Reproductive physiology of the female three-spined stickleback, *Gasterosteus aculeatus*

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Abstract

Reproduction in vertebrates, including fishes, is under control of the brain-pituitary-gonad (BPG) axis. The female three-spined stickleback, *Gasterosteus aculeatus*, produces egg clutches at intervals of a few days and spawns them in a nest built by male. Following ovulation, eggs are stored in the ovarian cavity surrounded by the ovarian fluid (OF). If spawning or spontaneous release do not occur, the eggs can undergo overripening, a phenomenon occurring both in nature and captivity.

In this PhD thesis, the changes of reproductive hormones and vitellogenesis were studied at overripening of eggs and over the natural spawning cycle. OF properties were also examined at overripening of eggs and after treatment with sex steroids.

Plasma levels of steroids: testosterone (T), estradiol (E2), 17,20β-dihydroxyprogren-4-en-3-one (17,20β-P) and 17,20β,21-trihydropregen-4-en-3-one (17,20β,21-P) were measured by radioimmunoassay, and relative mRNA levels of the pituitary gonatropins (fshβ/lhβ), brain gonadotropin-releasing hormones (gnrh2/gnrh3) and kisspeptin and its receptor (kiss2/gpr54) by qPCR. Overripening of eggs was accompanied with a significant reduction in most of endocrine parameters of BPG axis (T; E2; 17,20β-P; lhβ; kiss2, gpr54). Low level of hormones could be advantageous for the overripen egg-bound females, since this would reduce further ovulations giving higher chances to survive and reproduce again. Over the 3-day spawning cycle, T and E2 were highly correlated, showed cyclicity with low levels at ovulation and increasing from 24 and 6 hours post-spawning (hps), respectively. Spawning may give rise to this increase as these rises did not occur if release of the eggs does not happen (overripen females). A peak at pituitary lhβ mRNA levels appeared 48 hps, a day before the next ovulation. No significant changes were found for the other studied hormones.

Vitellogenesis was studied by measurement of the vitellogenin mRNA levels in the liver by qPCR. The levels were highest at 24 and 48 hps and were positively correlated to both E2 and T over the cycle. However, changes were small suggesting a rather continuous vitellogenesis over the stickleback spawning cycle which could be an advantage for a multiple spawner with a limited spawning season. Overripening reduced vitellogenin mRNA levels but did not abolish it.

OF amount was diminished in overripen females and had a lower viscosity but higher dry weight and protein levels than in non-overripen ovulated females, suggesting that changes in OF properties are related to the egg overripening. The effects of steroids were studied using Silastic capsules. T and 17,20β-P induced an increase of OF amount, but protein levels were only increased in 17,20β-P-treated females, proposing a role of this steroid in the control of OF secretion. 1-D SDS-PAGE showed that OF contained several proteins, some of them came from eggs, but no consistent differences between groups.

Concluding, the knowledge of the reproductive physiological changes is important for understanding their essential roles in the production of viable eggs in this species but also in the reproductive physiology of female fishes in general.

Keywords: overripening of eggs, spawning cycle, brain-pituitary-gonad (BPG) axis, vitellogenin, ovarian fluid, sex steroids, reproduction, three-spined stickleback, Gasterosteus aculeatus, fish.

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Chrysoula Roufidou
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*Gasterosteus aculeatus*

Chrysoula Roufidou
To my families
The thesis is based on the following articles, which are referred to in the text by their Roman numerals:


III Roufidou, C., Mentor, A. & Borg, B. Vitellogenesis in the three-spined stickleback, *Gasterosteus aculeatus*, over the spawning cycle and at egg overripening. – Manuscript.


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* Contribution Explanation
- Minor: contributed in some way, but contribution was limited.
- Significant: provided a significant contribution to the work.
- Substantial: took the lead role and performed the majority of the work.
I am also a co-author of the following articles, which are not included in this thesis:


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Abbreviations

1-D SDS PAGE  One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
17α-OHP  17α-hydroxyprogesterone
17,20β-P  17,20β-dihydroxyprogren-4-en-3-one
17,20β,21-P  17,20β,21-trihydroxyprogren-4-en-3-one
18s  Ribosomal protein 18s
actb  β-actin protein
BPG axis  Brain-pituitary-gonad axis
BSA  Bovine serum albumin
E2  Estradiol
E  Egg clutch
ESIₐ  Egg clutch somatic index with ovarian fluid
ESIₐ  Egg clutch somatic index without ovarian fluid
FOM  Final oocyte maturation
FSH, fsh-β  Follicle stimulating hormone, beta-subunit
GnRH, gnrh2/3  Gonadotropin releasing hormone(s)
gpr54  G protein-coupled receptor-54
GSI  Gonadosomatic index
GTHs  Gonadotropins
hCG  Human chorionic gonadotropin
hps  Hours post-spawning
HSI  Hepatosomatic index
ISI  Inter-spawning interval
kiss2  Kisspeptin
LH, lh-β  Luteinizing hormone, beta-subunit
MIH  Maturation-inducing hormone
O  Entire ovary
OF  Ovarian fluid
P4  Progesterone
POFs  Post-ovulatory follicles
qPCR  Real-time quantitative polymerase chain reaction
RIA  Radioimmunoassay
rpl8  Ribosomal protein rpl8
T  Testosterone
Vtg  Vitellogenin

Italic type fonts refer to the mRNA of the corresponding proteins
Introduction

Reproduction

Reproduction is an essential biological process which allows species to perpetuate themselves. In vertebrates, the most common mode is sexual reproduction and involves the fusion of a female (oocyte/egg) and male gamete (sperm). The origination of the gametes, termed gametogenesis, occurs in gonads; oogenesis in the female ovaries and spermatogenesis in the male testes. Gametogenesis is a crucial and great cascade in vertebrate reproduction, where diverse secreted substances take place in it. The reproductive success of vertebrates is regulated by the brain-pituitary-gonad (BPG) axis (Fig. 1) which is controlled both by external (e.g. photoperiod, temperature) and internal factors (e.g. hormones, neuropeptides) (Norris and Carr, 2013). Brain, pituitary and gonads communicate and interact to each other controlling from gametogenesis to behaviour.

Endocrine control of reproduction in fish

In teleost fishes, as in other vertebrates, the endocrine control of reproduction is regulated by the BPG axis (Fig. 1). The gonads produce gametes and sex steroid hormones. These functions are controlled by two gonadotropins (GTHs) secreted from the pituitary gland (hypophysis); the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) (Kawauchi et al., 1989). The stimulating gonadotropin-releasing hormone (GnRH) from the hypothalamic region in the brain is one of the factors controlling the synthesis and secretion of the GTHs. Most vertebrates, including fish, have two or three types of GnRHs. Other neurotransmitters and neuropeptides, such as dopamine and kisspeptin, are also present in the brain and regulate reproduction. Kisspeptins and their G protein-coupled receptors (gpr54) are known to be important factors triggering reproduction in fishes and in controlling the action of GnRHs (Zohar et al., 2010), however their specific role is still unknown. The BPG axis is under feedback effects by gonadal sex steroids (Fig. 1), where both positive (stimulation) and negative (suppression) effects have been reported in fishes (e.g. Billard et al., 1977; Crim and Evans, 1979; Hellqvist et al. 2008; Shao et al., 2015), having a key role in the regulation of reproduction.
Figure 1. The brain-pituitary-gonad (BPG) axis. GnRH: gonadotropin-releasing hormone, FSH: follicle-stimulating hormone, LH: luteinizing hormone. Sex steroids: androgens, estrogens, progestogens. +: stimulation, -: suppression, \(\rightarrow\): control/effect (drawing by Chrysoula Rouf).n

Oogenesis in fish

The oocyte production and development in teleost ovary is a process that involves a complex of physiological regulatory mechanisms which ensure the production of viable eggs, crucial for reproductive success. Oogenesis is the developmental procedure by which oogonia are transformed into mature oocytes, called eggs, ready to be ovulated, spawned and fertilized (Tyler and Sumpter, 1996; Wallace and Selman, 1981). Initially, the primordial germ cells differentiate into oogonia under the influence of gene cascades and steroid signalling which proliferate by mitosis. The next developmental steps of oocytes include the oocyte growth and vitellogenesis, and the maturation and ovulation controlled by diverse hormones (Lubzens et al., 2010; Urbatzka et al., 2010).
In female oviparous fishes, the developmental process of oogenesis is under the control of GTHs. FSH principally determines the earlier stages of oocyte growth and vitellogenesis, while LH mainly regulates the later stages which lead to oocyte maturation and ovulation (Lubzens et al., 2010). FSH and LH are released from the pituitary into the blood and exert their biological actions by binding to their receptors (FSHR and LHR) in the gonads (Levavi-Sivan et al., 2010). The synthesis and secretion of GTHs is regulated by different neurotransmitters and neuropeptides, as the GnRHs and kisspeptin in the brain (Zohar et al., 2010). Although the GTHs principally regulate the timing of the events during oogenesis, their actions are exerted through the sex steroid hormones: androgens, estrogens and progestogens (Pankhurst, 2008).

The androgen testosterone (T) is present at high levels in females of many teleosts, sometimes higher than in the males (Borg, 1994). T can be converted to the estrogen estradiol (E2) (Nagahama and Yamashita, 2008). In most salmonids, such as in amago salmon, Oncorhynchus rhodurus, T is produced in the theca cells of oocyte follicle and then secreted to the granulosa cells, where it can be converted to the E2 by the enzyme cytochrome P450 aromatase (Nagahama, 1983; 1994). The biological role of unconverted T acting as androgen is still not well known in fish (Borg, 1994).

E2 is produced in the ovarian follicles in response to pituitary gonadotropins and is responsible for inducing vitellogenin (Vtg) synthesis from the liver (Nagahama and Yamashita, 2008). Vitellogenesis is the principal physiological process during oogenesis responsible for the oocyte growth in oviparous animals, including fish (Mommsen and Walsh, 1988; Wallace, 1985). Vtg, a phospholipoglycoprotein, is the principal yolk protein precursor which provides the main nutritional reserves necessary for the embryonic development (Nagahama, 1983; 1994; Tyler and Sumpter, 1996). It is produced in the liver of mature females under estrogen stimulation, released into the bloodstream and then incorporated into the growing oocytes where it is cleaved into the yolk proteins (Bergink and Wallace, 1974; Hara et al., 2016; Mommsen and Walsh, 1988; Wallace, 1985).

Progestogens are mainly responsible for inducing the final oocyte maturation (FOM) and subsequent ovulation. Progesterone (P4) is the major progestogen in many vertebrates, including humans; however this is not the case for teleosts. In teleost fishes, the FOM is firstly stimulated by a peak in LH from the pituitary leading to synthesis of a maturation-inducing hormone (MIH) by the oocyte follicle cells (Nagahama and Yamashita, 2008). Theca cells produce 17α-hydroxyprogesterone (17α-OHP) which is then converted into the MIH by the granulosa cells (Nagahama, 1994; 1997). The 17,20β-dihydroxyprog-4-en-3-one (17,20β-P) and 17,20β,21-trihydroxyprog-4-en-
3-one (17,20β,21-P) are the two probable MIHs in teleosts. The 17,20β-P has been characterized as a MIH in a wide range of teleost fishes (Pankhurst, 2008) including salmonids and cyprinids. The closely related steroid, 17,20β,21-P, may be the MIH in others (e.g. Atlantic croaker, *Micropogonias undulatus*, Trant et al., 1986; Atlantic cod, *Gadus morhua*, Tveiten et al., 2010), whereas in other fish species (e.g. wrasse, *Pseudolabrus japonicus*, Matsuyama et al., 1998) both progestogens may play a role as MIHs (Nagahama and Yamashita, 2008; Scott et al., 2010).

