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Overripening of eggs and changes in reproductive hormones in the threespine stickleback, *Gasterosteus aculeatus*

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ABSTRACT

Background: Female threespine sticklebacks, *Gasterosteus aculeatus*, are batch spawners. As in most teleosts, the ovulated eggs are kept in the ovarian cavity until spawning. If spawning or spontaneous release of the eggs does not take place, they can become overripe and harden, and in most cases remain in the ovary. The overripe eggs are lost for reproduction and also block further spawnings. Reproductive hormones regulate egg production and may be involved in the mechanism of overripening.

Question: What are the reproductive endocrinological parameters characterizing overripening of ovulated eggs in the threespine stickleback?

Organism: Wild-caught adult threespine sticklebacks from the southern Baltic at Skåre in southern Sweden and the island of Askö in northwestern Baltic Proper in Sweden.

Experiments: We collected blood samples for hormone measurements, as well as pituitaries and brains for measurement of mRNA from both sexually mature non-overripe (non-ovulated and/or ovulated) and overripe (egg-bound) females. For the Skåre fish, sexual maturation was induced under laboratory conditions by exposure to a long photoperiod and we compared the non-overripe (including non-ovulated, with oocytes in different maturing or ripening stages, and ovulated females) with the overripe females. The Askö fish were sampled directly from nature, during the natural summer breeding season and we compared the non-overripe (including non-ovulated, with oocytes in different maturing or ripening stages, and ovulated females) with the overripe females.

Methods: In the fish collected from Skåre, we used radioimmunoassay to measure the plasma levels of four steroids: testosterone, estradiol, 17,20β-dihydroxypregn-4-en-3-one (17,20β-P), and 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P). We also measured the mRNA levels of gonadotropins [GTHs: follicle-stimulating hormone (*fsh-β*) and luteinizing hormone (*lh-β*)] in the pituitary, and of gonadotropin-releasing hormones (GnRHs: *gnrh2*, *gnrh3*) and kisspeptin (*kiss2*) and its G protein-coupled receptor (*gpr54*) in the brain by real-time

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quantitative PCR. In the fish collected from Askö, we measured only progestogens (17,20*β*-P and 17,20*β*,21-P).

Results: In the fish from Skåre, overripe female sticklebacks had significantly lower levels of circulating plasma steroid hormones (testosterone, estradiol, 17,20*β*-P), as well as of pituitary *lh-β* and brain *kiss2* and *gpr54* mRNA than the non-overripe females. In the fish caught from Askö, overripe females had lower 17,20*β*-P levels than the non-overripe non-ovulated females, but there was no difference between the non-overripe ovulated and the overripe females. The 17,20*β*,21-P plasma levels were under the limit of detection in all groups.

Keywords: testosterone, estradiol, 17,20*β*-dihydroxypregn-4-en-3-one, 17,20*β*,21-trihydroxypregn-4-en-3-one, *fsh-β*, *lh-β*, *gnrh2*, *gnrh3*, *kiss2*, *gpr54*, ovulation, spawning, overripe, reproduction, stickleback, teleosts.

INTRODUCTION

In teleost fishes, ovulated eggs are stored in the ovarian lumen, or in the abdominal cavity in salmonids, until spawning. If spawning does not occur, the ovulated eggs undergo an ageing process, known as overripening, leading to reduced egg quality (Springate *et al.*, 1984; Gillet, 1991; Bromage *et al.*, 1992). The overripe eggs can harden and are then not only lost for reproduction, but can cause physiological stress and obstruct further spawnings as the female becomes egg-bound. In extreme cases, this can result in the death of the female and in aquaculture, egg hardening is a major welfare issue. In many fishes, such as Atlantic halibut, *Hippoglossus hippoglossus* (Bromage *et al.*, 1994) and turbot, *Scophthalmus maximus* (McEvoy, 1984), the ovulated eggs decrease in overall quality within a few hours. However, salmonids, such as rainbow trout, *Oncorhynchus mykiss*, can hold the ovulated eggs in their body cavity for several days without loss of egg viability (Springate *et al.*, 1984).

Overripening of ovulated eggs is mostly known from fish in captivity, but has also been observed in nature, such as in the threespine stickleback, *Gasterosteus aculeatus* (Lam *et al.*, 1978). The stickleback is a batch spawner with indeterminate fecundity, where egg clutches are spawned at intervals of a few days over an extended breeding season in late spring and summer (Fletcher and Wootton, 1995). When females do not spawn in a nest, they usually 'drop' their ovulated eggs (in our experience usually within less than 24 hours at 20°C) and another cycle of egg maturation can begin (Wallace and Selman, 1979; for a review, see Wootton, 1985). Sometimes, however, the ovulated eggs remain in the ovarian cavity and become overripe if kept more than a few days. Hardened eggs give the fish a 'berried' appearance, which in the stickleback can be visible when there is only one batch of overripe eggs (Lam *et al.*, 1978).

Even though overripening is a well-known phenomenon, the physiological basis controlling this process is largely unknown. Circulating levels of reproductive hormones over extended periods after ovulation have been studied in the rainbow trout (Scott *et al.*, 1983; Chyb *et al.*, 1999), in which a decline in gonadal steroids could be related to a decline in viability of ovulated eggs. The aim of the present study was to determine whether overripening of ovulated eggs is related to changes in reproductive hormones in sticklebacks. To that end, plasma levels of four steroids, which are the major sex hormones in female fish reproduction (Nagahama and Yamashita, 2008) – namely, testosterone, estradiol, 17,20*β*-dihydroxypregn-4-en-3-one (17,20*β*-P) and 17,20*β*,21-trihydroxypregn-4-en-3-one (17,20*β*,21-P) – were measured by radioimmunoassay (RIA). Estradiol is responsible for stimulating vitellogenesis, while 17,20*β*-P and 17,20*β*,21-P are the steroids responsible for inducing final oocyte maturation

and subsequent ovulation (Nagahama and Yamashita, 2008). Although testosterone is the androgen present at the highest levels in female fish, its biological role is largely unknown (Borg, 1994). We also measured the mRNA levels of gonadotropins [GTHs: follicle-stimulating hormone (*fsh-β*) and luteinizing hormone (*lh-β*)] in the pituitary, and of neuropeptides important in the control of reproduction (Zohar *et al.*, 2010) – gonadotropin-releasing hormones (GnRHs: *gnrh2*, *gnrh3*) and kisspeptin (*kiss2*) and its G protein-coupled receptor (*gpr54*) in the brain – using real-time quantitative PCR (qPCR) in non-overripe and overripe female sticklebacks. Our hypothesis was that these hormones and neuropeptides would decline during overripening, since persistently high levels would not provide an advantage for the overripe fish.

