMSE = 1.899;

curvefit.results.calcDosePros.x = 0:1:2000;

a.calc = curvefit.results.calcDosePros.a;
b.calc = curvefit.results.calcDosePros.b;
c.calc = curvefit.results.calcDosePros.c;
d.calc = curvefit.results.calcDosePros.d;
x.calc = curvefit.results.calcDosePros.x;

% Create CPM precent vector
for i=1:length(curvefit.results.calcDosePros.x)
    curvefit.results.calcDosePros.y(i) = a.calc*exp(b.calc*x.calc(↩
        i))...
        + c.calc*exp(d.calc*x.calc(i));
end


%%   Result for defined dose in precent
curvefit.results.defDosePros.a = 82.7;
curvefit.results.defDosePros.b = -0.03272;
curvefit.results.defDosePros.c = 24.93;
curvefit.results.defDosePros.d = -0.0004476;

curvefit.results.defDosePros.goodness.SSE = 22.281;
curvefit.results.defDosePros.goodness.Rsquare = 0.9973;
curvefit.results.defDosePros.goodness.AdjustedRsquare = 0.9945;
curvefit.results.defDosePros.goodness.RMSE = 2.725;

curvefit.results.defDosePros.x = 0:1:2000;
```

```
a.def = curvefit.results.defDosePros.a;
b.def = curvefit.results.defDosePros.b;
c.def = curvefit.results.defDosePros.c;
d.def = curvefit.results.defDosePros.d;
x.def = curvefit.results.defDosePros.x;
% Create CPM precent vector
for i=1:length(curvefit.results.defDosePros.x)
    curvefit.results.defDosePros.y(i) = a.def*exp(b.def*x.def(i)) ↩
        + ...
        c.def*exp(d.def*x.def(i));
end



%% Start ploting

figure(1);
title('Standard Curve calculated dose');
semilogx(data.RIA.stdCurve.calcDosePros(:,1), ...
    data.RIA.stdCurve.calcDosePros(:,2),'-b');
hold on;
semilogx(curvefit.results.calcDosePros.x,...
    curvefit.results.calcDosePros.y, 'r');

% Remove % after intercept have been done

%axis([3 1000 0 105]);
%legend('Calculated Dose', 'Curvefitted standard curve');
%xlabel('Melatonin Concentration [pg/mL]')
%ylabel('Percentage of bound antigen [%]')


%% Calculating the results
% Based on equation (1), using the meassured data, we retrieve the
% final concentration
clear B;
B = data.RIA.experiment.result.CPM;
for i=1:length(B);
    data.RIA.experiment.result.CPM(i) = ((B(i) - NSB) / (B_0 - NSB↩
        )) * 100;
end

y = data.RIA.experiment.result.CPM;

for j=1:length(y)
   data.RIA.experiment.result.pgml(j) = (log(y(j)) - log(a.calc)- ↩
        ...
        log(c.calc))/(b.calc + d.calc);
end
%
% results = [data.RIA.experiment.result.pgml' y];
% results(:,1)
```

```
%
% semilogx(results(:,1), results(:,2), 'ko');
%X = [1; 1000];
%Y = [92.1713; 92.1713];
hold on;
X = [29.09 42.53 158.6 72.65 76.59 56.61 46.27 85.72 640.35 ↩
    135.6...
    115.2 85.56 6.192];
Y = [58.7395 47.0212 21.9104 32.3978 31.3639 38.7986 44.5593 ↩
    29.0497...
    16.6913 22.9444 24.5692 29.0005 92.1713];

semilogx(X, Y, 'ko');

axis([3 1000 0 105]);
legend('Calculated Dose', 'Curvefitted standard curve', 'Results')↩
    ;
xlabel('Melatonin Concentration [pg/mL]')
ylabel('Percentage of bound antigen [%]')

% figure(2);
% title('Standard Curve defined dose');
% semilogx(data.RIA.stdCurve.defDosePros(:,1), ...
%           data.RIA.stdCurve.defDosePros(:,2),'-bo');
% hold on;
% semilogx(curvefit.results.defDosePros.x, ...
%       curvefit.results.defDosePros.y, 'r');
% axis([3 1000 0 105]);
% legend('Defined Dose', 'Curvefitted standard curve');
% xlabel('Melatonin Concentration [pg/mL]')
% ylabel('Percentage of bound antigen [%]')



%% Print results to display

disp('Results:');
disp(' ');
disp(['Standard A :' num2str(X(13))]);
disp(' ');
disp(['PL1 :' num2str(X(1))]);
disp(['PL2 :' num2str(X(2))]);
disp(['PL4 :' num2str(X(3))]);
disp(['PL5 :' num2str(X(4))]);
disp(['PL6 :' num2str(X(5))]);
disp(['PL7 :' num2str(X(6))]);

disp(['PM11 :' num2str(X(7))]);
disp(['PM12 :' num2str(X(8))]);
disp(['PM13 :' num2str(X(9))]);
disp(['PM14 :' num2str(X(10))]);
disp(['PM15 :' num2str(X(11))]);
```