Ovulation is the release of a mature fertilizable oocyte (egg) by rupturing of its surrounding follicles into the ovarian cavity, or into the abdominal cavity in salmonids (Bobe et al., 2008). Following ovulation, the eggs are stored there until spawning. If spawning does not happen, the eggs undergo an aging process, described as overripening, if retained for extended time period after ovulation (Kjørsvik et al., 1990; Springate et al., 1984) (more details later). Ovulated eggs are surrounded by the ovarian fluid (OF) (or coelomic fluid). The epithelium of the ovarian lumen has been shown to have a secretory activity in several fishes, such as in medaka, *Oryzias latipes* (Takano, 1968; Yamamoto, 1963), goldfish, *Carassius auratus* (Takahashi and Takano, 1971), bleak, *Alburnus alburnus* (Lahnsteiner et al., 1997) and three-spined stickleback, *Gasterosteus aculeatus* (Lam et al., 1978). Lam et al. (1978) have suggested that the OF may play a role in protecting the ovulated eggs within the female sticklebacks, probably by maintaining their viability in the ovarian cavity protecting them by overripening (see later). Moreover, the OF may protect the eggs outside the females until the eggs will be fertilized by sperm of males. The OF has been shown to play an important role in sperm motility prolongation in sticklebacks in fresh water (Elofsson et al., 2003) and it is its ionic content that is responsible for this (Elofsson et al., 2006).

**Spawning strategies and hormonal changes**

There are three different patterns of ovarian oocyte development in teleosts: the synchronous, group synchronous and asynchronous (Wallace and Selman, 1981). In the synchronous spawners, such as salmonids, which are mostly annual spawners, the oocytes develop in the ovaries synchronously. Ovulation happens only once during the spawning period and they only develop one batch of eggs per season, or indeed lifetime (semelparous species). In group synchronous spawners, such as goldfish, medaka and three-spined stickleback, several distinct cohorts of oocytes, each at different developmental stage, are present in the ovaries the same time. Ovolutions occur several times over the spawning period, with successive egg clutches being produced. In the asynchronous spawners, such as the killifish mummichog *Fundulus heteroclitus* and zebrafish, *Danio rerio*, the oocytes develop asynchro-
nously and are present at all developmental stages in the ovaries. Ovulations and spawnings can happen at short intervals, even daily, over the spawning period (Wallace and Selman, 1981). The endocrine control of oocyte development can be more complicated in the group synchronous and asynchronous (multiple spawners) than in the synchronous spawners. In the latter, vitellogenesis and FOM occur at different time-points, whereas in the former several phases of oogenesis may occur simultaneously controlled by different hormones (reviewed by Wootton and Smith, 2015).

The seasonal and annual patterns of the reproductive hormones have been studied both in circulating and mRNA levels in a variety of fishes belonging in the three different above groups, such as in rainbow trout, *Oncorhynchus mykiss* (Scott et al., 1980), Atlantic salmon, *Salmo salar* (Andersson et al., 2013), goldfish (Kobayashi et al., 1986; Sohn et al., 1999), tench, *Tinca tinca* (Antonopoulou et al., 2001; Pinillos et al., 2003), gudgeon, *Gobio gobio* (Rinchard et al., 1993), European sea bass, *Dicentrarchus labrax* (Prat et al., 1990) and three-spined stickleback (Hellqvist et al., 2006). The hormonal profiles during the final gonadal maturation, peri-ovulatory period or spawning period have mostly been studied in the synchronous spawners, such as cyprinids (e.g. Santos et al., 1986; Scott et al., 1984; Stacey et al., 1984) and salmonids (e.g. Fitzpatrick et al., 1986; Pavlidis et al., 1994; Scott et al., 1983; Slater et al., 1994), as well as in some multiple spawners such as the striped bass, *Morone saxatilis* (Mylonas et al., 1997) and goldfish (Canosa et al., 2008; Kagawa et al., 1983; Kobayashi et al., 1987; Stacey, 1979). Nevertheless, the changes in reproductive hormones over the (short) spawning or ovulatory cycles of multiple-spawning fishes (group synchronous and asynchronous spawners) have only been studied in a few species. These are e.g. kanehira bitterling, *Acheilognathus rhombea* (Shimizu et al., 1985), chichibu-goby, *Tridentiger obscurus* (Kaneko et al., 1986), goldfish (Kobayashi et al., 1987; 1988), red sea bream, *Pagrus major* (Matsuyama et al., 1988) and gilthead sea bream, *Sparus aurata* (Gothilf et al., 1997), and even though the spawning patterns are quite diverse among the different fishes, the hormonal profiles are quite consistent at least for the circulating steroids.

Overripening of eggs

The production of viable eggs is crucial for a successful reproduction in teleosts (Luzbens et al., 2010; Urbatcka et al., 2011) while in aquaculture it constitutes a great issue. Egg quality, viability and subsequent embryo and larval development can be affected by the post-ovulatory ageing (Bromage et al., 1992; Kjørsvik et al., 1990). As it was previously mentioned, if spawning (or spontaneous release) does not occur, the ovulated eggs can become overripe, if retained for extended time period after ovulation (Kjørsvik et al., 1990; Springate et al., 1984).
The natural period, during which the ovulated eggs can be retained in the ovarian cavity, without becoming overripe, varies among fishes from an hour to several days (reviewed by Bromage et al., 1994) and it can be associated to various spawning strategies or water temperature (Flett et al., 1996; Gillet, 1991). In most fishes, ovulated eggs undergo a rapid decrease of their quality after a few hours, such in goldfish (Formacion et al., 1995), Atlantic halibut, Hippoglossus hippoglossus, (Bromage et al., 1994) and turbot, Scophthalmus maximus, (McEvoy, 1984). In contrast, salmonids such as rainbow trout can hold the ovulated eggs in the body cavity for several days without loss of egg viability (Aegerter and Jalabert, 2004; Springate et al. 1984).

The overripe eggs can harden and are then not only lost for reproduction, but can cause physiological stress to the female, as well as obstruct further spawns as the female becomes egg-bound. Sometimes, this can result in the death of the female and in aquaculture, as well as in hobby aquaria, egg hardening is a major welfare issue. Overripe eggs are characterized by common features of an egg gradual deterioration leading to increased transparent yolk, cytoplasm aggregation and oil droplets at the animal pole, and larger size than normal ovulated eggs. The phenomenon of overripening has been observed and characterized in several teleosts, such as goldfish (Formacion et al., 1993), turbot (McEvoy, 1984) and three-spined stickleback (Lam et al., 1978). The overripening of ovulated eggs is mostly known from fish in captivity, but it has also been observed in nature, e.g. in the three-spined stickleback (Lam et al., 1978). However, the underlying causes of egg retention and subsequent overripening are poorly known. Many factors have been suggested to cause or associate with the keeping of ovulated eggs by female fish, such as lack of mates or spawning sites (e.g. male nests), sex ratio, overcrowding, environmental factors and/or pheromones (reviewed by Rideout et al., 2005).

Hormones and ovarian fluid

Although egg overripening is a well-known phenomenon, the physiological basis associated with this process is largely unknown. Reproductive hormonal changes have been studied over the peri-ovulatory period at spontaneous or hormone induced ovulation in number of fishes (e.g. rainbow trout, Scott et al., 1983; goldfish, Kobayashi et al., 1988; common carp, Cyprinus carpio, Santos et al., 1986). In rainbow trout, circulating levels of reproductive hormones over extended periods after ovulation have been studied (Chyb et al., 1999; Scott et al., 1983) in which it is suggested that a decrease in sex steroids could be associated with a decline in egg viability after ovulation. However, endocrine changes over extended periods after ovulation, as well
as in non-overripe and overripe female fishes have still not been well studied. Formacion et al. (1995) have studied the changes in steroid hormones in goldfish during overripening, however, the fish were not naturally ovulated but ovulation was induced after human chorionic gonadotropin (hCG) injections. hCG remains in high concentrations in the blood for a long time after injection in fish (Ohta and Tanaka, 1997). This could affect the overripening and the levels of steroids.

A possible relationship between the post-ovulatory follicles (POFs, or corpora lutea) and their possible steroid secretion at egg overripening has been suggested in goldfish (Formacion et al., 1995) and three-spined stickleback (Lam et al., 1978; 1979). Formacion et al. (1995) showed that immersion in vivo of just ovulated female goldfish (ovulation induced by hCG) in a solution containing P4 (0.05 ppm) increased the fertilization rates of stripped eggs at 12 hours while 17,20β-P (0.025 or 0.05 ppm) did not have significant effects. Also, immersions in vitro of just ovulated eggs in OF containing anti-serum against P4 decreased the fertilization rates after 6 hours incubation suggesting a role of P4 (or other progestogens cross reacting with the antiserum) in delay of overripening (Formacion et al., 1995). Sticklebacks with overripe eggs have less fluid in the ovarian lumen than normal non-overripe ovulated females, which is associated with POFs degeneration and regression of the epithelium lining the ovarian cavity (Lam et al., 1978). Lam et al. (1979) also treated females that had laid their eggs with 0.5 ppm P4 or 1 ppm E2 or solvent alone via the water. At the termination of the experiments the ovaries of the P4 treated fish contained more fluid than the controls; in the overripe fish P4 also increased gonadosomatic index (GSI). E2 increased ovarian and liver weights, but it is doubtful if it increased fluid production. They suggested that steroid secretion by POFs may be crucial to maintain the viability of eggs in the ovarian cavity after ovulation, through stimulation of OF secretion by the ovarian epithelium.

During the overripening, both eggs and OF undergo morphological, physiological and biochemical changes (e.g. Bahrekazemi et al., 2009; Craik and Harvey; 1984; Formacion et al., 1993; Lahnsteiner, 2000; Lahnsteiner et al., 2001; Lam et al., 1978; Rime et al., 2004;) resulting in a progressive egg quality decrease (Springate et al., 1984). Some of these changing parameters, such as pH, enzyme activities and protein concentration of OF have also been proposed to be used as egg quality indicators in aquaculture (e.g. Fauvel et al., 1993; Lahnsteiner et al., 1999, 2001; Rime et al., 2004). The composition of the OF has been studied in salmonids (Hatef et al., 2009; Lahnsteiner et al., 1995) and cyprinids (Lahnsteiner et al., 1997, 2001). OF contains ions, glucose, fructose, cholesterol, phospholipids, proteins, free amino acids and enzymes. Changes in OF properties in relation to egg viability and overripening have been reported in e.g. turbot (Fauvel et al., 1993),
Caspian brown trout, *Salmo trutta caspius* (Bahrekazemi et al., 2009), lake trout, *Salmo trutta lacustris* (Lahnsteiner et al., 1999) and rainbow trout, *Salmo trutta*, where protein concentrations were usually elevated at overripening. However, OF protein composition is poorly known. Both egg and OF protein composition has been studied by Lahnsteiner (2007) in brown trout, *Salmo trutta*, relative to egg quality. Rime et al. (2004) have studied the proteome of OF in rainbow trout and found a progressive accumulation of some proteins during the post-ovulatory ageing.

### Three-spined stickleback

The three-spined stickleback, *Gasterosteus aculeatus*, is a small fish, widely spread in the cool temperature water areas of the Northern hemisphere in fresh, brackish and marine waters. Sticklebacks are characterized by a marked and well-described seasonal reproductive cycle. Their breeding condition is stimulated by long day photoperiod (e.g. Baggerman 1957).