MATERIALS AND METHODS

Fish and experimental set-up

We caught adult wild threespine sticklebacks, *Gasterosteus aculeatus*, using drop nets in the southern Baltic at Skåre, southern Sweden (55°22′35″N,13°3′8″E) in the late autumn (Capture A) and early winter (Capture B) of 2013. After transport to Stockholm University, the fish were kept in 700- or 1200-litre aquaria containing filtered and aerated brackish water (0.5% salinity). Temperature was gradually increased to 20°C. The photoperiod was long (16/8 hour light/dark cycle) to stimulate sexual maturation. Fine gravel covered the bottom of the aquaria and clay pots provided hiding places. We fed the fish daily with frozen bloodworms or mysids. After about 3 weeks, the matured males showed breeding colorations and aggressive behaviour while females started to have swollen abdomens due to large ovaries.

To prevent nest building by males, and thus diminish egg laying by females, algae were not present in the aquaria. We carefully sorted the stock fish groups, where both sexually mature females and males were present, every week over the course of a month for each autumn (Capture A) or winter (Capture B) capture. Sticklebacks can be in sexual breeding condition for several weeks. An equal number of sexually mature females and females that were suspected of being or becoming overripe were placed together in a 200-litre aquarium under similar conditions as before. To minimize stress effects, we kept these fish together for at least 48 hours before sampling.

In addition to fish that we caught from Skåre (Capture A and B) and kept in captivity, adult wild sticklebacks were also caught in the northwestern Baltic Proper at the island of Askö (Askö Laboratory), Sweden (58°49′N, 17°38′E). We collected the fish samples from Askö directly from nature (samples were taken within an hour) during the natural breeding season in early summer 2015; these samples were only used for progestogen analysis.

The study received permission from the Stockholm Northern Animal Experiment Ethical Committee (N 492/11, N 45/14).

Experimental groups and overripening assessment

We divided the fish from Skåre into two groups: non-overripe and overripe female sticklebacks. In the group of non-overripe females, both females with non-overripe, non-ovulated oocytes, in different maturing or ripening stages, and with non-overripe, ovulated eggs were included. Since there were only three females with non-overripe, ovulated eggs, we combined

these two groups of non-overripe females for all analyses in the fish from Skåre. The fish from Askö were split into three groups: (1) non-overripe, non-ovulated, (2) non-overripe, ovulated and (3) overripe females, as numbers in all groups were sufficient for statistical analysis.

We verified the overripening condition under a dissection microscope, using the criteria described by Lam *et al.* (1978). The females with normal non-overripe, ovulated eggs have a soft and smooth swollen abdomen (Fig. 1A) and ovary (Fig. 1C), whereas females with overripe, ovulated eggs have a berry-like abdomen (Fig. 1B) and ovary (Fig 1D), which is rough and feels hard to the touch. Furthermore, overripe eggs have aggregated cytoplasm and oil droplets at the animal pole and are more transparent (Fig. 1F) than normal non-overripe, ovulated eggs (Fig. 1E) (Lam *et al.*, 1978). The non-overripe, non-ovulated females were neither ovulated nor overripe but they had clearly maturing or ripening eggs in their ovaries. Intermediate fish that could not be classified clearly as sexually mature non-overripe or overripe were excluded. The possible presence of additional ovulated eggs and the general appearance of the oocytes were also noted. We also excluded females with a gonadosomatic index (GSI) of < 2.5 or that were otherwise suspected of being non-breeding or post-breeding.

Sampling

Fish were anaesthetized in a 0.025% solution of sodium-bicarbonate-buffered tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and weighed. The sample sizes (n) of fish that we used for each measurement are presented in Table 1. We collected the

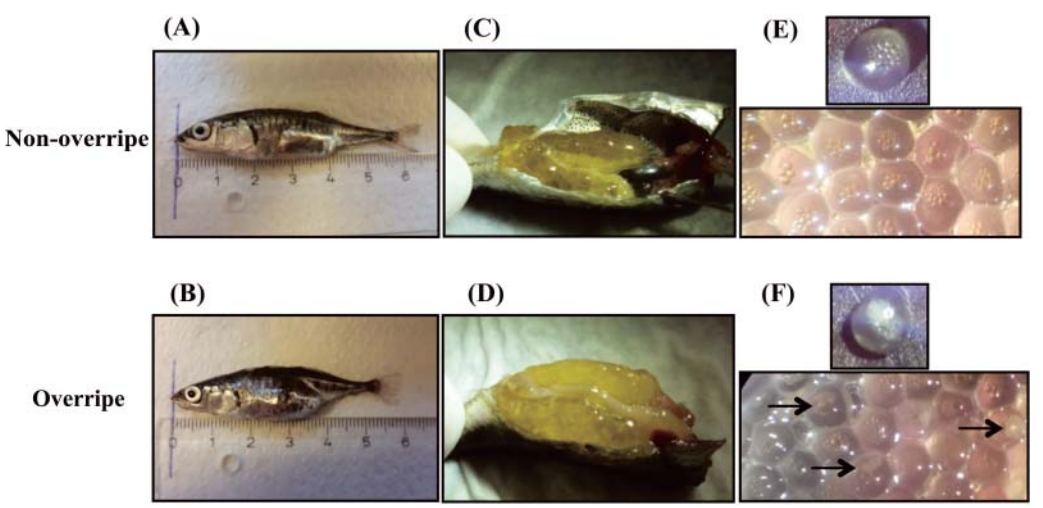

Fig. 1. Non-overripe and overripe female threespine sticklebacks, *Gasterosteus aculeatus*. (A) A non-overripe female stickleback with normal ovulated eggs has a soft and smooth swollen abdomen, whereas (B) a female with overripe, ovulated eggs has a 'berried' abdomen that is rough and feels hard to the touch. (C) Ovary with normal non-overripe, ovulated eggs in a non-overripe, ovulated female, and (D) ovary in an overripe female. (F) Overripe eggs have aggregated cytoplasm (black arrows) and oil droplets at the animal pole and are more transparent than (E) normal non-overripe, ovulated eggs (Lam *et al.*, 1978). Photos by Chrysoula Roufidou.