```
disp(['PM16 :' num2str(X(12))]);
```

## B.3 Parallelism curve for Melatonin RIA

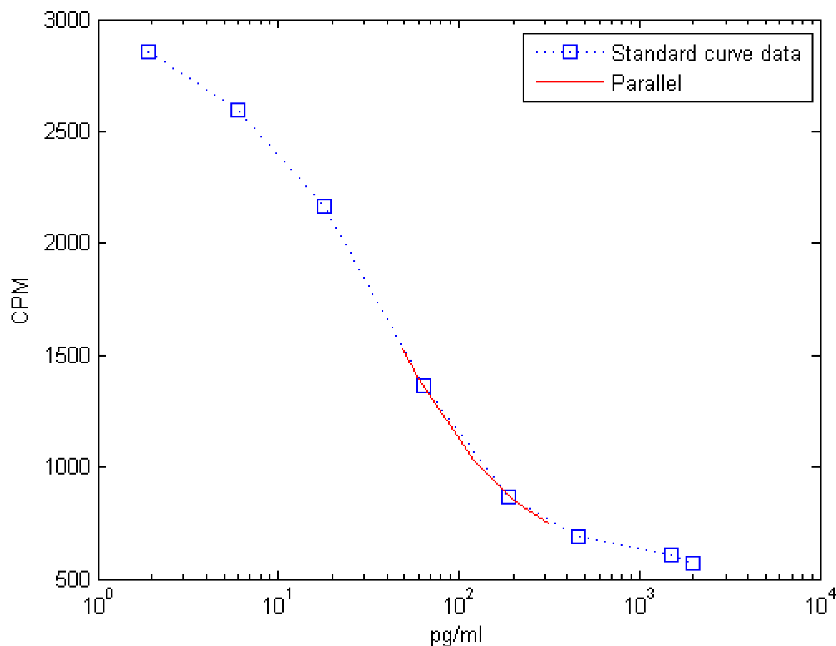


Figure B.2: Parallelisme curve and the standard curve for Melatonin RIA

## B.4 Script:polyFitandParallel.m

The following script plots the results from the parallelism validation.

upquote

```
% polyFitandPlot.m
%
%   Generic script for calculating the polynom of standard curve, ↩
    and
```

```
%   plotting the results of n-order polynomials
%
%   Author   : Guri Skauen
%   Date     : 06.02.2013
%
%   History :
%           1.0 - First version
%


close all;
clear;
clc;

%% Define dataset and important variables
%            CPM    pg/ml
dataset = [2856 1.9; 2592 6; 2164 18; 1361 64; 869 188;688 457; ↩
    604 1501; 570 2000];
%dataset = [10234 0; 9621 30; 8413 100; 6389 300; 4171 1000; 2863 ↩
    3000; 2077 10000];



start = round(dataset(1, 2));
slutt = round(dataset(length(dataset), 2));

CPM = dataset(:,1);
pgml = dataset(:,2);

y = CPM;
x = pgml;


semilogx(x, y, ':s');
xlabel('pg/ml');
ylabel('CPM');
legend('Standard curve data', 'Location', 'NorthEast');
hold on;



% Calculate the 3rd order polynomial
poly3order = polyfit(pgml, CPM, 3)
for i = start:1:slutt
    dataPoly(i-(start-1),1)= i;
    dataPoly(i-(start-1),2) = polyval(poly3order, i);
end
% polyval(poly1order, 1462)
%plot(dataPoly(:,1), dataPoly(:,2), 'r');


% Extrapolate for low CPM, by utilizing three known values for low↩
```

```
    CPM
dataToExtrapolate = [688 457; 570 1501; 570 2000];
a = polyfit(dataToExtrapolate(:,1),dataToExtrapolate(:,2), 1);
CPMtoFind = 572
CPMtoFind*a(1)+a(2)