Stickleback is a multiple batch spawner (group-synchronous), where egg clutches are produced and spawned by females over an extended breeding season in late spring and summer (Baggerman, 1957). Females produce eggs at intervals of a few days and lay them in a nest built by the male (Wootton, 1974) (Fig. 2). Males have bright breeding colors, with red abdomen and blue eyes, and show nest building behaviour. Their kidney hypertrophies under androgen stimulation and produces the glue-protein spiggin which is used as a glue to build the nest (Borg, 2007; Jakobsson et al., 1999).

The inter-spawning interval (ISI) is largely depended on food ration, which controls the ISI rather than the amount of eggs per spawning (Wootton, 1973). When the supply of food is high, the females spawn at shorter intervals than when the food ration is low (Wootton, 1973, 1977). In our experience, and with an *ad libitum* food, the ISI is usually 3 days at 20 °C.

When females do not find a suitable male nest to spawn, they usually “drop” their ovulated eggs and another cycle of egg maturation can start (Wallace and Selman, 1979; reviewed by Wootton, 1985). However, the ovulated eggs can sometimes be retained in the ovarian cavity for more than a few days and become overripe. As a result, the eggs get hardened giving a
‘berried’ look and the females can finally die under this condition (Lam et al., 1978). Even though overripening is harmful, it is a natural phenomenon in the sense that it does not only occur in captivity, but is also common in nature in some fishes, including the stickleback.

Although aspects of reproductive physiology (e.g. Baggerman, 1957; Wallace and Selman, 1979) and endocrine changes under different seasons, photoperiods, as well as after castration and steroid hormone treatments have been extensively studied in this species (e.g. Borg, 2007; Hellqvist et al., 2006; 2008; Shao et al., 2013; 2015), the hormonal changes at the overripening of eggs and over the short spawning cycle are still unknown. Moreover, even though stickleback vitellogenin has often been used as biomarker for estrogenic effects (e.g. Andersson et al., 2007; Katsiadaki et al., 2007; 2010), the endogenous vitellogenin levels in untreated sticklebacks have been less studied under different physiological conditions. Finally, a possible relationship between the overripening, steroids by POFs and OF has been previously suggested by Lam et al. (1978; 1979), the changes in OF properties at overripening and after sex steroid treatments have not been investigated.

This thesis investigates the reproductive physiology of the female sticklebacks, focusing on the overripening of eggs and spawning cycle.
Aim of the thesis

The overall aim of the thesis was to investigate the reproductive physiology of female fish and the phenomenon of overripening of eggs using the three-spined stickleback as a model species. The specific aims were to:

- Examine whether overripening of eggs is associated with changes in the reproductive hormones of the BPG axis in the female stickleback (Paper I)

- Investigate the hormonal profiles of the BPG axis over the natural spawning cycle of the female stickleback, clarifying their biological roles and their possible cyclicity over the cycle (Paper II)

- Characterize the natural vitellogenesis in female sticklebacks over the spawning cycle and at egg overripening measuring the expression of the *vitellogenin* mRNA in the liver (Paper III)

- Study the relationship between changes in ovarian fluid properties and overripening of eggs, as well as the effects and role of sex steroids on ovarian fluid characteristics in female sticklebacks (Paper IV)
Materials and Methods

All studies were performed following permission from the Stockholm Northern Animal Experiment Ethical Committee (N 492/11, N 45/14).

Fish

Adult non-breeding three-spined sticklebacks, *Gasterosteus aculeatus*, were caught using drop nets in the southern Baltic at Skåne County, southern Sweden in the late autumns to early springs during 2013-2015. After transportation to Stockholm University aquaria facilities, the fish were kept in 700- or 1200-liter aquaria containing filtered and aerated brackish water (0.5% salinity) while fine gravel, clay pots and sometimes algae were present. Temperature was gradually increased at 20 °C. Breeding condition was stimulated by setting the photoperiod to long day (16 hours light: 8 hours dark; LD 16:8). Fish were fed daily with frozen red midge larvae (blood worms) or mysids. After about 3 weeks, the sexually mature males showed breeding colouration while females began to have swollen abdomens due to large ovaries filled with mature eggs.

In *Papers I, III and IV*, additional breeding sticklebacks were caught by drop nets in the northwestern Baltic Proper in Sweden, at the island of Askö (Askö Laboratory) during the natural breeding season in June 2014 and 2015. The fish were sampled within an hour after capture at Askö Laboratory.

Experiments

Overripening of eggs

To investigate the changes in reproductive hormones (*Paper I*) and in ovarian fluid properties (*Paper IV*) at overripening of eggs, fish from both Skåne (named as Skåre in *Paper I*, because the fish were caught from the harbour of Skåre in Skåne County) and Askö were used, whereas to study the vitellogenesis at overripening (*Paper III*), only fish from Askö used. The fish from Skåne were kept in aquaria where both sexes were present but no algae in order to prevent nest building by males, and thus decrease egg laying by females. The fish were sorted every week over a course of a month for each
of the captures. An equal number of sexually mature females (non-overripe ovulated or sometimes judged close to ovulation in Paper IV) and females which were suspected of being or becoming overripe were placed together in a 200-liter aquarium under similar conditions as described above. The females were kept together for 48 hours (Paper I) or sometimes less (Paper IV) before sampling. The fish from Askö were sampled directly from nature, within an hour after capture.

In Paper I, the female sticklebacks from Skåne (Skåre harbour) were divided in two groups: non-overripe and overripe. In the former, both females with non-overripe non-ovulated oocytes, in different maturing or ripening stages, and with non-overripe ovulated (3 individuals) were included. A few males were also included in the progestogen analyses. The females from Askö were divided into three groups: non-overripe non-ovulated, non-overripe ovulated and overripe, as the number of individuals was sufficient for statistical analyses. These fish were used only for gonad weights and progestogen analysis in Paper I, as well as for vitellogenin mRNA levels in Paper III. In Paper IV, the females from both Skåne and Askö were divided in two groups: non-overripe ovulated and overripe, as the scope was the ovarian fluid which does not exist in non-overripe non-ovulated females.

In all cases, overripening condition was verified under a dissection microscope based on the criteria by Lam et al. (1978) (Fig. 3). Briefly, overripe ovulated had a rough berry-like abdomen and ovary which feel hard to touch, whereas in non-overripe ovulated females the swollen abdomen and ovary are soft and smooth. Moreover, overripe eggs are more transparent than normal ovulated eggs, have aggregated cytoplasm and oil droplets at the animal pole (Lam et al., 1978) (Fig. 3). The non-overripe non-ovulated females were neither ovulated nor overripe but they had maturing or ripening eggs. Intermediate fish that could not be classified clearly at each group were not included, while parasitized fish and fish suspected for being non-breeding or post-breeding were excluded.
Spawning cycle

To investigate the changes of reproductive hormones (Paper II) and the vitellogenesis (Paper III) over the spawning cycle, fish from Skåne were used. The females were sampled over different time-points before or after spawning. Spawning was accomplished naturally using males that had placed individually in 50-litre aquariums under similar conditions as above, where fine gravel and algae were provided for nest building. Females with swollen abdomens ( gravid ) were then allowed to mate with the nesting males. Maximum time allowed for spawning was 30 minutes; if the female had not spawned by the end of this period, she was removed. The five groups of females were: A) "ready to spawn" females (Ready) which were ovulated and displayed a positive courtship response (e.g. head-up posture) to a nesting male but spawning was interrupted by netting out the female and immediately sampled (before spawning). The other four groups were all allowed to spawn naturally and then transferred to a 50-litre aquarium under similar conditions as for the males until sampling (after spawning). They were sampled at: B) 6 ± 1 hours post-spawning (hps) (6 hps), C) 24 ± 1 hps (24 hps), D) 48 ± 1 hps (48 hps) and E) 72 ± 1 hps (72 hps), when most female had ovulated again.
Treatment with sex steroids

To investigate the effects and role of sex steroids hormones on the ovarian fluid properties (Paper IV), fish from Skåne were used. Breeding males were placed individually in 50-litre aquaria for nest building, as described above. Gravid females were then allowed to spawn with the nesting males in the morning (max time 30 min, see above). After spawning, females were operated and implanted with Silastic tubes or capsules, filled with different sex steroid hormones (see details later), in the afternoon the same day.

Operations were done after fish had been anaesthetised in a 0.025% solution of sodium-bicarbonate-buffered tricaine methanesulfonate (MS-222, Sigma Aldrich, St. Louis, MO, USA). Females were firstly marked according to treatment by spine clipping. The implant was carefully inserted into the abdominal cavity through a small incision (c. 1.5 mm) in the rear lateral part of the abdomen. The opening was closed using a surgical suture. Operation per fish did not take more than a few minutes. After operation, the treated fish was placed in a container with aquarium water to recover. Females operated the same day were placed together in the same 200-litre aquarium, under similar conditions as above, until sampling.

The sampling was done when the fish had ovulated again which was at least three days after operation. Females were checked for ovulation 2-3 times per day from day three onwards. Determination of ovulation was done in fish with round and soft abdomens by checking their genital opening, and they were regarded as ovulated when only big transparent eggs were visible via the opening surrounded by ovarian fluid and were ready to be expelled. If they were ovulated, they were sampled. Otherwise, the non-ovulated yet were placed back to the aquarium until the next ovulation control. A few females that were found to have already dropped the eggs before the first ovulation control of the day, regarded as having ovulated that day in the calculations of intervals, also the liver data were collected, but not the ovarian and fluid data. After seven days, the remaining females, all non-ovulated, were dissected. These fish were regarded as having an interval of > 7 days, and they were included only in the calculations of intervals and liver weights. A few females that were found not to be fully ovulated or to be sick when sampled, and a few non-ovulated females treated with T which were dissected at Day 3 after ovulation for other purposes, were not included in the analyses.
Implants

In the sex steroid treatment experiment (Paper IV), five different implants made from medical grade silicone tubing (ID 0.64 x OD 1.19 mm, Silclear™ Tubing, Degania Silicone Ltd, Degania Bet, Israel) were used: control, testosterone (T) (Fluka, Sigma-Aldrich, Steinheim, Germany), estradiol (E2) (Merck, Darmstadt, Germany), 17,20β-dihydroxyprog-4-en-3-one (17,20β-P) (kindly synthesized and purified by Dr. Mats Thelin, Department of Organic Chemistry, Stockholm University, Sweden) and progesterone (P4) (ICN Biomedicals Inc., Aurora, Ohio, USA). Controls contained only melted cocoa butter (Crearome, Sweden), while the T, P4 and 17,20β-P were dissolved in melting cocoa butter in 1% w/v final concentration. 17,20β-P was not totally soluble in just cocoa butter and chloroform was used for dissolving it which was then been evaporated under the fume hood overnight and melted again next day. The melted cocoa butter containing or not steroids was then transferred into silicone tubes and left to solidify at room temperature. Tubes were divided into 1 cm length and both ends left opened. E2 was not soluble in cocoa butter, not even after adding chloroform or absolute ethanol. Thus, capsules with crystalline E2 powder were used in 0.3 cm length with both ends sealed with silicone glue. All implants were stored at +4 °C until use.