Table 1. Sample sizes (*n*) of fish used for GSI calculations and hormone studies

		Fish from Skåre			Fish from Askö		
		Non-overripe females			Non-overripe females		
Tissue		Non-ovulated (Capture A/B (*n*))	Ovulated (Capture A/B (*n*))	Overripe females (Capture A/B (*n*))	Non-ovulated (*n*)	Ovulated (*n*)	Overripe females (*n*)
Gonads	GSI	11/15	0/3	9/9	14	14	12
Plasma (circulating levels)	Testosterone	11/15	0/3	9/9	—	—	—
	Estradiol	11/15	0/3	9/9	—	—	—
	17,20*β*-P	9/15	0/2	9/8	14	14	12
	17,20*β*,21-P	9/15	0/2	9/8	14	14	12
Pituitary (mRNA levels)	*fsh-β*	0/7	0/1	0/6	—	—	—
	lh-β	0/9	0/3	0/9	—	—	—
Brain (mRNA levels)	*gnrh2*	0/5	0/3	0/5	—	—	—
	gnrh3	0/5	0/3	0/5	—	—	—
	kiss2	0/5	0/3	0/5	—	—	—
	gpr54	0/5	0/3	0/5	—	—	—

Note: Values are the fish sample size (*n*) used for analyses. Stickleback were caught in the southern Baltic at Skåre, southern Sweden in the late autumn (Capture A) and early winter (Capture B) of 2013, or in the northwestern Baltic Proper at the island of Askö, Sweden during the natural breeding season in early summer 2015. GSI = gonadosomatic index, 17,20*β*-P = 17,20*β*-dihydroxypregn-4-en-3-one, 17,20*β*,21-P = 17,20*β*,21-trihydroxypregn-4-en-3-one, *fsh-β* = follicle-stimulating hormone *β* chain mRNA, *lh-β* = luteinizing hormone *β* chain mRNA, *gnrh2* and *gnrh3* = gonadotropin-releasing hormone 2 and 3 mRNA, *kiss2* = kisspeptin 2 mRNA, *gpr54* = G protein-coupled receptor mRNA. — = not measured.

The GSI and all circulating sex hormones (testosterone, estradiol, 17,20*β*-P, 17,20*β*,21P) were measured in all individual fish from Skåre except in two non-overripe, non-ovulated fish from Capture A and one non-overripe, ovulated fish from Capture B, as well as in one overripe female from Capture B where the amount of plasma was only sufficient for measuring testosterone and estradiol levels. The *fsh-β*/*lh-β* and *gnrh2*/*gnrh3*/*kiss2*/*gpr54* mRNA levels were measured in the pituitaries and brains of the same individuals as for GSI and sex steroids measurements from Capture B only. *gnrh2*/*gnrh3*/*kiss2*/*gpr54* were measured in the same brains. The same fish and also some additional ones were used for measuring *fsh-β*/*lh-β* in the pituitary, although the *fsh-β* qPCR measurements failed in some.

blood samples in heparinized microhaematocrit tubes (BRAND®, Wertheim, Germany) from the severed caudal peduncle. The tubes were sealed with haematocrit sealing compound (Vitrex Medical, Herlev, Denmark) and centrifuged (Haematokrit 20, Hettich Zentrifugen, Tuttlingen, Germany) at 13,000 rpm for 2 minutes. We collected the plasma in pre-weighed (± 0.01 mg) 0.5-mL Eppendorf tubes, which were weighed again to determine the amount of plasma, and then stored at −80°C.

The pituitary and brain were collected after decapitation. We carefully removed the skullcap and the pituitary was separated from the brain using fine dissecting forceps. Pituitaries and brains were individually immersed in RNA*later*® solution (Ambion, Austin, TX, USA), incubated at 4°C overnight, and stored at −80°C until RNA extraction.

We weighed the ovaries to the nearest 0.01 g. The gonadosomatic index was calculated as: GSI = gonad weight/total fish body weight × 100.

Radioimmunoassay

We measured the plasma levels of four steroid hormones: testosterone, estradiol, 17,20*β*-dihydroxypregn-4-en-3-one (17,20*β*-P) and 17,20*β*,21-trihydroxypregn-4-en-3-one (17,20*β*,21-P) by means of specific radioimmunoassays (RIAs). We also included a few samples from territorial breeding males in the assays.

Heat-treated samples, as described by Schulz *et al.* (1994) and Scott *et al.* (1984), were used for the analysis. Briefly, we diluted the individual plasma samples (6–37 μL) with 300 μL RIA buffer, then vortexed and heat treated them at 80°C for 60 minutes. After heat treatment, we kept the samples at 4°C for at least 30 minutes to overnight. Following centrifugation at 13,000 rpm for 20 minutes at 4°C, the supernatant was stored at 4°C.

We added an aliquot of 100 μL sample (50–100 μL for the 17,20*β*-P and 17,20*β*,21-P RIA assays, adjusted to 100 μL with RIA buffer) of heat-treated diluted plasma to individual incubation tubes together with 50 μL of ^{3}H-labelled testosterone or ^{3}H-labelled estradiol (both from PerkinElmer, USA) and 200 μL testosterone or estradiol antiserum, respectively (gift from Dr. Helge Tveiten, University of Tromsø) (Frantzen *et al.*, 2004), or 100 μL buffer-radiolabel-antibody cocktail of ^{3}H-labelled 17,20*β*-P or ^{3}H-labelled 17,20*β*,21-P (Lower *et al.*, 2004; Scott *et al.*, 2005; Sebire *et al.*, 2007). The tubes were vortexed and incubated overnight at 4°C. We included the respective assay standards for each steroid. The following morning, 300 μL (or 1000 μL for the 17,20*β*-P and 17,20*β*,21-P assays) of cold dextran-coated charcoal suspension was added to all tubes, incubated for 5 minutes (or 30 minutes for the 17,20*β*-P and 17,20*β*,21-P assays) at 4°C and then centrifuged at 4000 rpm for 5 minutes (or 2500 rpm for 12 minutes for the 17,20*β*-P and 17,20*β*,21-P assays) at 4°C. We decanted the supernatant to scintillation tubes together with 4 mL (or 7 mL for the 17,20*β*-P and 17,20*β*,21-P assays) of scintillation fluid (Ultima Gold™, PerkinElmer, Waltham, MA, USA) and conducted counting in a scintillation counter. Since the stickleback is a small fish, the blood volume that can be drawn is also small. To achieve the best measurements possible, we used all the plasma obtained from each individual. This resulted in different dilution factors between samples, which were normalized before statistical analysis. The detection limit of the RIAs (3 pg per assay tube for testosterone and estradiol, and 1.95 pg per assay tube for 17,20*β*-P and 17,20*β*,21-P) was also normalized to reflect that this adjustment depends on the amount of the steroid/assay tube, which in turn is directly related to the amount of plasma used. When calculating means and medians, fish that had non-detectable steroid values had theirs set as 0.30 ng·mL^{-1} for testosterone and estradiol, and 0.37 ng·mL^{-1} for the progestogens, as

these were the lowest detectable levels after plasma dilutions. All samples for which we reported detectable values were within the standard curve for each assay.

mRNA quantification

We measured the mRNA levels of gonadotropins (GTHs: follicle-stimulating hormone, *fsh-β* and luteinizing hormone, *lh-β*) in the pituitary, and of gonadotropin-releasing hormones (GnRHs: *gnrh2*, *gnrh3*) and kisspeptin (*kiss2*) and its G protein-coupled receptor (*gpr54*) in the brain by real-time quantitative PCR (qPCR).