% plot parallelity
%pl = [746 314; 852 200; 1028 122; 1396 60; 1531 49];
pl = [746 314; 852 200; 1028 122; 1396 60; 1531 49];
plot(pl(:,2), pl(:,1), 'r')
legend('Standard curve data', 'Parallel', 'Location', 'NorthEast')↩
    ;
```

# C. ANOVA tables

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 58356.2 | 7 | 8336.61 | 7.35 | 1.13073e-05 |
| **Error** | 45352.3 | 40 | 1133.81 | | |
| **Total** | 103708.6 | 47 | | | |

Figure C.1: Vtg mRNA expression. All groups.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 20258.4 | 6 | 3376.39 | 2.61 | 0.0341 |
| **Error** | 45350.3 | 35 | 1295.72 | | |
| **Total** | 65608.6 | 41 | | | |

Figure C.2: Vtg mRNA expression. All groups other than the control.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 17788.4 | 5 | 3557.69 | 4.85 | 0.0023 |
| **Error** | 22004 | 30 | 733.47 | | |
| **Total** | 39792.4 | 35 | | | |

Figure C.3: Vtg mRNA expression. Groups treated with cortisol, and cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 10502.6 | 1 | 10502.6 | 13.1 | 0.0047 |
| Error | 8017.2 | 10 | 801.7 | | |
| Total | 18519.8 | 11 | | | |

Figure C.4: Vtg mRNA expression. 10 nM cortisol and 10 nM cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 1958.69 | 1 | 1958.69 | 6.13 | 0.0327 |
| Error | 3193.42 | 10 | 319.34 | | |
| Total | 5152.11 | 11 | | | |

Figure C.5: Vtg mRNA expression. 100 nM cortisol and 100 nM cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 4157.5 | 1 | 4157.54 | 3.85 | 0.0781 |
| Error | 10793.4 | 10 | 1079.34 | | |
| Total | 14950.9 | 11 | | | |

Figure C.6: Vtg mRNA expression. 1000 nM cortisol and 1000 nM cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 538.337 | 7 | 79.9053 | 7.71 | 6.92279e-06 |
| Error | 339.065 | 40 | 9.9766 | | |
| Total | 937.402 | 47 | | | |

Figure C.7: ER mRNA expression. All groups other than the control.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 129.874 | 5 | 25.9749 | 2.37 | 0.0635 |
| Error | 329.427 | 30 | 10.9809 | | |
| Total | 459.302 | 35 | | | |

Figure C.8: ER mRNA expression. Groups treated with cortisol, and cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 29.714 | 1 | 29.7145 | 1.46 | 0.2555 |
| Error | 204.213 | 10 | 20.4213 | | |
| Total | 233.927 | 11 | | | |

Figure C.9: ER mRNA expression. 10 nM cortisol and 10 nM cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 0.9625 | 1 | 0.96251 | 0.29 | 0.6041 |
| Error | 33.5797 | 10 | 3.35797 | | |
| Total | 34.5422 | 11 | | | |

Figure C.10: ER mRNA expression. 100 nM cortisol and 100 nM cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 44.342 | 1 | 44.3419 | 4.84 | 0.0525 |
| Error | 91.635 | 10 | 9.1635 | | |
| Total | 135.977 | 11 | | | |

Figure C.11: ER mRNA expression. 1000 nM cortisol and 1000 nM cortisol + MEL.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 84493.8 | 3 | 28164.6 | 1.92 | 0.1585 |
| **Error** | 292999.4 | 20 | 14650 | | |
| **Total** | 377493.2 | 23 | | | |

Figure C.12: Melatonin in blood plasma. All groups.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 42984.3 | 1 | 42984.3 | 1.67 | 0.2258 |
| **Error** | 257982.2 | 10 | 25798.2 | | |
| **Total** | 300966.4 | 11 | | | |

Figure C.13: Melatonin in blood plasma. Periphere blood system, light and dark.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 694.4 | 1 | 694.38 | 0.2 | 0.6656 |
| **Error** | 35017.2 | 10 | 3501.72 | | |
| **Total** | 35711.6 | 11 | | | |

Figure C.14: Melatonin in blood plasma. Vena portae, light and dark.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | SS | df | MS | F | Prob>F |
| **Columns** | 205186.5 | 3 | 68395.5 | 4.48 | 0.0147 |
| **Error** | 305673.1 | 20 | 15283.7 | | |
| **Total** | 510859.5 | 23 | | | |

Figure C.15: Cortisol in blood plasma. All groups.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 37000.7 | 1 | 37000.7 | 1.86 | 0.2025 |
| Error | 198918.7 | 10 | 19891.9 | | |
| Total | 235919.4 | 11 | | | |

Figure C.16: Cortisol in blood plasma. Periphere blood system, light and dark.

| ANOVA Table | | | | | |
|---|---|---|---|---|---|
| Source | SS | df | MS | F | Prob>F |
| Columns | 2399.6 | 1 | 2399.6 | 0.22 | 0.6456 |
| Error | 106754.4 | 10 | 10675.4 | | |
| Total | 109153.9 | 11 | | | |

Figure C.17: Cortisol in blood plasma. Vena portae, light and dark.

# D. Experimental Data and MATLAB scripts

This Appendix contain the additional MATLAB scripts written during the work on this thesis, including the experimental data for the individual experiments. The data from each experiment is found in the script associated with the individual experiment.

## D.1 Script: Experiment1A.m

upquote

```
%      Experiment1A.m
%
%      Generic script for analysing data for experiment 1A,
%      using the ANOVA method.
%
%
%      Author :  Guri Skauen
%      Date   :  26.04.2013
%
%
%      Ref:
%            [1]
%
%
clear all;
close all;
clc;