Sampling

Before sampling, all fish were anesthetized in a 0.025% MS-222 solution and weighed. Blood samples were collected in heparinized microhaematocrit tubes (BRAND®, Wertheim, Germany) from the severed caudal peduncle. After centrifuging at 13,000 rpm for 2 min (Haematocrit 20, Hettich Zentrifugen, Tuttingen, Germany), plasma was collected in a pre-weighed (+ 0.01 mg) 0.5 mL tube, weighed again to determine plasma amount, and then stored at -80 °C. Pituitary and brains were collected after decapitation and skullcap removal under RNase free conditions. Pituitaries and brains were individually fixed in 100 and 500 μl, respectively RNAlater® solution (Ambion, Austin, TX, USA), followed by overnight storage at +4 °C and then at -80 °C until RNA extraction. Ovaries and liver were excised and weighed to the nearest mg. A part of the liver was fixed in 500 μl RNAlater® solution, and stored similarly as above until analysis. Gonadosomatic index (GSI) was calculated as: GSI = (gonad weight/total fish body weight) x 100, Hepatosomatic indices (HSIs) with gonad weight [(liver weight / total fish body weight) x 100], or without gonad weight {[(liver weight / (total fish body weight-gonad weight))] x 100}. As for the ovarian fluid (OF) sampling, this is described below.
Ovarian fluid collection

For the collection of the OF (Paper IV), the non-overripe ovulated and steroid treated ovulated females were gently stripped (gently pressure on the abdomen) and the egg clutch (E) together with OF were pressed out and collected into a pre-weighed 5 mL plastic container and weighed before OF sampling. In overripe females, however, stripping of eggs was not possible because overripe eggs were not running as they were berried and stuck. Instead, the entire ovary (O) was dissected and placed in the pre-weighed container and weighed before OF sampling. A sufficient amount of OF was carefully and gradually obtained from the E or O using a pipette with a cropped tip end (because of its high viscosity in most cases) and transferred to a 1.5mL tube, frozen on dry ice and stored at -80°C until analysis. On the OF sampling day, the fish were not fed and entire procedure was done carefully to avoid contamination with urine, feces or blood or accidental breakage of eggs. When this was not successful, OF samples were not used. In addition, the appearance of the OF was noted, or whether it was viscous or not (or any finer grading). After OF collection, the females that had been stripped were also dissected and their O was weighed. GSIs were calculated as described before.

Amount of the ovarian fluid

Since OF had a very high viscosity, at least in ovulated females with non-overripe eggs, it was not impossible to remove all of it from the E or O with the pipette. Thus, in Paper IV, OF amount was determined gravimetrically transferring the E and O (after OF sample collection) on a filter paper to absorb the remaining OF; a small amount of 0.6% NaCl solution was also used to wash out the remnants of OF. The E and O were weighed again afterwards using the same containers as before and the OF amount % was calculated using the subtracted weights relative to the E and/or O weights. For the overripe females, the OF amount was calculated only relative to the O weight since it was not possible to expel all overripe ovulated eggs without damaging them. In steroid treated females, the amount of OF was further calculated relative to the fish body weight. Also, the egg clutch somatic indices (ESIs) were calculated as: ESI_A = egg clutch somatic index with ovarian fluid [(egg clutch weight with fluid/total fish body weight) x 100] and ESI_B = egg clutch somatic index without ovarian fluid {[(egg clutch weight with fluid – OF weight)/total fish body weight) x 100].
Dry weight of the ovarian fluid

The percentage of OF dry weight from non-overripe ovulated and overripe females (Group A, see Paper IV) was calculated after drying out an amount on pre-weighed containers of aluminium foil at 70 °C for 24 hours.

Radioimmunoassay (RIA)

The circulating levels of T, E2, 17,20β-P and 17,20β,21-P were measured in the plasma using specific radioimmunoassay (RIAs) (Paper I and II). Briefly, individual plasma samples were diluted with RIA buffer, vortexed and heat-treated as described by Schulz et al. (1994) and Scott et al. (1984). Aliquots of heat-treated plasma were incubated with 3H-labelled T or 3H-labelled E2 (both from PerkinElmer, USA) and the respective antiserum (gift from Dr. Helge Tveiten, University of Tromsø), or buffer-radiolabel-antibody cocktail of 3H-labelled 17,20β-P or 3H-labelled 17,20β,21-P (for a detailed RIA protocol see Paper I). The specificity of the antibodies can be found in Frantzen et al. (2004) for the T/E2 and in Lower et al. (2004), Scott et al. (2005) and Sebire et al. (2007) for the 17,20β-P/17,20β,21-P. The detection limits of the RIAs were 3 pg/assay tube for T/E2 and 1.95 pg/assay tube for 17,20β-P/17,20β,21-P. All samples for which detectable values are reported, they were within the standard curve for each assay.

Real-time quantitative PCR (qPCR)

The relative mRNA levels of gonadotropins fsh-β and lh-β (beta-subunits) in the pituitary, GnRHs: gnrh2 and gnrh3, as well as kisspeptin (kiss2) and its G protein-coupled receptor (gpr54) in the brain (Paper I and II), and vitellogenin in the liver (Paper III) were measured by qPCR. Total RNA was isolated from pituitaries using TRizol® RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) (Paper I and II), from whole brains using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) (Paper I) or RNeasy Plus Universal Mini Kit with gDNA Eliminator Solution (Qiagen) (Paper II) and from livers using the TRizol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) and TRizol Reagent (Invitrogen, Carlsbad, CA, USA) (Paper III). RNA samples were treated with DNase (2 U) using TURBO DNA-free™ Kit (Ambion, Austin, TX, USA) in Paper I and II, and with PureLink DNase (Invitrogen, Carlsbad, CA, USA) in Paper III to remove possible genomic contamination. RNA concentration and purity were determined using NanoDrop™ (Thermo Fisher Scientific, Delaware, USA). cDNA synthesis was performed by reverse transcription (RT) following the corresponding manufacturers’ protocols (for details see Paper I - III). cDNA samples were stored at -20 °C until analysis.
The qPCR analyses were performed using either the Mx3000P® real-time PCR machine (Stratagene, La Jolla, CA, USA) and Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (fsh-β / lh-β: Paper I and II), or the Applied Biosystems StepOnePlus System and Fast SYBR Green Master Mix (Applied Biosystems) (gnrh2 / gnrh3 and kiss2/gpr54: Paper I) or the LightCycler® 480 System (Roche Diagnostics, Mannheim, Germany) and LightCycler®480 SYBR Green I Master (gnrh2/ gnrh3 and kiss2/gpr54: Paper II), or the Rotor-Gene 6000 DNA amplification system (Qiagen, Hilden, Germany) and the iQ SYBR Green Supermix kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) (vitellogenin: Paper III). In Paper I and II, the ribosomal proteins 18s rRNA and rpl8, as well as β-actin protein (actb) (only in Paper I) were used as reference genes and data normalization. In Paper III, two potential reference genes were tested; ubiquitin and 18s. Only the levels of 18s were stable among samples and groups and thus, this gene was used as reference gene. Sequences and concentrations of the gene specific primers are shown in Table 1. Specificity of the amplification and absence of primer dimers were verified by a melting curve analysis. Negative controls, with nuclease-free water (no template), and reverse transcriptase controls in which no reverse transcriptase was added during cDNA synthesis were included. The PCR reaction efficiencies were determined using standard curves prepared from cDNA consisting of a mix of samples (Paper I and II), or using the LinRegPCR program (version 2016.0, Ruijter et al. 2009) (Paper III). In Paper I and II, the relative mRNA levels of fsh-β/ lh-β were obtained relative to their standard curves and normalized to the reference gene 18s rRNA (e.g. Shao et al., 2013) while those of gnrh2/gnrh3 and kiss2/gpr54 were obtained through the efficiency-corrected method (Roche Applied Science 2001; Weltzien et al., 2005) and normalized against the geometric means of the expressions of the reference genes rpl8 and actb (Paper I) or against the reference gene rpl8 (Paper II) (e.g. Shao et al., 2015). In Paper III, the relative mRNA levels of vitellogenin were obtained through the Pfaffl method (Pfaffl 2001), normalized against the expression of the reference gene 18s rRNA.
Table 1. Sequences and concentrations of the forward (Fw) and reverse (Rv) primer used in qPCRs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Nucleotide sequence (5´→3´)</th>
<th>Concentration (nM)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>18s rRNA*</td>
<td>Fw</td>
<td>CTCAACACCGGAAAACCTCAC</td>
<td>200</td>
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<tr>
<td></td>
<td>Rv</td>
<td>AGACAAATCGCTCCACCAAC</td>
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<td></td>
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<tr>
<td>rpl8*</td>
<td>Fw</td>
<td>CGACCGTGACCTGCTCAAGAA</td>
<td>50</td>
<td>Shao et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Rv</td>
<td>GCACATTGCAATGTCAAGCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>actb*#</td>
<td>Fw</td>
<td>ATGGCCAGAGGAGAGCTTA</td>
<td>50</td>
<td>Shao et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Rv</td>
<td>TCACAATACCGTGCTCAATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ubiquitin**</td>
<td>Fw</td>
<td>AGACGGGCATAGACTTGC</td>
<td>1000</td>
<td>Hibbeler et al., 2008;</td>
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<tr>
<td></td>
<td>Rv</td>
<td>CAGGACAGGAGAGCATCC</td>
<td></td>
<td>Svensson et al., 2013</td>
</tr>
<tr>
<td>fsh-β</td>
<td>Fw</td>
<td>CATCCACACCCACCATCTGCT</td>
<td>100</td>
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<td></td>
<td>Rv</td>
<td>TGCTCACACCTCTGGGACCA</td>
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<td></td>
</tr>
<tr>
<td>lh-β</td>
<td>Fw</td>
<td>GGTCACTGCTACCAAGGA</td>
<td>100</td>
<td>Shao et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Rv</td>
<td>GAGCGGAGCTGCTCTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gnrh2</td>
<td>Fw</td>
<td>CCGTCGGAGATTTGCAGAGAGATT</td>
<td>50</td>
<td>Shao et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Rv</td>
<td>TCTAAGCTCTGCTGGTCAAGGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gnrh3</td>
<td>Fw</td>
<td>AGCGATGGTCAGGTTGATGTT</td>
<td>50</td>
<td>Shao et al., 2015</td>
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<tr>
<td></td>
<td>Rv</td>
<td>TTAAATCTCTCTGCTGGTCGGC</td>
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<tr>
<td>kiss2</td>
<td>Fw</td>
<td>CAGGACCGGAGAGAATCAAT</td>
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<td></td>
<td>Rv</td>
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<tr>
<td></td>
<td>Rv</td>
<td>TCCGTGTAACGTCAACCAATGT</td>
<td></td>
<td>Svensson et al., 2013</td>
</tr>
</tbody>
</table>

* 18s rRNA was used as reference gene for the fsh-β/lh-β and vitellogenin measurements, while rpl8 and actb (# only in Paper I) were used as reference genes for the gnrh2/gnrh3 and kiss2/gpr54 measurements. ** ubiquitin was not stable and not used as reference gene in vitellogenin measurements.

Protein analysis of ovarian fluid

Protein concentration

Due to the high viscosity of OF, forming a jelly appearance, the OF samples in Paper IV were treated with enzyme mix of β-Glucuronidase/aryl sulfatase from snail *Helix pomatia* (EC 3.2.1.31 + EC 3.1.6.1, cat no. 104114, Merck Millipore, Darmstadt, Germany) to make them more soluble before protein measurements, and the OF weight was used instead of its volume. In brief, OF sample was mixed 1:1 (weight:volume) with the 10-fold diluted snail enzyme in acetate buffer (20 mM, pH 4.5; optimum for the β-Glucuronidase) and incubated at 25 °C for 3 hours. Protease inhibitors (Halt™ Protease In-
hibitor Cocktail, EDTA-Free, Thermo Fisher Scientific, Waltham, MA, USA) were also added (for a detailed protocol see Paper IV). Control samples with snail enzyme alone but not OF, were also included.