Total RNA was isolated from pituitaries using TRIzol® RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) and from whole brains using the RNeasy® Lipid Tissue Mini Kit (Qiagen cat. 74804, Hilden, Germany) according to the manufacturers' protocols. We treated RNA samples with DNase (2 U) using the TURBO DNA-*free*™ Kit (Ambion, Austin, TX, USA) to remove possible genomic contamination. We determined the total RNA level and 260/280 nm ratio of each sample using NanoDrop™ (Thermo Fisher Scientific, Wilmington, DE, USA). We performed cDNA synthesis of 200 ng total RNA for pituitary and 4000 ng total RNA for brain by reverse transcription (RT) using random primers (or $Oligo(dT)_{20}$ for brain samples) and SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA samples were stored at −20°C until analysis.

We measured relative mRNA levels by qPCR using either the Mx3000P® real-time PCR machine (Stratagene, La Jolla, CA, USA) and the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilient Technologies, Stratagene) (pituitary *fsh-β*/*lh-β*), or the Applied Biosystems StepOnePlus™ System and the Fast SYBR® Green Master Mix (Applied Biosystems) (brain *gnrh2*/*gnrh3* and *kiss2*/*gpr54*), according to the manufacturers' instructions and to methods similar to previous studies [*fsh-β*/*lh-β* (Shao *et al.*, 2013); *gnrh2*/*gnrh3*, *kiss2*/*gpr54* (Shao *et al.*, 2015)].

We used the ribosomal proteins *18s* rRNA and *rpl8*, as well as *β*-actin protein (*actb*) as reference genes depending on target gene analysis. For *fsh-β*, *kiss2*, and *gpr5*, pairs of specific forward and reverse primers were designed using the Primer 3 software. The specific primers for *kiss2* and *gpr54* target genes were based on the published sequences obtained from the nucleotide NCBI Database (KT202354 and KT261496, respectively). All other primers were designed in previous studies [*18s* rRNA/*lh-β* (Shao *et al.*, 2013); *rpl8*/*actb*, *gnrh2*/*gnrh3* (Shao *et al.*, 2015)]. The sequences of each forward and reverse primer for each gene, as well as the primer concentrations, are shown in Table 2. For each reaction, either 1 μL of diluted cDNA template (1:60 for *18s* rRNA, 1:15 for *fsh-β*/*lh-β*) was used for each reaction and run in duplicate in a total reaction volume of 15 μL, or 40 ng (for *rpl8*, *actb*) or 4 ng (for *gnrh2*/*gnrh3* and *kiss2*/*gpr54*) was used and run in triplicate in a final volume of 20 μL. We performed the following thermal protocols: 95°C for 3 minutes, followed by 40 cycles at 95°C for 5 seconds, and 60 or 62°C for 20 seconds (60°C for *18s* rRNA/*fsh-β*, 62°C for *lh-β*); or 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (for *rpl8*/*actb*, *gnrh2*/*gnrh3* and *kiss2*/*gpr54*). The specificity of the products was verified by a melting curve analysis. We included negative controls of nuclease-free water instead of cDNA in each plate to identify any signal background, primer dimer or DNA contamination. The lowest Ct-difference for the negative control and the highest recorded Ct sample value was > 10 Cts. We determined the reaction efficiencies using standard curves prepared from cDNA consisting of a mix of samples obtained from

Table 2. List of forward (Fw) and reverse (Rv) primer sequences and their concentrations used in qPCRs

Gene	Primer name	Nucleotide sequence (5′ → 3′)	Concentration (nM)	Reference
18s rRNA*	Fw	CTCAACACGGGAAACCTCAC	200	Shao *et al.* (2013)
	Rv	AGACAAATCGCTCCACCAAC		
fsh-β	Fw	CATCCACACCACCATCTGCT	100	Present study – Primer 3
	Rv	TGTGTCACCTCGTAGGACCA		
lh-β	Fw	GGTCACTGCCTCACCAAGGA	100	Shao *et al.* (2013)
	Rv	GGAGCGCGATCGTCTTGTA		
*rpl8**	Fw	CGACCCGTACCGCTTCAAGAA	50	Shao *et al.* (2015)
	Rv	GGACATTGCCAATGTTCAGCTGA		
*actb**	Fw	ATGGGCCAGAAGGACAGCTA	50	Shao *et al.* (2015)
	Rv	TCACAATACCGTGCTCAATGG		
gnrh2	Fw	CCGTCGGAGATTTCAGAGGAGATT	50	Shao *et al.* (2015)
	Rv	TCTGAAGCTCTCTGGCTAAGGCAT		
gnrh3	Fw	AGCGATGGTGCAGGTGTTGATGTT	50	Shao *et al.* (2015)
	Rv	TTAAGTCTCTCTTGGGTCTGGGCA		
kiss2	Fw	CAACGACCGCAGGAATCAAT	50	Present study – Primer 3
	Rv	CGCTCTTCTGTAAATGTAGCGTTTC		
gpr54	Fw	GTCCACCTTCTGTCCGAGAGAA	50	Present study – Primer 3
	Rv	CCAGCAAACGGCGAAAAG		

* *18s* rRNA was used as the reference gene for the *fsh-β*/*lh-β* measurements in the pituitary and both *rpl8* and *actb* were used as reference genes for the *gnrh2*/*gnrh3* and *kiss2*/*gpr54* measurements in the brain.

the RT reactions. Standards covered a concentration span of at least three orders of magnitude. R-square (R^2) values were consistently > 0.95 and efficiencies were > 0.90% for all reactions.

We obtained relative expression mRNA levels of *fsh-β*/*lh-β* relative to their standard curves, which covered a concentration span of at least three orders of magnitude, using the MxPro qPCR software v.4.10 (Stratagene, La Jolla, CA, USA) and normalized against the reference gene, *18s* rRNA, as previously used in sticklebacks (e.g. Shao *et al.*, 2013). The relative expression mRNA levels of *gnrh2*/*gnrh3* and *kiss2*/*gpr54* were obtained through the efficiency-corrected method using the StepOne™ Software (Applied Biosystems) and were normalized against the geometric means of the expressions of two reference genes, *rpl8* and *actb* (Roche Applied Science, 2001; Weltzien *et al.*, 2005), as previously used by Shao *et al.* (2015).

Statistical analyses

Statistical significance was set at $P < 0.05$. We tested normality and homogeneity of variance using the Kolmogorov-Smirnov and the Bartlett method, respectively. We did log or square-root transformations if the data were not normally distributed or the variance was not homogeneous. When these criteria were fulfilled, we used Welch's two-sample *t*-test for two group comparisons or a one-way ANOVA followed by *post-hoc* Tukey HSD multiple comparisons for more than two groups. If these criteria were still not met after transformation, the non-parametric Mann-Whitney-Wilcoxon (MWW) test was used. As

there were many non-detectable values for steroid levels, the above tests could not be used. Instead, the proportions of non-detectable and detectable values were compared using Fisher's exact test for count data. We performed all analyses using the R statistical software v.3.2.2 (R Development Core Team, 2015). In the text, the data are presented as means ± standard errors.