% Add data and create variables
VPL = [...
        40.375;
```

```
        20.343;
        201.410;
        192.620;
        81.007;
        98.052
        ];

PL = [...
        29.090;
        42.530;
        317.200;
        145.300;
        76.590;
        113.220
        ];

VPD = [...
        91.497;
        155.430;
        84.341;
        51.064;
        85.287;
        74.905
        ];

PD = [...
        93.220;
        171.440;
        640.350;
        135.600;
        230.400;
        171.120
        ];


%% Constructing data matrices

data = [VPL(:,1) PL(:,1) VPD(:,1) PD(:,1)];
data_VP = [VPL(:,1) VPD(:,1)];
data_P = [PL(:,1) PD(:,1)];

%% Plot setup
%

dataToCalc = {...                % [fileName SaveName]
                data 'result_all';
                data_VP 'result_VP';
                data_P 'result_P';
                };

%% DO NOT EDIT BELOW THIS LINE
%
```

```
% Calculate and plot ANOVA
for i=1:length(dataToCalc(:,1))
    results(i) = anova1(dataToCalc{i});
    grid on;
end

% Save results
figHandles = get(0,'Children');
cd figures2A;
for i=1:length(dataToCalc(:,1))
    saveas(figHandles(i*2-1), [dataToCalc{(length(dataToCalc)-i+1)↩
        , 2} '_plot'], 'png');
    saveas(figHandles(i*2), [dataToCalc{(length(dataToCalc)-i+1), ↩
        2} '_table'], 'png');
end
cd ..;
figHandles = get(0,'Children');
results = 'OK';
for i=1:(length(figHandles)-1)
    if figHandles(i) < figHandles(i+1)
        results = 'failed';
    end
end
disp(['Save figures and tables ' results]);
close all;
%% End of script
```

## D.2 Script: Experiment1B.m

upquote

```
%     Experiment1B.m
%
%     Generic script for analysing data for experiment 1B,
%     using the ANOVA method.
%
%
%     Author :  Guri Skauen
%     Date   :  23.04.2013
%
%
%     Ref:
%           [1]
%
%
clear all;
close all;
clc;
```

```
% Add data and create variables
VPL = [...
        280.63;
        287.51;
        162.08;
        335.91;
        521.94;
        380.97
        ];

PL = [...
        454.43;
        474.2;
        403.94;
        666.95;
        638.88;
        576.32
        ];

VPD = [...
        278.17;
        358.93;
        268.95;
        254.03;
        203.31;
        435.96
        ];

PD = [...
        289.34;
        508.18;
        413.46;
        313.95;
        300.99;
        722.46
        ];


%% Constructing data matrices

data = [VPL(:,1) PL(:,1) VPD(:,1) PD(:,1)];
data_VP = [VPL(:,1) VPD(:,1)];
data_P = [PL(:,1) PD(:,1)];

%% Plot setup
%

dataToCalc = {...               % [fileName SaveName]
                data 'result_all';
                data_VP 'result_VP';
                data_P 'result_P';
```

```
                    };

%% DO NOT EDIT BELOW THIS LINE
%
% Calculate and plot ANOVA
for i=1:length(dataToCalc(:,1))
    results(i) = anova1(dataToCalc{i});
end

% Save results
figHandles = get(0,'Children');
cd figures2B;
for i=1:length(dataToCalc(:,1))
    saveas(figHandles(i*2-1), [dataToCalc{(length(dataToCalc)-i+1)↩
        , 2} '_plot'], 'png');
    saveas(figHandles(i*2), [dataToCalc{(length(dataToCalc)-i+1), ↩
        2} '_table'], 'png');
end
cd ..;
figHandles = get(0,'Children');
results = 'OK';
for i=1:(length(figHandles)-1)
    if figHandles(i) < figHandles(i+1)
        results = 'failed';
    end
end
disp(['Save figures and tables ' results]);
close all;
%% End of script
```

## D.3 Script: Experiment2A.m

upquote

```
%   Experiment2A.m
%
%     Generic script for analysing data for experiment 2A,
%     using the ANOVA method.
%
%
%     Author :  Guri Skauen
%     Date   :  22.04.2013
%
%
%     Ref:
%           [1]
%
%
```

```
clear all;
close all;
clc;