The total protein concentration was measured by the Bradford method (Bradford, 1976) at 595 nm in a spectrophotometer (Genova, Jenway, UK) using 0.5 μg/μl bovine serum albumin (BSA) as standard. Total protein concentration was expressed per OF weight in μg/mg. Enzyme concentration was subtracted from the final protein concentration of OF. Also, a few samples of OF and eggs from non-overripe and overripe females that had not been treated with enzyme were diluted with cold acetate buffer containing protease inhibitors, but not enzyme, on ice (no incubation at 25 °C) before protein measurements. The eggs had further been homogenized using pellet pestles, kept on ice for 5 minutes, centrifuged at 7000 rpm at 4°C for 10 minutes and supernatant was used. However, protein concentration measurements were done approximately for non-treated OF samples and were only used for examine the protein composition later. This was due to that the OF could not be completely dissolved in acetic buffer if it was not enzyme treated and incubated at 25 °C for 3 hours (a jelly pellet appeared), and the protein concentrations spread considerably within duplicates. Thus, only protein levels in enzyme treated samples are shown, in which OF was not viscous anymore.

Protein composition

To investigate the general differences in protein profiles in Paper IV, OF samples and few egg samples were run using one-dimensional (1-D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reduced conditions using the XCell SureLock® Mini-Cell (Novex™, Invitrogen, Life Technologies, Carlsbad, CA, USA). Samples estimated to contain c. 3 μg protein were mixed with NuPAGE® LDS Sample Buffer (4X) and sample reducing agent (10X) (Novex™, Invitrogen, Life Technologies, Carlsbad, CA, USA). They were incubated in water bath at 70°C for 10 minutes and loaded into each gel lane. The proteins were separated using the NuPAGE® system with 4-12% gradient Bis-Tris protein gels, MOPS SDS Running Buffer and Antioxidant (Novex™, Invitrogen, Life Technologies, Carlsbad, CA, USA). Molecular weight standard Novex® Sharp Unstained Protein Standard (Invitrogen, Life Technologies, Carlsbad, CA, USA) and sometimes control samples with snail enzyme alone (~ 5 μg, as much as was present in enzyme treated OF samples) were also included. Electrophoresis was performed at constant 200 V for c. 50 min. To visualize the protein bands, gels were stained with Coomassie® Blue G-250 using the SimplyBlue™ SafeStain (Novex™, Invitrogen, Life
Technologies, Carlsbad, CA, USA), following the maximum sensitivity manufacturer’s protocol.

**Data analysis**

The statistical analyses were performed using the R statistical software v. 3.2.2 (R Development Core Team, 2015) (Paper I-III) or the GraphPad Prism v. 5.00 (GraphPad Software Inc., CA, USA) (Paper IV). The Kolmogorov-Smirnov and histogram plots were tested for data normal distribution. The Barlett methods, Fligner-Killeen test or F-test were used to test homogeneity of variances. Log or square-root transformations were done if necessary. When those criteria were fulfilled, a two-sample unpaired \( t \)-test was used for two group comparisons, with Welch’s correction when variances did differ, and a one-way ANOVA followed by post-hoc Tukey HSD for multiple comparisons for more than two groups. If these criteria were still not met after transformations, the non-parametric Mann-Whitney Wilcoxon test was used. In Paper I, there were many non-detectable values for the steroid levels and the proportions of non-detectable and detectable values were compared using Fisher’s exact test for count data. In Paper II and III, the relationships between T and E2 plasma levels (II), or those between vitellogenin mRNA and E2 and T levels (III) were tested by a Spearman rank test. In Paper II, the effect of time of day at sampling on levels of T and E2 levels were tested using the Generalized Linear Model (GLM). In Paper IV, the non-parametric Kruskal-Wallis test with post hoc Dunn was used for the distribution analysis of the days until next ovulation in the steroid treatment experiment. The statistical significance level was set at \( p < 0.05 \). The data are as medians with ranges in box-and-whisker plots, while means are presented as solid circles (Paper I and II), or as mean ± standard error of the mean (SEM) (Paper III and IV). To optimize high-resolution and sensitivity images of the Coomassie® Blue-stained gels, they were imaged using the ChemiDoc™ XRS+ system with Image LAB™ Software (version 3.0, Bio-Rad Laboratories Inc., Hercules, CA, USA).
Results

Paper I

To investigate whether egg overripening is related to changes in hormones of the BPG axis in female sticklebacks, the plasma levels of T, E2, 17,20β-P and 17,20β,21-P, and the relative mRNA levels of the pituitary fsh-β/lh-β, brain gnrh2/3 and kiss2/gpr54 were analysed. Overripe females from Skåne (Skåre harbour) had higher GSI than the non-overripe; however most of the overripe had lower GSI than the non-overripe ovulated ones (see Paper I: Table 3; Fig. 2-A). This suggests that some overripe fish had not ovulated again after overripening occurred. Overripe females from Askö had higher GSI than both non-overripe non-ovulated and ovulated, and many of the overripe displayed additional ovulations (Paper I: Fig. 2-B). The main results of this paper were that the overripe females from Skåne (Skåre harbour) had lower levels of plasma steroids (T, E2, 17,20β-P) (Fig. 4), as well as of pituitary lh-β and brain kiss2 and gpr54 mRNA levels (Fig. 5), compared to the non-overripe females. No significant differences were found for the fsh-β, gnrh2 and gnrh3, even though the latter showed a tendency to decline in the overripe (Paper I: Fig. 5-A and 6). Additionally, T and E2 circulating levels were lower (undetectable) in the overripe with a low GSI than in the overripe females with a high GSI and several egg clutches. The overripe fish from Askö had lower 17,20β-P levels than the non-overripe non-ovulated but not than the non-overripe ovulated ones (Paper I: Fig. 4-B). Circulating 17,20β,21-P was under the limit of detection in all females.

Figure 4. Plasma levels of (A) testosterone, (B) estradiol, (C) 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) in non-overripe (n = 29) and overripe (n = 18) female sticklebacks from Skåne (Skåre harbour) (* p < 0.05, *** p < 0.001).
Figure 5. Relative mRNA levels of (A) luteinizing hormone (lh-β) in the pituitary and of (B) kisspeptin 2 (kiss2) and (C) its G protein-coupled receptor (gpr54) in the brain of non-overripe \((n = 10-12)\) and overripe \((n = 8-9)\) female sticklebacks from Skåne (Skåre harbour) \((^* p < 0.05)\).

**Paper II**

To investigate the profiles of the BPG axis hormones over the natural spawning cycle, same hormones as in **Paper I** were analysed in female sticklebacks sampled at different time-points pre- (“ready to spawn”) and post-spawning (6, 24, 48 and 72 hps). The spawning cycle (or inter-spawning interval) was usually at 3 days with *ad libitum* food since most of females (15 out of 19) were ovulated again at 72 hps. The GSI peaked in the “ready to spawn” females, dropped to a minimum immediately after spawning and then again increased progressively from 6 to 72 hps (**Paper II**: Fig. 1-A). HSIs show no particular changes (**Paper II**: Fig. 1-B).

The circulating levels of sex steroids showed fluctuations over the spawning cycle (Fig. 6). T showed peaks at 24 and 48 hps while significantly decreased at 72 hps, when the females were ovulated and at similarly low levels as “ready to spawn” females (Fig. 6-A). One of the females at 72 hps, that were analysed for T, was non-ovulated and had T levels at 69.29 ng/ml, i.e. similar as the levels in the non-ovulated females at 48 hps. E2 increased significantly a little earlier than T, already 6 hps and remained at high levels up to 48 hps. No difference was found between the females at 72 hps and “ready to spawn” (Fig. 6-B). A strong positive correlation between T and E2 levels appeared over the cycle (Fig. 6-C). Both 17,20β-P and 17,20β,21-P were non-detectable in most of the samples from this study (not included in **Paper II**).

The pituitary lh-β mRNA levels showed a peak at 48 hps, a day before ovulation (Fig. 6-D), while rest of studied hormones did not change over the cycle (**Paper II**: Fig. 4-A and 5).
Figure 6. Plasma levels of (A) testosterone and (B) estradiol \((n = 11-15/\text{group})\), (C) their correlation \((n = 61)\) and (D) Relative mRNA levels of luteinizing hormone \((lh-f)\) in the pituitary \((n = 13-16/\text{group})\), normalized against the 18s rRNA reference gene, over the spawning cycle in female sticklebacks. Groups: Ready: "ready to spawn" females, ovulated and showed a positive courtship to a nesting male, 6hps: 6 ± 1 hours post-spawning (hps), 24hps: 24 ± 1 hps, 48hps: 48 ± 1 hps, 72hps: 3 ± 1 hps females. (* \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\)).

Paper III

In this paper, natural vitellogenesis was studied over the spawning cycle and at egg overripening in the stickleback, analysing the *vitellogenin* liver mRNA levels. The *vitellogenin* mRNA was present at all different time-points and relative levels differed over the 3-day spawning cycle (Fig. 7-A). Those were higher at 24 and 48 hps than both "ready to spawn" females and the females at 6 hps. No further differences were observed (Fig.7-A). Even though the significant increases, the magnitude of change was small at c. 1.3-1.4 times suggesting a rather continuous vitellogenesis. *vitellogenin* mRNA levels were positively correlated with both E2 and T circulating levels over the cycle (Paper III: Fig. 2 and 3). The latter ones had been measured in Paper II in the same individual fish.

Overripe females had lower *vitellogenin* mRNA levels than the non-overripe non-ovulated, but not than the non-overripe ovulated ones. Non-overripe groups did not differ to each other (Fig. 7-B). Similarly to the cycle above, the magnitude of change was small at c. 1.5 times suggesting a decreased but not eliminated vitellogenin production at overripening.
Figure 7. Relative mRNA levels of vitellogenin in the liver of (A) female sticklebacks over the spawning cycle (Ready: “ready to spawn”, 6hps: 6 ± 1 hours post-spawning (hps); 24hps: 24 ± 1 hps, 48hps: 48 ± 1 hps, 72hps: 72 ± 1 hps, n = 10/group) and (B) non-overripe, non-ovulated (n = 9), non-overripe, ovulated (n = 10) and overripe (n = 10) female sticklebacks. The relative mRNA levels are normalized against the 18s rRNA reference gene (* p < 0.05, ** p < 0.01).

Paper IV

The changes in OF properties were studied at overripening of eggs and the effects of different sex steroids hormones were also investigated in female sticklebacks, measuring the amount of the OF and studying its protein content and composition. Overripe females had a diminished amount of OF in their ovaries (Fig. 8-A) (see also Paper IV: Fig. 1), that was more turbid, had a higher percentage of dry weight (Fig. 8-B) and a lower viscosity than the non-overripe ovulated females. In addition, OF from overripe females had higher protein levels than the non-overripe ones (Fig. 8-C) (see also Paper IV: Fig. 4). The 1-D SDS-PAGE analysis of protein profiles showed that OF contains a number of proteins; some appeared to correspond to protein from the eggs while some others are coming from other sources (Paper IV: Fig. 6). The band at c. 100 kDa was often the dominating one in the lanes, probably leaking out from the oocytes, since this band was the major band in all egg samples and likely represent yolk proteins. No consistent differences were found between the two conditions while in general the protein composition was highly variable not only between, but also within conditions (Paper IV: Fig. 6 and 7).