RESULTS

Skåre fish caught in autumn (Capture A) and winter (Capture B) had similar variance in gonadosomatic index (GSI) and steroid levels, so were combined in analyses. Pituitary and brain mRNA levels were only measured in the fish from Capture B (see Table 1). For statistical analyses, we combined non-ovulated (with oocytes in different maturing or ripening stages) and ovulated females for comparison with the overripe females, except for GSIs and progestogens from the Askö fish. However, all data for the non-ovulated, non-overripe, the ovulated, non-overripe, and the overripe females, as well as territorial males, are shown for each group in Table 3.

Askö fish caught in the early summer were only used for analysis of GSI and progestogens and were divided into three groups for analysis.

Gonads

Overripe females from Skåre had a significantly higher GSI (mean ± standard error: 22.38 ± 2.32, median = 19.35) than non-overripe females (12.50 ± 1.05, median = 10.16) ($n_{\text{non-overripe}} = 29$, $n_{\text{overripe}} = 18$; Welch's t-test for log transformed data: $t_{35} = 4.3565$, $P < 0.001$) (Fig. 2A). Among non-overripe females, ovulated fish had a GSI of about 24 (Table 3). Most females with overripe hardened eggs had a lower GSI than this (11 of 18 had GSI ≤ 20.3), whereas others had a considerably higher value (up to 44) and some were intermediate.

Overripe females from Askö had a significantly higher GSI (32.68 ± 1.65; median = 33.92) than both non-overripe, non-ovulated (15.99 ± 1.25, median = 14.52) and non-overripe, ovulated females (25.14 ± 1.10, median = 24.81). Moreover, non-overripe, ovulated females had a higher GSI than non-overripe, non-ovulated females ($n_{\text{non-overripe, non-ovulated}} = 14$, $n_{\text{non-overripe, ovulated}} = 14$, $n_{\text{overripe}} = 12$; one-way ANOVA: $F_{2,37} = 38.93$, $P < 0.001$ with *post-hoc* Tukey $P < 0.001$ in all cases) (Fig. 2B). Many overripe females displayed additional ovulations.

Steroids

Testosterone levels were non-detectable (n.d.) in 12 of 18 overripe and 4 of 29 non-overripe females. Testosterone was lower in overripe females (5.22 ± 2.28 ng·mL^{-1}, median = n.d.) than in non-overripe females from Skåre (19.16 ± 3.66 ng·mL^{-1}, median = 10.53 ng·mL^{-1}) ($n_{\text{non-overripe}} = 29$, $n_{\text{overripe}} = 18$) (Fig. 3A). The higher proportion of non-detectable levels in overripe fish was significant (Fisher's exact test: $P < 0.001$).

Similar results were found for plasma estradiol levels, which were non-detectable in 12 of 18 overripe and one of 29 non-overripe females. Overripe females from Skåre had lower estradiol (2.33 ± 0.80 ng·mL^{-1}, median = n.d.) than non-overripe females (10.05 ± 1.04 ng·mL^{-1}, median = 8.67 ng·mL^{-1}) ($n_{\text{non-overripe}} = 29$, $n_{\text{overripe}} = 18$) (Fig. 3B). The higher

Table 3. Gonadosomatic index, plasma steroid hormone levels, and pituitary and brain mRNA levels in fish from Skåre, with non-overripe female stickleback divided into non-ovulated and ovulated groups

		Fish from Skåre			
		Non-overripe females			
Tissue		Non-ovulated	Ovulated	Overripe females	Territorial males
Gonads	GSI	11.19 ± 0.84; 10.01 (n = 26)	23.85 ± 0.15; 23.76 (n = 3)	22.38 ± 2.32; 19.35 (n = 18)	—
Plasma (ng·mL^{-1})	Testosterone	21.11 ± 3.91; 11.07 (n = 26; 3 n.d.)	2.29 ± 1.09; 2.50 (n = 3; 1 n.d.)	5.22 ± 2.28; n.d. (n = 18; 12 n.d.)	—
	Estradiol	10.14 ± 1.53; 8.31 (n = 26; 1 n.d.)	9.21 ± 1.41; 9.32 (n = 3)	2.33 ± 0.80; n.d. (n = 18; 12 n.d.)	—
	17,20β-P	1.15 ± 0.16; 0.89 (n = 24; 6 n.d)	1.46 and 4.24 (n = 2)	0.86 ± 0.26; n.d. (n = 17; 10 n.d.)	1.54 ± 0.45; 0.82 (n = 10; 4 n.d.)
	17,20β,21-P	(n = 24; all n.d.)	(n = 2; all n.d.)	(n = 17; all n.d.)	2.00 (n = 5; 4 n.d.)
Pituitary (fold change)	*fsh-β*	1.13 ± 0.55; 0.60 (n = 7)	6.10 (n = 1)	0.84 ± 0.23; 0.74 (n = 6)	—
	lh-β	0.58 ± 0.19; 0.43 (n = 9)	0.62 ± 0.10; 0.56 (n = 3)	0.19 ± 0.10; 0.003 (n = 9)	—
Brain (fold change)	*gnrh2*	0.99 ± 0.25; 0.72 (n = 5)	0.98 ± 0.29; 1.11 (n = 3)	0.76 ± 0.14; 0.82 (n = 5)	—
	gnrh3	0.99 ± 0.018; 0.99 (n = 5)	1.17 ± 0.22; 1.03 (n = 3)	0.70 ± 0.11; 0.68 (n = 5)	—
	kiss2	1.20 ± 0.18; 1.13 (n = 5)	1.48 ± 0.29; 1.69 (n = 3)	0.82 ± 0.09; 0.76 (n = 5)	—
	gpr54	1.05 ± 0.031; 1.09 (n = 5)	0.89 ± 0.18; 0.90 (n = 3)	0.68 ± 0.08; 0.70 (n = 5)	—

Note: Values are means ± standard error; median. GSI = gonadosomatic index, 17,20β-P = 17,20β-dihydroxypregn-4-en-3-one, 17,20β,21-P = 17,20β,21-trihydroxypregn-4-en-3-one, *fsh-β* = follicle-stimulating hormone β chain mRNA, *lh-β* = luteinizing hormone β chain mRNA, *gnrh2* and *gnrh3* = gonadotropin-releasing hormone 2 and 3 mRNA, *kiss2* = kisspeptin 2 mRNA, *gpr54* = G protein-coupled receptor mRNA. n.d. = non-detectable; where limit of detection: 3 pg per assay tube for testosterone/estradiol and 1.95 pg per assay tube for 17,20β-P/17,20β,21-P. — = not measured. For statistical analyses, the groups non-ovulated and ovulated were combined to compare non-overripe with overripe females.