% Add data and create variables

a = [...
        0.745355992;
        0.915607042;
        1.158292185;
        0.36600247;
        1.600361165;
        2.159767401];

b = [...
        219.2042004;
        76.54398837;
        49.54609533;
        52.5456398;
        157.7234503;
        75.2060731];

c = [...
        121.7801113;
        96.83226481;
        183.7668285;
        127.6433422;
        71.33101224;
        123.2201737];

d = [...
        111.5027275;
        97.97731637;
        90.23756945;
        100.3082006;
        89.18297159;
        56.38575306;
        ];

e = [...
        56.38575306;
        103.299939;
        73.45849262;
        145.26404;
        116.1862049;
        98.91888592;
        ];

f = [...
        73.8915433;
        50.42751985;
        53.16699743;
```

```
        84.09200133;
        51.93154392;
        56.05529726];

g = [...
        68.85921014;
        92.92894663;
        68.85921014;
        65.38058402;
        42.775127;
        53.48042619];

h = [...
        45.63218696;
        57.72717238;
        45.09888794;
        101.4943555;
        103.299939;
        16.89899002];

%% Constructing data matrices

data= [a(:,1) b(:,1) c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];
data_ab = [a(:,1) b(:,1)];
data_b2h = [b(:,1) c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];
data_c2h = [c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];

data_cf = [c(:,1) f(:,1)];
data_dg = [d(:,1) g(:,1)];
data_eh = [e(:,1) h(:,1)];

%% Plot setup
%

dataToCalc = {...                % [fileName SaveName]
                data 'result_a2h';
                data_b2h 'result_b2h';
                data_c2h 'result_c2h';
                data_cf 'result_cf';
                data_dg 'result_dg';
                data_eh 'result_eh'
                };

%% DO NOT EDIT BELOW THIS LINE
%
% Calculate and plot ANOVA
for i=1:length(dataToCalc(:,1))
    results(i) = anova1(dataToCalc{i});
end

% Save results
figHandles = get(0,'Children');
```

```
cd figures1A;
for i=1:length(dataToCalc(:,1))
    saveas(figHandles(i*2-1), [dataToCalc{(length(dataToCalc)-i+1)↩
        , 2} '_plot'], 'png');
    saveas(figHandles(i*2), [dataToCalc{(length(dataToCalc)-i+1), ↩
        2} '_table'], 'png');
end
cd ..;
figHandles = get(0,'Children');
results = 'OK';
for i=1:(length(figHandles)-1)
    if figHandles(i) < figHandles(i+1)
        results = 'failed';
    end
end
disp(['Save figures and tables ' results]);
close all;
%% End of script
```

## D.4 Script: Experiment2B.m

upquote

```
%       Experiment2B.m
%
%       Generic script for analysing data for experiment 2B,
%       using the ANOVA method.
%
%
%       Author :  Guri Skauen
%       Date   :  22.04.2013
%
%
%       Ref:
%             [1]
%
%
clear all;
close all;
clc;

% Add data and create variables

a = [...
        0.995108597;
        1.357927104;
        0.944739202;
        0.955394146;
```

```
            1.311945214;
            0.624946519
            ];

b = [...
            5.592469648;
            4.67033477;
            12.17208888;
            12.93264584;
            12.81095688;
            10.66614949
            ];

c = [...
            5.491070398;
            12.59646811;
            7.482021442;
            6.117521465;
            15.99785888;
            11.42330334
            ];

d = [...
            8.682714811;
            8.518307618;
            11.06234699;
            10.22639602;
            8.62596337;
            13.05912208
            ];

e = [...
            13.93600142;
            7.085877306;
            7.963967603;
            12.05839469;
            10.32998545;
            10.60568624
            ];

f = [...
            10.495268;
            5.359828695;
            12.65263318;
            19.80516318;
            14.74827702;
            14.93022834
            ];

g = [...
            9.845373626;
            9.144016428;
```

```
        11.47503531;
        6.103456688;
        9.462716001;
        10.74569795
        ];

h = [...
        8.170998424;
        10.49515921;
        9.350038151;
        2.129753668;
        5.885179307;
        2.88143332
        ];

%% Constructing data matrices

data= [a(:,1) b(:,1) c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];
data_ab = [a(:,1) b(:,1)];
data_b2h = [b(:,1) c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];
data_c2h = [c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];

data_cf = [c(:,1) f(:,1)];
data_dg = [d(:,1) g(:,1)];
data_eh = [e(:,1) h(:,1)];

%% Plot setup
%

dataToCalc = {...                % [fileName SaveName]
                data 'result_a2h';
                data_b2h 'result_b2h';
                data_c2h 'result_c2h';
                data_cf 'result_cf';
                data_dg 'result_dg';
                data_eh 'result_eh'
                };

%% DO NOT EDIT BELOW THIS LINE
%
% Calculate and plot ANOVA
for i=1:length(dataToCalc(:,1))
    results(i) = anova1(dataToCalc{i});
end

% Save results
figHandles = get(0,'Children');
cd figures1B;
for i=1:length(dataToCalc(:,1))
    saveas(figHandles(i*2-1), [dataToCalc{(length(dataToCalc)-i+1)↩
        , 2} '_plot'], 'png');
    saveas(figHandles(i*2), [dataToCalc{(length(dataToCalc)-i+1), ↩
```