T-treated females had a delayed next ovulation compared to the controls and to other treatments (Paper IV: Table 2). Both T and 17,20β-P induced increase in OF amount (Fig. 9-A) (see also Paper IV: Fig. 2); however OF protein levels were only elevated in 17,20β-P-treated females (Fig. 9-B) suggesting that OF secretion is under the control of this steroid. OF was much more viscous in T-treated that in controls, whereas in female treated with 17,20β-P the OF was much less viscous than controls. The protein
composition by 1-D SDS-PAGE showed high individual variability but no consistent differences among treatments, except for E2-treated fish where the protein band at c. 100kDa was mostly absent or very weak (Paper IV: Fig. 8).

Figure 8. Changes in ovarian fluid (OF) properties at overripening of eggs. (A) Amount of OF relative to entire ovary (O) weight, (B) dry weight of the OF and (C) protein content of OF (expressed per OF weight) in the non-overripe ovulated (n = 10-15) and overripe (n = 8-15) female sticklebacks from Group A (Skåne fish) (** p < 0.01, *** p < 0.001).

Figure 9. Effects of different sex steroid treatment on the ovarian fluid (OF) (A) Amount relative to egg clutch (E) weight and (B) protein content. Treatments of females are presented as: Control: only cocoa butter (n = 18/10), T: testosterone (n = 7), E2: estradiol (n = 13/10), 17,20β-P: 17,20β-dihydroxypregn-4-en-3-one (n = 10/9), P4: Progesterone (n = 10/8) (** p < 0.01, *** p < 0.001).
Discussion

The fact that, under optimal conditions, the stickleback can mature, ovulate spontaneously and spawn naturally in a nest built by the male in the laboratory is a great advantage when studying the hormonal and physiological changes over the natural spawning cycle. As a multiple spawner, stickleback produces several egg clutches over the breeding period (Baggerman, 1957) at intervals of a few days (Wootton, 1973; 1974). However, if spawning does not occur, the eggs can be spontaneously dropped and lost, or remain in the ovarian cavity and undergo overripening, which not only makes these eggs a loss, but also blocks further clutches. This phenomenon of overripening of eggs is present in the stickleback both in captivity and nature (Lam et al., 1978; present thesis).

This PhD thesis focuses on the reproductive physiology of the female threespined stickleback, with a particular interest in the phenomenon of overripening of eggs and in the spawning cycle. Both the profiles of the reproductive hormones of the BPG axis and vitellogenesis were investigated under the above different physiological conditions (Paper I-III). In addition, the changes in the OF properties were studied at egg overripening and after the treatment with different sex steroid hormones using Silastic capsules (Paper IV).

Overripe females found to have a significant reduction in most of endocrine parameters of BPG axis and vitellogenesis, while OF properties changed at egg overripening (Paper I, III, IV). The spawning cycle usually lasted 3-days, and ovulation occurred sometime between 48 and 72 hps. Some reproductive hormones underwent changes showing cyclicity, whereas others remain constant over this 3-day cycle (Paper II). Even though vitellogenin mRNA levels showed small changes and positively correlated to E2 and T over the cycle, it seems that vitellogenesis is rather continuous (Paper III). Some of the sex steroids changed OF properties in ovulated females, with the 17,20β-P to be the most likely responsible for stimulation of OF secretion (Paper IV).
Ovarian weights and circulating sex steroids

The stickleback, as a repeat spawner, produces egg in clutches over the breeding period (Baggerman, 1957) and spawns at intervals of a few days (Wootton, 1973; 1974). Thus, in Paper II, there was a progressive increase in the GSI over the spawning cycle. The GSI increase is mainly due to yolk accumulation in the beginning and at the end also to ovarian fluid accumulation into the ovary and swelling of the ovulating eggs due to an increase in both dry matter content and water content (Wootton, 1974). The follicles enlarge by yolk accumulation during an active vitellogenesis at first, while the further enlargement is because of the hydration during the steroid-induced maturation (Wallace and Selman, 1979). Similar GSI patterns as in Paper II have been found in other multiple spawners, e.g. in the cyprinid kanehira bitterling (ISI = ~ 5 days) (Shimizu et al., 1985) and in the chichibu-goby (ISI = ~ 10 days) (Kaneko et al., 1986) with the highest GSI when fish are ovulated, before spawning In the Paper I, GSI was higher in overripe than in the non-overripe females (both non-ovulated and ovulated). However, we found overripe females both with low GSIs which had ovulated only once after overripening, as has also been found by Lam et al. (1978), and high GSIs which had ovulated again after overripening, having several layers of overripe eggs (mainly in fish from Askö). Thus, sometimes the females can become overripe, and subsequently egg-bound, with just one clutch of eggs and then may stop to produce further clutches, but others continue to ovulate again resulting in a huge abdomen. We also found in Paper I that among the overripe females from Skåne (Skåre harbour), the GSI was significantly lower in those ones which had both T and E2 in low levels (undetectable) than in those with several overripe egg clutches and a high GSI.

The circulating levels of T were lower in overripe than non-overripe sticklebacks, however not lower than in the few non-overripe ovulated females (see Paper I: Table 3). In Paper II, we showed that T levels increased at 24 and 48 hps, i.e. in non-ovulated females and decreased markedly at 72 hps, when most of females were ovulated again. This is in accordance with the results in Paper I where very low T levels appeared in non-overripe ovulated and overripe (post-ovulated) females compared to the high levels in mature non-overripe non-ovulated ones. The results of both Paper I and II could demonstrate the possible importance of T during vitellogenesis and maturation, while the drop afterwards could be related to ovulation. The observed T pattern in Paper II is also largely consistent with those in other multiple spawning fishes (e.g. kanehira bitterling, Shimizu et al., 1985; goldfish, Kobayashi et al., 1987, 1988; red sea bream, Matsuyama et al., 1988) where T appears in higher levels before ovulation, but drops by the time of ovulation. Furthermore, a decline in T levels (similar to the overripe females in Paper I) appears in rainbow trout females with retained post-ovulatory eggs (Chyb
et al., 1999; Scott et al., 1983). It is known that androgen T can be converted via aromatization to the estrogen E2 and has been found in high levels in females of many teleosts, including breeding female sticklebacks (Borg, 1994; Borg and Mayer, 1995, present thesis). However, the biological role of the unconverted form, acting as an androgen, is still not well known in fish, even though androgen receptors occur in both sexes and in female sticklebacks the androgen receptor-beta mRNA levels are as high in the ovaries as in the testes (Hoffmann et al., 2012).

In Paper II, T and E2 largely follow a rather similar pattern over the stickleback spawning cycle with a strong positive correlation between individual T and E2 levels over the cycle, which do not suggest any major role of T other than being an E2 precursor.

E2 is known to be responsible for stimulation of vitellogenin production by the liver (Nagahama and Yamashita, 2008) and thus, in Paper I, the low E2 levels at overripening could be connected to a vitellogenesis reduction (which was shown in Paper III). A decrease in E2 at -and after the ovulation also appears in rainbow trout (Chyb et al., 1999; Scott et al., 1983). In Paper II, we found that E2 declined in ovulated females (72 hps) as well. After spawning, E2 increased already after 6 hours and remained high at 24 and 48 hps. Similar patterns with high E2 levels in pre-spawning females and a decline before ovulation have been found in other fishes (e.g. kanehira bitterling, Shimizu et al., 1985; chichibu-goby, Kaneko et al., 1986; common carp, Santos et al., 1986; white sucker, Catostomus commersoni, Scott et al., 1984; rainbow trout, Scott et al., 1983). The early post-spawning increase of E2 in the stickleback in Paper II, already 6 hps, suggests an early renewal of vitellogenesis.

Besides the reduction in 17,20β-P levels at overripening in Paper I, the 17,20β-P was higher in non-overripe females judged close to ovulation while 17,20β,21-P was non-detectable in all females. These results could suggest that the 17,20β-P is the more likely MIH in the stickleback, the progestogen responsible for the oocyte maturation and ovulation under the control of the pituitary LH (Nagahama and Yamashita, 2008). Over the stickleback cycle 17,20β-P was detectable only in a few females, mostly at 48 hps, i.e. a day before ovulation and 17,20β,21-P was also detectable in a few samples, but not in a specific group of females (not included in Paper II). An often short-lived peak of the 17,20β-P in the maturational phase before ovulation followed by a decline at ovulation has also been observed in other fishes (e.g. kanehira bitterling, Shimizu et al., 1985; goldfish, Kobayashi et al., 1987; common carp, Santos et al., 1986) while in rainbow trout, 17,20β-P remains high even after few days after ovulation (Scott et al., 1983). In the tench there is a post-spawning peak of both progestogens (Antonopoulou et al.,
suggesting a yet unknown role besides the oocyte final maturation. The often undetectable levels of progestogens in the stickleback could be due to that levels are never so high but also to a short-lived peak, which could often have been missed (e.g. Gothilf et al., 1997; Murayama et al., 1994). The decrease in the 17,20β-P levels in sticklebacks with post-ovulatory overripe eggs in the Paper I, could suggest an additional role of this steroid in egg viability (maybe via stimulation of OF secretion, as shown in Paper IV). Generally, there is a decrease of sex steroid hormones after ovulation in both stickleback (Paper I) and rainbow trout (Chyb et al., 1999; Scott et al., 1983) which could be related to the loss of viability of ovulated egg, which happens during overripening. The steroid secretion by the POFS has been proposed to be crucial in maintaining egg viability after ovulation in the stickleback since it may stimulate the OF secretion (Lam et al., 1979) (effects of sex steroids were tested in Paper IV and discussed later).

Pituitary gonadotropins

In Paper I, the low pituitary lh-β mRNA levels at overripening could be responsible for the low levels of plasma 17,20β-P in overripe females. In Paper II pituitary lh-β mRNA levels showed a peak at 48 hps, i.e. a day before the next ovulation. It is known in fish that a peak in LH leads to the MIH synthesis for final egg maturation induction (Nagahama and Yamashita, 2008). Similarly to the results of Paper II, circulating LH ("GTH") increases during maturation phase having a peak just prior ovulation and then drops rapidly in cyprinids (e.g. Kobayashi et al., 1987, 1988; Santos et al., 1986; Scott et al., 1984; Shimizu et al., 1985), while in salmonids circulating LH ("GTH") increases for several weeks after ovulation (Scott et al., 1983).

Both in Paper I and II, pituitary fsh-β mRNA levels did change neither at overripening nor over the spawning cycle in the stickleback. However, fsh-β mRNA levels change under different seasons, photoperiods or by castration and steroid treatment in the stickleback (Hellqvist et al., 2006; 2008 Shao et al., 2013) or over the cycle in other fishes (e.g. Gothilf et al., 1997; Ohta et al., 2008). The role of FSH is only clear in salmonids where it has a primary role in the early stages of oocyte growth during vitellogenesis, inducing E2 production (e.g. Swanson et al., 2003). Our results suggest that FSH synthesis is probably not affected much by overripening (Paper I) and that a continuous FSH synthesis, probably required, appears over the cycle (Paper II).
Brain neuropeptides

Regarding the studied neuropeptides and neurotransmitters in the brain, the grnh2/grnh3 mRNA levels did not differ at egg overripening - even though grnh3 had a tendency to decline in overripe females - (Paper I), and remain constant over the spawning cycle (Paper II). In other fishes, grnhs vary over the spawning cycle or season (e.g. Canosa et al., 2008; Gothilf et al., 1997; Shahjahan et al., 2010a). The GnRHs control the GTHs (Zohar et al., 2010) and in sticklebacks appear to have roles in the control of seasonal reproduction (Shao et al., 2015).