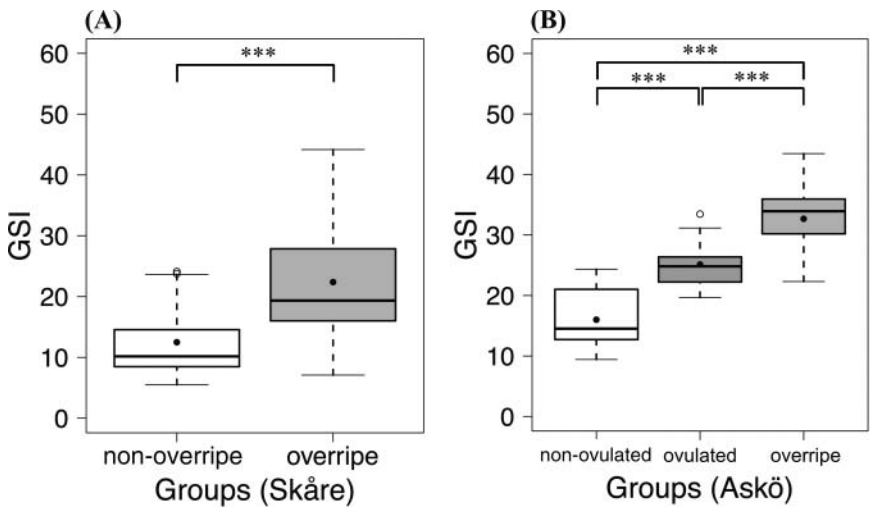

Fig. 2. Gonadosomatic index (GSI = gonad weight/total fish body weight × 100) in (A) non-overripe (non-ovulated, with oocytes in different maturing or ripening stages, combined with ovulated) ($n = 29$) and overripe ($n = 18$) female sticklebacks from Skåre and (B) non-overripe, non-ovulated (non-ovulated) ($n = 14$), non-overripe, ovulated (ovulated) ($n = 14$), and overripe ($n = 12$) female sticklebacks from Askö. The median (lines), the 25th to 75th percentiles (boxes), and the 10th to 90th percentiles (ranges/whiskers) are shown. Open circles show values more than 1.5 times outside the interquartile range of the box and the means are indicated by the solid circles. ***$P < 0.001$.

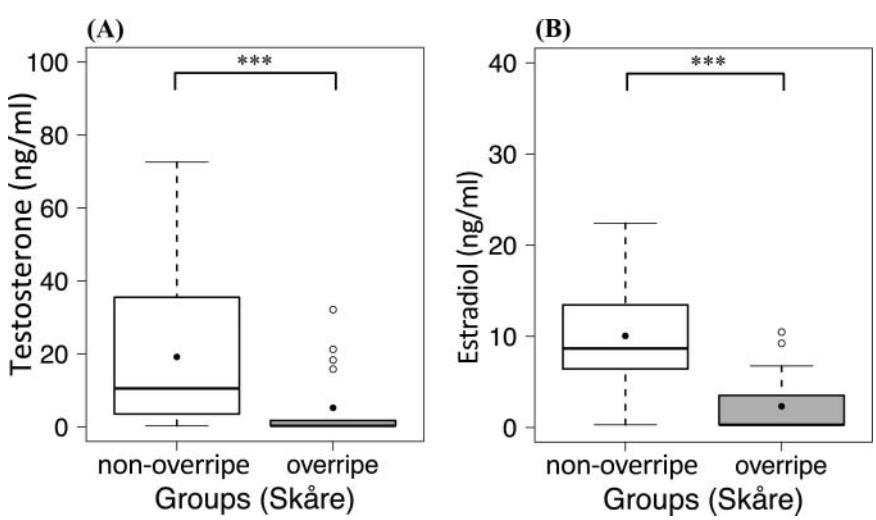

Fig. 3. Plasma levels of (A) testosterone and (B) estradiol in non-overripe (non-ovulated, with oocytes in different maturing or ripening stages, combined with ovulated) ($n = 29$) and overripe ($n = 18$) female sticklebacks from Skåre. The median (lines), the 25th to 75th percentiles (boxes), and the 10th to 90th percentiles (ranges/whiskers) are shown. Open circles show values more than 1.5 times outside the interquartile range of the box and the means are indicated by the solid circles. ***$P < 0.001$.

proportion of non-detectable levels in overripe fish was significant (Fisher's exact test: $P < 0.001$).

In the RIA analysis, circulating plasma 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) levels were non-detectable in 10 of 17 overripe and 6 of 26 non-overripe females. Overripe females from Skåre had lower circulating plasma 17,20β-P (0.86 ± 0.26 ng·mL^{-1}, median = n.d.) than non-overripe females (1.28 ± 0.19 ng·mL^{-1}, median = 1.07 ng·mL^{-1}) ($n_{\text{non-overripe}} = 26$, $n_{\text{overripe}} = 17$) (Fig. 4A). The higher proportion of non-detectable levels in overripe fish was significant (Fisher's exact test: $P < 0.0258$). Plasma levels of 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P) were under the limit of detection in all females.

Among overripe fish from Skåre, the GSI was lower in females whose testosterone levels were non-detectable (18.45 ± 2.07, median = 16.58) than those with a detectable value (30.24 ± 4.21, median = 30.22) ($n_{\text{detectable}} = 6$, $n_{\text{non-detectable}} = 12$; Mann-Whitney-Wilcoxon test: $W = 10$, $P = 0.0169$). Similar results were observed for estradiol (detectable: 28.40 ± 3.99, median = 25.37; non-detectable: 19.38 ± 2.54, median = 16.58) ($n_{\text{detectable}} = 6$, $n_{\text{non-detectable}} = 12$; MWW test: $W = 14$, $P = 0.0441$), but not for 17,20β-P (detectable: 23.26 ± 3.55, median = 19.45; non-detectable: 21.22 ± 3.46, median = 17.09) ($n_{\text{detectable}} = 7$, $n_{\text{non-detectable}} = 10$; MWW test: $W = 27$, $P = 0.475$).