```
        2} '_table'], 'png');
end
cd ..;
figHandles = get(0,'Children');
results = 'OK';
for i=1:(length(figHandles)-1)
    if figHandles(i) < figHandles(i+1)
        results = 'failed';
    end
end
disp(['Save figures and tables ' results]);
close all;
%% End of script
```

## D.5 Script: plotScript.m

upquote

```
%   plotScript.m
%
%     Script for generating plots from the master thesis ↩
    experiments
%
%
%     Author :  Guri Skauen
%     Date   :  12.05.2013
%
%
%     Ref:
%            [1]
%
%

clear all;
close all;
clc;

%% Experiment 2A

% Add data and create variables

a = [...
        0.745355992;
        0.915607042;
        1.158292185;
        0.36600247;
        1.600361165;
        2.159767401];
```

```
b = [...
        219.2042004;
        76.54398837;
        49.54609533;
        52.5456398;
        157.7234503;
        75.2060731];

c = [...
        121.7801113;
        96.83226481;
        183.7668285;
        127.6433422;
        71.33101224;
        123.2201737];

d = [...
        111.5027275;
        97.97731637;
        90.23756945;
        100.3082006;
        89.18297159;
        56.38575306;
        ];

e = [...
        56.38575306;
        103.299939;
        73.45849262;
        145.26404;
        116.1862049;
        98.91888592;
        ];

f = [...
        73.8915433;
        50.42751985;
        53.16699743;
        84.09200133;
        51.93154392;
        56.05529726];

g = [...
        68.85921014;
        92.92894663;
        68.85921014;
        65.38058402;
        42.775127;
        53.48042619];

h = [...
```

```
        45.63218696;
        57.72717238;
        45.09888794;
        101.4943555;
        103.299939;
        16.89899002];

%% Constructing data matrices

data= [a(:,1) b(:,1) c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];

width = 0.5;

% Calculating the standard diviation and the standard diviation of↩
    the
% error mean

a_std = std(a)/sqrt(length(a));
b_std = std(b)/sqrt(length(b));
c_std = std(c)/sqrt(length(c));
d_std = std(d)/sqrt(length(d));
e_std = std(e)/sqrt(length(e));
f_std = std(f)/sqrt(length(f));
g_std = std(g)/sqrt(length(g));
h_std = std(h)/sqrt(length(h));

% calculate mean
a_mean = mean(a);
b_mean = mean(b);
c_mean = mean(c);
d_mean = mean(d);
e_mean = mean(e);
f_mean = mean(f);
g_mean = mean(g);
h_mean = mean(h);

data = [a_mean b_mean c_mean f_mean d_mean g_mean e_mean h_mean];
data_std = [a_std b_std c_std f_std d_std g_std e_std h_std];

data_cf = [c_mean f_mean];
data_cf_std = [c_std f_std];
data_dg = [d_mean g_mean];
data_dg_std = [d_std g_std];
data_eh = [e_mean h_mean];
data_eh_std = [e_std h_std];

data_white = [0];
data_white_std = [0];

data_combi = [c_mean f_mean data_white d_mean g_mean data_white ↩
    e_mean h_mean];
data_combi_std = [c_std f_std data_white_std d_std g_std ↩
```

```
    data_white_std e_std h_std];

colorMap = [0.4 0.4 0.4; 0.8 0.8 0.8; 1 1 1; 0.4 0.4 0.4; 0.8 0.8 ↩
    0.8; 1 1 1; 0.4 0.4 0.4; 0.8 0.8 0.8];
colorMap_data = [1 1 1; 0 0 0; 0.4 0.4 0.4; 0.8 0.8 0.8; 0.4 0.4 ↩
    0.4; 0.8 0.8 0.8; 0.4 0.4 0.4; 0.8 0.8 0.8;];
%% Start Plot Experiment 1A

% Plot mean values and standard diviation
figure(1);
handles = barweb(data, data_std, [0.5], [], [], [], [], ↩
    colorMap_data, [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1A_all_data', 'png');

% Plot subplots
figure(2);
handles = barweb(data_cf, data_cf_std, [0.3], [], [], [], [], [0.4↩
     0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1A_cf', 'png');

figure(3);
handles = barweb(data_dg, data_dg_std, [0.3], [], [], [], [], [0.4↩
     0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1A_dg', 'png');

figure(4);
handles = barweb(data_eh, data_eh_std, [0.3], [], [], [], [], [0.4↩
     0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1A_eh', 'png');

figure(5);
handles = barweb(data_combi, data_combi_std, [0.5], [], [], [], ↩
    [], colorMap, [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1A_combi', 'png');



%% Experiment 2B
clear;
% Add data and create variables

a = [...
        0.995108597;
        1.357927104;
        0.944739202;
        0.955394146;
        1.311945214;
```