Important factors that trigger reproduction in fishes, even though specific roles are still unknown, are the kisspeptins and their receptors (Zohar et al., 2010). In Paper I, we found low kiss2 and its receptor (gpr54) mRNA levels at overripening, suggesting that this decline can be a factor suppressing the BPG axis at overripening and have a role in the control of the female reproduction. On the other hand, both kiss2 and gpr54 remain constant over the stickleback cycle (Paper II). In several fishes, their expressions change seasonally with high levels in the reproductive period (e.g. Shahjahan et al., 2010b, Zmora et al., 2012), supporting the idea of their role in reproduction.

Vitellogenesis

The natural vitellogenesis was investigated by measuring the relative vitello-genin mRNA levels, based on a stickleback vitellogenin B partial gene sequence (Hogan et al., 2008) (Paper III). Though the levels are significantly higher at 24 and 48 hps than at other times, the magnitude of changes was small (~ 1.4 times) over the cycle. The pattern of vitellogenesis has been studied in other multiple spawners (mummichog, Cerdá et al. 1996; fathead minnow, Pimephales promelas, Jensen et al. 2001), where changes were similarly small and vitellogenesis relatively constant over their spawning cycle. In the stickleback, Wallace and Selman (1979) have studied the oogenesis histologically and suggested that the synthesis and secretion of vitellogenin by the liver is relatively continuous in breeding female sticklebacks over the reproductive period. They also suggest that the sequestration of vitellogenin is changing instead of the levels in the blood itself; when one oocyte group is entering maturation, and stops incorporate vitellogenin, a new oocyte group begins vitellogenin uptake. The results of Paper III are consistent with this since the changes were small suggesting a relatively constant vitellogenin production and that vitellogenesis is rather continuous over the spawning cycle in stickleback and other multiple-spawning fishes. That could be more advantageous for multiple spawners than to interrupt it for every spawning due to their limited spawning season during which they have to produce as many egg clutches as possible, and simultaneously hav-
ing vitellogenesis for an egg group and maturation for another group it saves time.

Although a positive correlation between the E2 and T and vitellogenin over the stickleback cycle, the magnitude of changes in circulating E2 and T was larger (Paper II) than in vitellogenin mRNA levels (Paper III) over the cycle. The significant increases in E2 already at 6 hps than in vitellogenin mRNA levels, at 24 hps, suggest some delay in the action of E2 on vitellogenin synthesis. Estrogens mediate their action of vitellogenesis via estrogen receptors in the liver and which activate the vitellogenin gene expression (Mommsen and Walsh 1988; Wallace 1985; Wahl 1988). The delay in vitellogenin mRNA in the stickleback could be due to that the process takes some time. Also, it has been shown in the rainbow trout that the expression of the estrogen receptor and vitellogenin genes are induced differentially by estrogens in the liver, showing different threshold responses to estrogen with the vitellogenin genes to require a higher estrogen level, and this suggests that the two genes are controlled differently by the estrogen-estrogen receptor complex (Flouriot et al. 1997b). In addition, a high expression of vitellogenin gene demands the presence of higher estrogen receptor levels to be induced and the vitellogenin transcriptional response is directly proportional to the amount of estrogen receptors that are synthesized (Flouriot et al. 1997a), which could explain the delay in vitellogenin mRNA levels in the stickleback.

Even though vitellogenesis was decreased at overripening, it was not eliminated. The lower vitellogenin mRNA levels in overripe females (Paper III) could probably be due to the low circulating E2 (and maybe also T) in overripe females (Paper I). However, the magnitude of changes in circulating E2 and T at overripening was at least 4 times (Paper I) which is much larger than that in the liver vitellogenin mRNA levels (~1.5 times), similarly low to the magnitude of changes found over the cycle (Paper III). Thus, the results do not suggest an abolishment of vitellogenesis in females with overripe eggs since vitellogenin is still present, proposing that even very low E2 levels can stimulate vitellogenin transcription in the liver, and/or that the stimulatory effects are persistent. The diminished vitellogenin production at overripening could be advantageous as keeping a lower vitellogenesis level could give higher chances to the overripe egg-bound females to survive for the next season, avoiding further egg clutches accumulation. To entirely avoid further vitellogenesis comes at a price, since if the ovulated females would wait to start vitellogenesis until after spawning, they will lose time for producing more egg clutches.
Ovarian fluid – egg overripening

The stickleback females with overripe eggs had less OF with a lower viscosity, a higher percentage dry weight and much higher protein levels compared to females with non-overripe ovulated eggs (Paper IV). A secretory activity of the ovarian lumen epithelium has been previously proposed not only in the stickleback (Lam et al., 1978) but also in other fishes, such as in medaka (Takano, 1968; Yamamoto, 1963), goldfish (Takahashi and Takano, 1971) and bleak (Lahnsteiner et al., 1997). Lam et al. (1978) have previously observed much less OF in the “berried” female (overripe) sticklebacks compared with the presence of abundant OF in the “nonberried” females (non-overripe ovulated), similarly to the present results (Paper IV). Lam et al. (1978) have observed that overripe eggs are larger in size and have higher percentage water content than normal non-overripe ovulated eggs. The authors proposed that the increased water content in overripe eggs may compensate the apparent loss of OF from the ovarian cavity, suggesting that the OF in the overripe females has been absorbed into the eggs without being replenished (Lam et al., 1978). In Paper IV, the OF percentage dry weight was ~ 4.8 times higher in overripe than in non-overripe ovulated females, which is much more than can be explained due to the diminished OF amount. Overripe eggs may absorb water from the OF while the same time dry components may be “leaking” out to the OF from the overripe eggs. The OF in non-overripe ovulated sticklebacks was transparent and quite viscous, while in overripe ones OF was turbid and much less viscous. The change in OF viscosity was a very marked and consistent phenomenon, even though it was not measured. As OF was much more viscous in non-overripe ovulated than in overripe females we expected it to contain more proteins and to have a higher percentage of dry weight than the overripe ovaries, but the opposite was the case. Dietrich et al. (2012) showed in walleye, *Sander vitreus*, that the OF amount positively influences viability of ovulated eggs after 42 hours of *in vitro* storage. OF turbidity increase after the *in vitro* storage is positive correlated with oocyte ageing and is probably due to elevated OF protein concentrations that increase with the percentage of “large” overripe eggs (Dietrich et al., 2012).

Stickleback OF in non-overripe ovulated females contained c. 2 mg/ml of protein. The protein levels were significantly higher in overripe sticklebacks which had protein c. 46 mg/ml i.e. ~ 23-24 times higher than in non-overripe ovulated females (Paper IV). OF protein levels increase during the post-ovulatory ageing and overripening in several fishes, such as turbot (Fauvel et al., 1993), lake trout (Lahnsteiner et al., 1999), rainbow trout (Lahnsteiner, 2000; Aegerter and Jalabert, 2004) and different cyprinids (Lahnsteiner et al., 2001). Even though there is a consistent increase in OF
protein levels at overripening, both the relative and the absolute increase were much higher in the stickleback than in the other fishes.

OF proteins may come from several sources such as actively secreted by the ovarian epithelial cells or released from rupturing follicles cells during ovulation or leakage from overripe eggs due to degradation and/or changes in egg cell membrane (Lahnsteiner et al., 1997, 1999, 2001; Lahnsteiner, 2000, 2007; Lahnsteiner et al., 1999). Using 2-D SDS-PAGE, Rime et al. (2004) have observed that some protein spots, including vitellogenin fragments, progressively accumulate in the OF during the overripening in the rainbow trout, and that some of them are not present at ovulation time suggesting a leakage of oocyte components due to weakening of the cellular membrane. Dietrich et al. (2012) have found that egg storage in OF in vitro cause eggs to release higher protein amounts, assuming that the yolk in the eggs was broken down and leaked out through the egg membrane. In Paper IV, 1-D SDS-PAGE analysis showed that stickleback OF contains several proteins; some of them probably come from the eggs (e.g. c 100 kDa) since they appeared to correspond to proteins from the eggs, but some others may be also delivered from sources other than eggs (e.g. c. 200 kDa, single band at c. 70-75 kDa) such as ovarian epithelium. The former were usually more prominent in OF in overripe females. Also, the variation in OF protein levels in overripe females was rather modest which is more consistent with leakage over membranes than with accidental eggs breaks. Changes in egg membranes of overripe eggs in the stickleback must occur, as overripe eggs are bigger in size than non-overripe ovulated eggs (Lam et al., 1978; own observations), however the protein composition profiles were largely similar between the non-overripe and the overripe fish, without consistent differences. The protein profiles varied considerably also between sticklebacks in the same condition. To conclude, as proteins appeared to leak out from the eggs, the increased OF protein levels in in overripe females may be a consequence rather than a cause of overripening.

Ovarian fluid – effects of sex steroids

In Paper IV, the effects of different steroids on OF properties were tested in female sticklebacks. Both T and 17,20β-P induced a significant increase in OF amount not only compared to the controls but also to E2 and P4 treatments. The OF was transparent in all treatments and controls (unlike in overripe fish), but it was much more viscous in T-treated females and much less viscous in 17,20β-P-treated ones compared to the controls. OF protein concentration was significantly increased only by 17,20β-P (Paper IV).

The secretory activity of the ovarian lumen epithelium has been suggested to be under the control of sex steroids in goldfish fry (Takahashi and Takano,
Immersion in vivo of just ovulated female goldfish (ovulation induced by hCG) in P4 solution (0.05 ppm) increase the fertilization rates of stripped eggs at 12 hours while 17,20β-P (0.025 or 0.05 ppm) do not have significant effects (Formacion et al., 1995). Also, immersions in vitro of just ovulated eggs in OF containing anti-serum against P4 decreased the fertilization rates after 6 hours incubation suggesting a role of P4 (or other progestogens cross reacting with the antiserum) in delay of overripening (Formacion et al., 1995). In the stickleback, Lam et al. (1978) proposed that steroids secreted by the POFs stimulate the epithelium to secrete OF, maintaining the egg viability. POFs are degenerated and the ovarian cavity epithelium is regressed in overripe sticklebacks (Lam et al., 1978). Lam et al. (1979) treated spawned sticklebacks with E2 (1 ppm) or P4 (0.5 ppm) via the water for 5 days and overripe female with P4 (0.5 ppm) for 9 days. P4 treatment resulted in copious amounts of OF, whereas the effect of E2 was less clear. A role of P4 on OF secretion and maintenance of egg viability was suggested by Lam et al. (1979).

P4 is the major progestogen in many vertebrates; however this is not the case for teleosts. In teleosts, the final egg maturation and ovulation are stimulated by a peak in LH from the pituitary leading to synthesis of a MIH by the follicle cells surrounding the oocyte (Nagahama and Yamashita, 2008). The progestogens 17,20β-P and 17,20β,21-P are the two probable MIHs in teleosts. The progestogen 17,20β-P is suggested to be a more likely MIH than the 17,20β,21-P in the stickleback (Paper I). In Paper IV, 17,20β-P-treated females were mostly ovulated again after 3 days (whereas T delayed ovulation), OF amount was much higher than in controls (T also increased OF amount). However, 17,20β-P females had OF with high protein levels but less viscosity than controls and other steroid treatments, suggesting a role of this steroid in ovulation, OF secretion and increased protein secretion. There appears to be a general and negative correlation between viscosity and protein levels of OF; this was not only so in 17,20β-P-treated females but also in overripe females.

What can be the cause of egg overripening?