In the RIA analysis for females from Askö, 17,20β-P levels were under the detection limit in 9 of 12 overripe, 10 of 14 non-overripe, ovulated, and 2 of 14 non-overripe, non-ovulated females. Overripe females had lower 17,20β-P (0.44 ± 0.04 ng·mL^{-1}, median = n.d.) than non-overripe, non-ovulated females (1.67 ± 0.57 ng·mL^{-1}, median = 0.66 ng·mL^{-1}), but there was no difference between non-overripe, ovulated (mean = 0.49 ± 0.07 ng·mL^{-1},

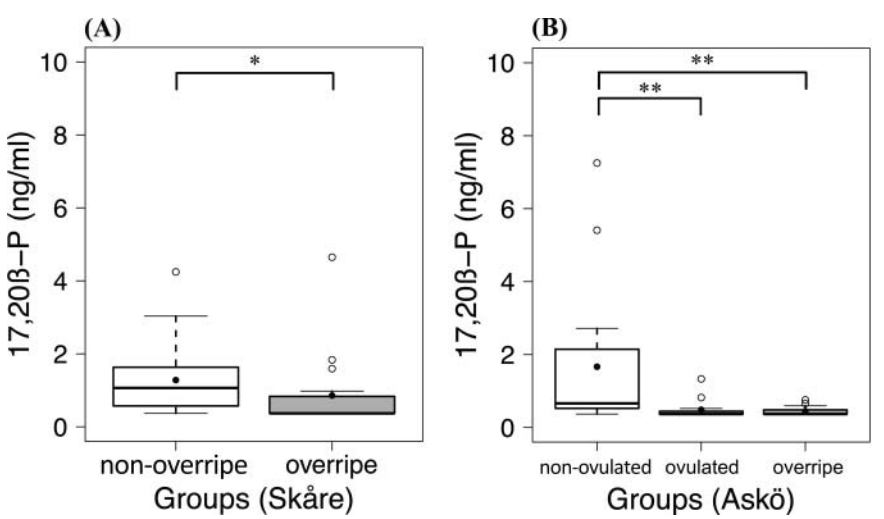

Fig. 4. Plasma levels of 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) in (A) non-overripe (non-ovulated, with oocytes in different maturing or ripening stages, combined with ovulated) ($n = 26$) and overripe ($n = 17$) female sticklebacks from Skåre and (B) non-overripe, non-ovulated (non-ovulated) ($n = 14$), non-overripe, ovulated (ovulated) ($n = 14$), and overripe ($n = 12$) female sticklebacks from Askö. The median (lines), the 25th to 75th percentiles (boxes), and the 10th to 90th percentiles (ranges/whiskers) are shown. Open circles show values more than 1.5 times outside the interquartile range of the box and the means are indicated by the solid circles. *$P < 0.05$, **$P < 0.01$

median = n.d) and overripe females. Furthermore, non-overripe, ovulated females had lower 17,20β-P than non-overripe, non-ovulated females ($n_{\text{non-overripe, non-ovulated}} = 14$, $n_{\text{non-overripe, ovulated}} = 14$, $n_{\text{overripe}} = 12$) (Fig. 4B). The highest individual values (7.25, 5.41, and 2.72 ng·mL^{-1}) were found in 3 of 7 non-overripe, non-ovulated females that were judged to be close to ovulation. The higher proportion of non-detectable levels in overripe fish than in non-overripe, non-ovulated fish was significant (Fisher's exact test: $P = 0.0043$), as was the difference between non-overripe, non-ovulated and non-overripe, ovulated fish (Fisher's exact test: $P = 0.0063$); however, this was not the case between the non-overripe, ovulated and overripe females (Fisher's exact test: $P = 1$). The 17,20β,21-P plasma levels were under the limit of detection (1.95 pg per assay tube) in all females.

fsh-β and *lh-β*

The relative mRNA levels of *fsh-β* in the pituitary were not significantly different between overripe (0.84 ± 0.23 fold change, median = 0.74 fold change) and non-overripe females from Skåre (1.75 ± 0.79, median = 0.76) ($n_{\text{non-overripe}} = 8$, $n_{\text{overripe}} = 6$; MWW test: $W = 24$, $P = 1$) (Fig. 5A). By contrast, the relative mRNA levels of *lh-β* in the pituitary were significantly lower in overripe (0.19 ± 0.10, median = 0.003) than non-overripe females (0.61 ± 0.14, median = 0.55) ($n_{\text{non-overripe}} = 12$, $n_{\text{overripe}} = 9$; MWW test: $W = 19$, $P = 0.012$) (Fig. 5B).

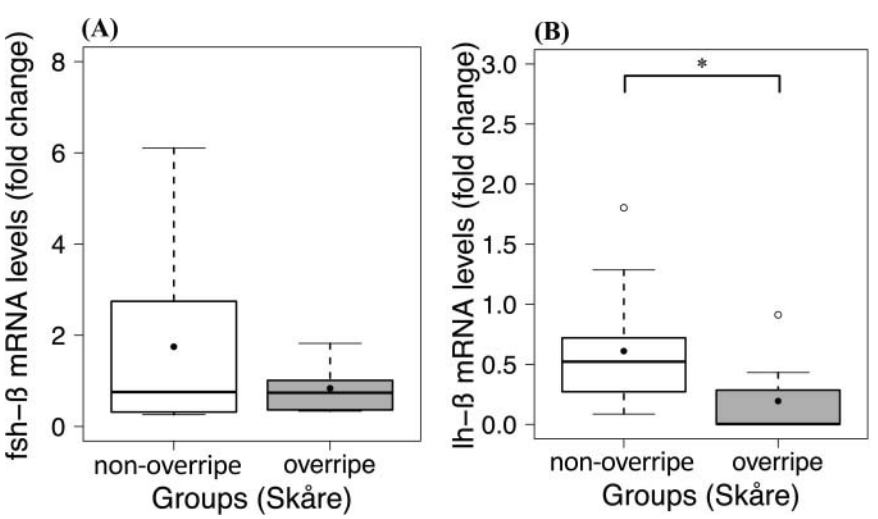

Fig. 5. Relative mRNA levels of (A) follicle-stimulating hormone (*fsh-β*) and (B) luteinizing hormone (*lh-β*) in the pituitary of non-overripe (non-ovulated, with oocytes in different maturing or ripening stages, combined with ovulated) ($n = 10$–12) and overripe ($n = 8$–9) female sticklebacks from Skåre. Relative mRNA levels are normalized against the *18s* rRNA reference gene. The median (lines), the 25th to 75th percentiles (boxes), and the 10th to 90th percentiles (ranges/whiskers) are shown. Open circles show values more than 1.5 times outside the interquartile range of the box and the means are indicated by the solid circles. *$P < 0.05$.

gnrh2 and *gnrh3*

The relative mRNA levels of *gnrh2* in the brain were not significantly different between overripe (0.76 ± 0.14 fold change, median = 0.82 fold change) and non-overripe females from Skåre (0.99 ± 0.18, median = 0.91) ($n_{\text{non-overripe}} = 8$, $n_{\text{overripe}} = 5$; Welch's t-test: $t_{11} = -1.0148$, $P = 0.332$) (Fig. 6A). The relative mRNA levels of *gnrh3* in the brain were not