```
          0.624946519
          ];

b = [...
          5.592469648;
          4.67033477;
          12.17208888;
          12.93264584;
          12.81095688;
          10.66614949
          ];

c = [...
          5.491070398;
          12.59646811;
          7.482021442;
          6.117521465;
          15.99785888;
          11.42330334
          ];

d = [...
          8.682714811;
          8.518307618;
          11.06234699;
          10.22639602;
          8.62596337;
          13.05912208
          ];

e = [...
          13.93600142;
          7.085877306;
          7.963967603;
          12.05839469;
          10.32998545;
          10.60568624
          ];

f = [...
          10.495268;
          5.359828695;
          12.65263318;
          19.80516318;
          14.74827702;
          14.93022834
          ];

g = [...
          9.845373626;
          9.144016428;
          11.47503531;
```

```
        6.103456688;
        9.462716001;
        10.74569795
        ];

h = [...
        8.170998424;
        10.49515921;
        9.350038151;
        2.129753668;
        5.885179307;
        2.88143332
        ];

%% Constructing data matrices

%data= [a(:,1) b(:,1) c(:,1) d(:,1) e(:,1) f(:,1) g(:,1) h(:,1)];

width = 0.5;

% Calculating the standard diviation and the standard diviation of↩
    the
% error mean

a_std = std(a)/sqrt(length(a));
b_std = std(b)/sqrt(length(b));
c_std = std(c)/sqrt(length(c));
d_std = std(d)/sqrt(length(d));
e_std = std(e)/sqrt(length(e));
f_std = std(f)/sqrt(length(f));
g_std = std(g)/sqrt(length(g));
h_std = std(h)/sqrt(length(h));

% calculate mean
a_mean = mean(a);
b_mean = mean(b);
c_mean = mean(c);
d_mean = mean(d);
e_mean = mean(e);
f_mean = mean(f);
g_mean = mean(g);
h_mean = mean(h);

data = [a_mean b_mean c_mean f_mean d_mean g_mean e_mean h_mean];
data_std = [a_std b_std c_std f_std d_std g_std e_std h_std];

data_cf = [c_mean f_mean];
data_cf_std = [c_std f_std];
data_dg = [d_mean g_mean];
data_dg_std = [d_std g_std];
data_eh = [e_mean h_mean];
data_eh_std = [e_std h_std];
```

```
data_white = [0];
data_white_std = [0];

data_combi = [c_mean f_mean data_white d_mean g_mean data_white ↩
    e_mean h_mean];
data_combi_std = [c_std f_std data_white_std d_std g_std ↩
    data_white_std e_std h_std];

colorMap = [0.4 0.4 0.4; 0.8 0.8 0.8; 1 1 1; 0.4 0.4 0.4; 0.8 0.8 ↩
    0.8; 1 1 1; 0.4 0.4 0.4; 0.8 0.8 0.8];
colorMap_data = [1 1 1; 0 0 0; 0.4 0.4 0.4; 0.8 0.8 0.8; 0.4 0.4 ↩
    0.4; 0.8 0.8 0.8; 0.4 0.4 0.4; 0.8 0.8 0.8;];

%% Start Plot Experiment 1B

% Plot mean values and standard diviation
figure(6);
handles = barweb(data, data_std, [0.5], [], [], [], [], ↩
    colorMap_data, [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1B_all_data', 'png');

% Plot subplots
figure(7);
handles = barweb(data_cf, data_cf_std, [0.3], [], [], [], [], [0.4↩
     0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1B_cf', 'png');

figure(8);
handles = barweb(data_dg, data_dg_std, [0.3], [], [], [], [], [0.4↩
     0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1B_dg', 'png');

figure(9);
handles = barweb(data_eh, data_eh_std, [0.3], [], [], [], [], [0.4↩
     0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1B_eh', 'png');

figure(10);
handles = barweb(data_combi, data_combi_std, [0.5], [], [], [], ↩
    [], colorMap, [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment1B_combi', 'png');


%% Experiment 1A
clear;
% Add data and create variables
```

```
% Add data and create variables
VPL = [...
        40.375;
        20.343;
        201.410;
        192.620;
        81.007;
        98.052
        ];

PL = [...
        29.090;
        42.530;
        317.200;
        145.300;
        76.590;
        113.220
        ];

VPD = [...
        91.497;
        155.430;
        84.341;
        51.064;
        85.287;
        74.905
        ];

PD = [...
        93.220;
        171.440;
        640.350;
        135.600;
        230.400;
        171.120
        ];

%% Constructing data matrices

width = 0.5;

% Calculating the standard diviation and the standard diviation of↩
    the
% error mean

VPL_std = std(VPL)/sqrt(length(VPL));
PL_std = std(PL)/sqrt(length(PL));
VPD_std = std(VPD)/sqrt(length(VPD));
PD_std = std(PD)/sqrt(length(PD));