The results of the present thesis regards the overripening of eggs suggest that changes in reproductive hormones (Paper I), vitellogenesis (Paper III) and in OF properties (Paper IV) are more likely consequences rather than causes of overripening. Lack of male nests or spawning sites has been suggested, among other factors, to be related to the failure of the female fishes to spawn (Rideout et al., 2005). We have carried out experiments showing that full overripening in stickleback females occurred only when there is lack of males with nests (unpublished preliminary results). The presence of overripe females is quite common in nature, especially at the end of the breeding sea-
son (Borg and van Veen, 1982, our own observations) and this could be explained by that female initiative in courtship behaviour is quite common, mainly late in the breeding season (Borg, 1985; Kynard, 1978; our own observations at Askö). The brood-caring males may be the limiting sex, and so not all females have an opportunity to lay their eggs. The reduction in hormones and vitellogenin production, as well as the OF changes at overripening (Paper I, III, IV) are likely an effect of rather than the cause of egg retention. To better understand a possible evolutionary significance of the changes at egg overripening, it is critical to know whether the overripe females can recover and spawn again in the next breeding season, and this is more likely to happen if both the levels of the reproductive hormones and vitellogenin production continue be low resulting in less further ovulations and higher possibilities for the overripe eggs to be reabsorbed (Paper I, III).
Concluding remarks

In this PhD thesis, I have investigated the reproductive physiology of the female sticklebacks, focusing on the phenomenon of egg overripening and the spawning cycle. I conclude that overripening of eggs is accompanied by a reduction in most of reproductive hormones of the BPG axis and in vitellogenin production, as well as by changes in ovarian fluid properties. These are more likely to be the effects of overripening of eggs rather than causes. Also, the first two consequences ought to be advantageous from an evolutionary perspective for the overripe fish since they increase their possibilities for survival. Even though the patterns in hormonal changes and vitellogenesis over the stickleback spawning cycle are largely consistent with others in other multiple spawning fishes, an early rise in both E2 and T levels after spawning is remarkable in the stickleback. These increases do not happen if spawning does not take place, as I observe in females with post-ovulatory overripe eggs into the cavity that have low (or undetectable) E2 and T levels. These combined results highlight that spawning is obviously responsible for this rise and considering this, it is also important to study by which mechanism this is regulated, but also whether it is the act of spawning or the remaining presence of eggs that is critical. Sex steroids seem to control not only the spawning cycle but also the OF secretion and properties, with the role of 17,20β-P to be more advanced maintaining the viability of ovulated eggs. To conclude, the knowledge of the reproductive physiological changes is important for understanding their essential roles in the production of viable eggs crucial for reproductive success in this species but also in other female fishes.
Swedish summary / Svensk sammanfattning


I denna doktorsavhandling studerades förändringarna i fortplantningshormoner och gulebildning i samband med övermognad av ägg och under den naturliga lekcykeln. Även ovariévätskans egenskaper undersöktes vid övermognad av äggen och efter behandling med könssteroider.

Plasma nivåer av steroiderna: testosteron (T), östradiol (E2), 17,20β-dihydroxyprog-4-en-3-one (17,20β-P) och 17,20β,21-trihydroxyprog-4-en-3-one (17,20β,21-P) mättes med hjälp av radioimmunoassay och relativa nivåer av mRNA gonadotropinerna (*fsh-β/lh-β*) i hypofysen och av gonadotropin-releasing hormonerna, (gnrh2/gnrh3) och kisspeptin och dess receptor (kiss2/gpr54) i hjärnan mättes med qPCR. Övermognad av äggen åtföljs av en signifikant minskning i de flesta endokrina parametrarna på hjärn-hypofys-gonad axeln (T, E2, 17,20β-P; lh-β; kiss2, gpr54). Låga nivåer av hormonerna kan vara fördelaktigt för de övermogna äggstina honorna, de detta kan minska ytterligare ovulationer och ge bättre chanser att kunna överleva och fört-planta sig på nytt. Över lekcykeln som varade tre dagar, var T och E2 starkt korrelerade och visade en cyklicitet med låga halter vid ovulationen och ökande nivåer från 24 och 6 timmar efter leken, respektive. Själva leken kan ge upphov till dessa ökningar då de inte sker om äggläggning inte sker (övermogna honor). En topp i hypofysens lh-β mRNA nivåer fanns 48 timmar efter leken, en dag innan nästa ovulation. Inga signifikanta förändringar påvisades för de andra studerade hormonerna.

Gulebildningen studerades genom mätning av *vitellogenin* mRNA nivåerna i levern med qPCR. Nivåerna var högst 24 och 48 timmar efter leken och var positivt korrelerade med både E2 och T över cykeln. Förändringarna var emellertid små, vilket tyder på en ganska kontinuerlig gulebildning över spiggens lekcykel vilket kan vara till fördel för en fisk som lägger ägg flera gånger under en begränsad lektid. Övermognad minkade *vitellogenin* mRNA nivåerna, men de försvann inte.
Mängden ovarievätska minskade hos de övermogna honorna och vätskan hade en lägre viskositet men högre torrvikthalt och proteininnehåll än hos icke-övermogna ovulerade honor, vilket tyder på förändringar i ovarievätskan som kan relateras till övermognad av äggen. Effekterna av steroider studerades genom användande av Silastic kapslar. T och 17,20β-P inducerade enökning av mängden av ovarievätska, men protein nivåerna ökades bare hos 17,20β-P-behandlade honor, vilket tyder på att denna steroid har en roll i att kontrollera sekretionen av ovarievätska. 1-D SDS-PAGE elektrofores visade att ovarievätskan innehåll flera proteiner av vilka en del kom från äggen, men det fanns inga konsekventa skillnader mellan grupperna.

Sammanfattningsvis är kunskap om förändringar i fortplantningsfysiologin viktig för att förstå dessa avgörande betydelse för produktionen av livsdugliga ägg hos denna art, men också för fortplantningsfysiologin hos honfiskar i allmänhet.
Αναπαραγωγική φυσιολογία του θηλυκού ακανθερού, *Gasterosteus aculeatus*


Στην παρούσα διδακτορική διατριβή, οι μεταβολές των αναπαραγωγικών ορμονών και της λεκιθογένεσης (vitellogenesis) μελετήθηκαν τόσο στην υπερωρίμανση των αβγών όσο και κατά τη διάρκεια του φυσικού κύκλου ωοτοκίας. Επίσης, οι ιδιότητες του OF εξετάστηκαν στην υπερωρίμανση των αβγών, καθώς και μετά τη χορήγηση στεροειδών ορμονών (sex steroids).

Τα επίπεδα των παρακάτω στεροειδών στο πλάσμα του αίματος μετρήθηκαν με τη μέθοδο του radioimmunoassay (radioimmunoassay). Τα στεροειδή που μελετήθηκαν ήταν: η τεστοστερόνη (testosterone, T), η οιστραδιόλη (estradiol, E2) και δύο μέλη προγεσταγόνων (17,20β-dihydroxypregn-4-en-3-one, 17,20β-P; 17,20β,21-trihydroxypregn-4-en-3-one, 17,20β,21-P). Τα σχετικά επίπεδα mRNA των γοναδοτροπινών της υπόφυσης (fsh-β / lh-β), των ορμονών εκλυτικής απελευθέρωσης των γοναδοτροπινών του εγκεφάλου (gnrh2 / gnrh3), καθώς και της κισπεπτίνης (kisspeptin, kiss2) και του υποδοχέα της (gpr54) στον εγκέφαλο μετρήθηκαν με τη μέθοδο qPCR.

Η υπερωρίμανση των αβγών συνοδεύτηκε με σημαντική μείωση των περισσότερων ενδοκρινικών παραμέτρων του BPG άξονα (T, E2, 17, 20β-P; lh-β; kiss2, gpr54). Τα χαμηλά επίπεδα αυτών των ορμονών θα μπορούσαν να θεωρηθούν ως πλεονέκτημα για τα θηλυκά άτομα με υπερώριμα αβγά, καθώς αυτά θα μπορούσαν να μειώνουν τον αριθμό τους περαιτέρω ωορρηξιών, παρέχοντας μεγαλύτερες πιθανότητες επιβίωσης και μελλοντικής αναπαραγωγικής επιτυχίας. Κατά τη διάρκεια του τριήμερου...
κύκλου ωοτοκίας, τα επίπεδα της T συσχετίστηκαν σε μεγάλο βαθμό με αυτά της E2. Συγκεκριμένα, παρουσίασαν μία κυκλική εναλλαγή με χαμηλά επίπεδα ορμονών κατά την ωορρηξία, τα οποία αυξήθηκαν στις 24 και 6 ώρες μετά την ωοτοκία (hours post-spawning, hps), αντίστοιχα. Είναι πιθανόν η ωοτοκία να προκαλεί την επαγωγή ορμονών, καθώς αυτές οι αυξήσεις δεν παρατηρήθηκαν στα θηλυκά με υπερώριμα αβγά (overripe), όπου δεν πραγματοποιήθηκε απελευθέρωση των αβγών. Στις 48 hps, μια ημέρα πριν από την επόμενη ωορρηξία, παρατηρήθηκε μια κυκλική εναλλαγή στα επίπεδα mRNA της lh-β της υπόφυσης. Περαιτέρω σημαντικές αλλαγές δεν παρατηρήθηκαν για τις υπόλοιπες ορμόνες που μελετήθηκαν.

Η λεκιθογένεση (vitellogenesis) μελετήθηκε με τη μέτρηση των σχετικών επιπέδων mRNA της λεκιθογενίνης (vitellogenin) στο ήπαρ με τη μέθοδο qPCR. Το epίπεδα ήταν υψηλά στις 24 και 48 hps και συσχετίστηκαν θετικά τόσο με το epίπεδα της E2 όσο και αυτά της T κατά τη διάρκεια του κύκλου ωοτοκίας. Παρόλα αυτά, οι αλλαγές ήταν μικρές υποδηλώνοντας μια μάλλον συνεχή λεκιθογένεση κατά τον κύκλο ωοτοκίας του ακανθερού, που μπορεί να αποτελεί ενα plenoκτημα για ένα ψάρι με πολλαπλή ωοτοκία με μια περιορισμένη περίοδο ωοτοκίας.

Η ποσότητα του OF μειώθηκε στα θηλυκά με μη-υπερώριμα αβγά (non-overripe) και είχε χαμηλότερο εξώδες, αλλά υψηλότερα epίπεδα ξηρού βάρους και πρωτεΐνης από ό, τι στα θηλυκά με μη-υπερώριμα ωοθυλακωμένα αβγά (non-overripe ovulated), γεγονός που υποδηλώνει ότι οι μεταβολές στις ιδιότητες του OF σχέτισται με την αναπαραγωγή των αβγών. Οι epιδράσεις των πρωτεΐνων φύλου μελετήθηκαν χρησιμοποιώντας εμφύτευμα (Silastic capsules). Οι ορμόνες T και 17,20β-Ρ προκάλεσαν αύξηση της ποσότητας του OF, αλλά τα epίπεδα της πρωτεΐνης σε αυτό αυξήθηκαν μόνο στα θηλυκά που τους χορηγήθηκε εμφύτευμα με 17,20β-Ρ, υποδηλώνοντας έναν πιθανό ρόλο του στον χωρισμό και κατανόηση της πρωτεΐνης. Η ανάλυση με ηλεκτροφόρηση σε πηκτή πολυακρυλαμίδη (1-D SDS-PAGE) έδειξε ότι το OF περιέχει αρκετές πρωτεΐνες, μερικές από αυτές προερχόμενες από τα αβγά, ωστόσο δεν υπήρχαν σταθερές διαφορές μεταξύ των διαφορετικών ομάδων.

Συνεπειακά, η γνώση των αναπαραγωγικών φυσιολογικών αλλαγών είναι σημαντική για την κατανόηση των βασικών τους ρόλων στην παραγωγή βιώσιμων αβγών σε αυτό το είδος ψαριών και γενικότερα στην αναπαραγωγική φυσιολογία των θηλυκών ψαριών.
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