% calculate mean
VPL_mean = mean(VPL);
```

```
PL_mean = mean(PL);
VPD_mean = mean(VPD);
PD_mean = mean(PD);

data_white = [0];
data_white_std = [0];

data = [PL_mean VPL_mean PD_mean VPD_mean];
data_std = [PL_std VPL_std PD_std VPD_std];

data_PL_PD = [PL_mean data_white PD_mean];
data_PL_PD_std = [PL_std data_white_std PD_std];
data_VPL_VPD = [VPL_mean data_white VPD_mean];
data_VPL_VPD_std = [VPL_std data_white_std VPD_std];

data_PL_VPL = [PL_mean VPL_mean];
data_PL_VPL_std = [PL_std VPL_std];
data_PD_VPD = [PD_mean VPD_mean];
data_PD_VPD_std = [PD_std VPD_std];

colorMap = [0.4 0.4 0.4; 0.4 0.4 0.4; 0.8 0.8 0.8; 0.8 0.8 0.8];

%% Start Plot Experiment 2A

% Plot mean values and standard diviation
figure(11);
handles = barweb(data, data_std, [0.5], [], [], [], [], [0.4 0.4 ↩
    0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2A_all_data', 'png');

% Plot subplots
figure(12);
handles = barweb(data_PL_PD, data_PL_PD_std, [0.5], [], [], [], ↩
    [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2A_PL_PD', 'png');

figure(13);
handles = barweb(data_VPL_VPD, data_VPL_VPD_std, [0.5], [], [], ↩
    [], [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2A_VPL_VPD', 'png');

figure(14);
handles = barweb(data_PL_VPL, data_PL_VPL_std, [0.4], [], [], [], ↩
    [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2A_PL_VPL', 'png');

figure(15);
handles = barweb(data_PD_VPD, data_PD_VPD_std, [0.4], [], [], [], ↩
```

```
    [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2A_PD_VPD', 'png');


%% Experiment 1B
clear;
% Add data and create variables

VPL = [...
        280.63;
        287.51;
        162.08;
        335.91;
        521.94;
        380.97
        ];

PL = [...
        454.43;
        474.2;
        403.94;
        666.95;
        638.88;
        576.32
        ];

VPD = [...
        278.17;
        358.93;
        268.95;
        254.03;
        203.31;
        435.96
        ];

PD = [...
        289.34;
        508.18;
        413.46;
        313.95;
        300.99;
        722.46
        ];


%% Constructing data matrices

width = 0.5;

% Calculating the standard diviation and the standard diviation of↩
    the
```

```
% error mean

VPL_std = std(VPL)/sqrt(length(VPL));
PL_std = std(PL)/sqrt(length(PL));
VPD_std = std(VPD)/sqrt(length(VPD));
PD_std = std(PD)/sqrt(length(PD));

% calculate mean
VPL_mean = mean(VPL);
PL_mean = mean(PL);
VPD_mean = mean(VPD);
PD_mean = mean(PD);

data_white = [0];
data_white_std = [0];

data = [PL_mean VPL_mean PD_mean VPD_mean];
data_std = [PL_std VPL_std PD_std VPD_std];

data_PL_PD = [PL_mean data_white PD_mean];
data_PL_PD_std = [PL_std data_white_std PD_std];
data_VPL_VPD = [VPL_mean data_white VPD_mean];
data_VPL_VPD_std = [VPL_std data_white_std VPD_std];

data_PL_VPL = [PL_mean VPL_mean];
data_PL_VPL_std = [PL_std VPL_std];
data_PD_VPD = [PD_mean VPD_mean];
data_PD_VPD_std = [PD_std VPD_std];



colorMap = [0.4 0.4 0.4; 0.4 0.4 0.4; 0.8 0.8 0.8; 0.8 0.8 0.8];

%% Start Plot Experiment 2B

% Plot mean values and standard diviation
figure(16);
handles = barweb(data, data_std, [0.5], [], [], [], [], [0.4 0.4 ↩
    0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2B_all_data', 'png');

% Plot subplots
figure(17);
handles = barweb(data_PL_PD, data_PL_PD_std, [0.5], [], [], [], ↩
    [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2B_PL_PD', 'png');

figure(18);
handles = barweb(data_VPL_VPD, data_VPL_VPD_std, [0.5], [], [], ↩
    [], [], [0.4 0.4 0.4], [], [], 1, 'plot');
```

```
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2B_VPL_VPD', 'png');

figure(19);
handles = barweb(data_PL_VPL, data_PL_VPL_std, [0.4], [], [], [], ↩
    [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2B_PL_VPL', 'png');

figure(20);
handles = barweb(data_PD_VPD, data_PD_VPD_std, [0.4], [], [], [], ↩
    [], [0.4 0.4 0.4], [], [], 1, 'plot');
set(gca,'YGrid','on');
saveas(gcf, 'Experiment2B_PD_VPD', 'png');
```