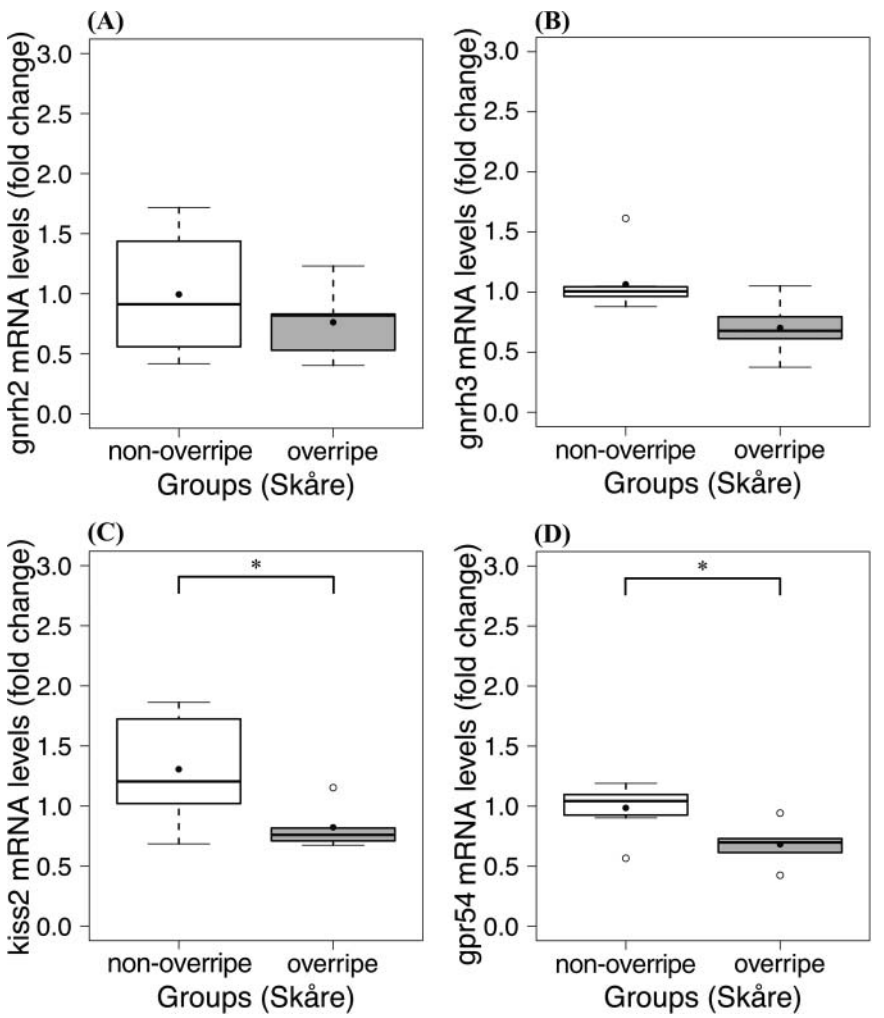

Fig. 6. Relative mRNA levels of (A, B) gonadotropin-stimulating hormone-2 (*gnrh2*) and -3 (*gnrh3*), (C) kisspeptin 2 (*kiss2*) and (D) its G protein-coupled receptor (*gpr54*) in the brain of non-overripe (non-ovulated, with oocytes in different maturing or ripening stages, combined with ovulated) ($n = 8$) and overripe ($n = 5$) female sticklebacks from Skåre. Relative mRNA levels are normalized against the geometric means of the expressions of *rpl8* and *actb* as reference genes. The median (lines), the 25th to 75th percentiles (boxes), and the 10th to 90th percentiles (ranges/whiskers) are shown. Open circles show values more than 1.5 times outside the interquartile range of the box and the means are indicated by the solid circles. $^*P < 0.05$.

significantly different between overripe (0.70 ± 0.11, median = 0.68) and non-overripe females (mean = 1.06 ± 0.08, median = 1.01), but there was a tendency towards lower levels in overripe than non-overripe females ($n_{\text{non-overripe}} = 8$, $n_{\text{overripe}} = 5$; MWW test: $W = 7$, $P = 0.065$) (Fig. 6B).

kiss2 and its receptor *gpr54*

The relative mRNA levels of *kiss2* in the brain were significantly lower in overripe (0.82 ± 0.09 fold change, median = 0.76 fold change) than non-overripe females from Skåre (1.31 ± 0.15, median = 1.204) ($n_{\text{non-overripe}} = 8$, $n_{\text{overripe}} = 5$; Welch's t-test: $t_{10.373} = -2.7888$, $P = 0.019$) (Fig. 6C). The relative mRNA levels of its receptor *gpr54* in the brain were also significantly lower in overripe (0.68 ± 0.08, median = 0.70) than non-overripe females (0.99 ± 0.07, median = 1.04) ($n_{\text{non-overripe}} = 8$, $n_{\text{overripe}} = 5$; Welch's t-test: $t_9 = -2.8091$, $P = 0.021$) (Fig. 6D).

DISCUSSION

In the threespine stickleback, gonad weight (expressed as the gonadosomatic index, GSI) was higher in overripe than non-overripe females, and a significant decline was observed in the plasma levels of all measurable steroid hormones, namely testosterone, estradiol, and 17,20*β*-dihydroxypregn-4-en-3-one (17,20*β*-P). The mRNA levels of luteinizing hormone (*lh-β*) in the pituitary were lower in overripe than non-overripe females, as were the mRNA levels of kisspeptin (*kiss2*) and its G protein-coupled receptor (*gpr54*) in the brain. However, there were no significant differences in mRNA levels in the pituitary for the other gonadotropin, follicle-stimulated hormone (*fsh-β*), or in the brain for the gonadotropin-releasing hormones (GnRHs) *gnrh2* and *gnrh3*, although *gnrh3* showed a tendency towards a decline in the overripe group. Hence, we observed a reduction in a number of components of the brain–pituitary–gonad (BPG) axis in overripe females with hardened eggs.

Most overripe females from Skåre had a lower GSI than non-overripe ovulated females (Table 3), indicating that they had not ovulated after overripening had occurred. This finding is similar to the overripe stickleback females studied by Lam *et al.* (1978), which appeared to have ovulated only once and had low GSIs. However, some of the overripe females from Skåre, and most overripe females from Askö, had ovulated again, as judged by high GSIs and the presence of additional ovulations.

Testosterone levels are high in females of many teleost fishes, including the stickleback (Borg, 1994). While testosterone can be converted to the estrogen estradiol, the biological role of unconverted testosterone, acting as an androgen, is still not well established in fish. In the present study, testosterone levels were lower in overripe than non-overripe sticklebacks (Fig. 3A), and within the former, the levels were lower in females with a low GSI (had not ovulated again after overripening). However, testosterone levels in the overripe sticklebacks were not lower than in the few non-overripe ovulated females (Table 3). Changes in hormone levels over the ovulatory and post-ovulatory period have been studied most extensively in salmonids. Both Scott *et al.* (1983) and Chyb *et al.* (1999) observed a decline in plasma testosterone in rainbow trout females that retained their eggs for long periods after ovulation. Salmonids, however, are not representative of fishes in general in this respect, since they can keep viable eggs for a long time (weeks) and the ovulated eggs are not stored

in the ovarian lumen, but in the abdominal cavity. Thus, the biological importance of the decline in testosterone in ovulated and post-ovulated stickleback and rainbow trout is difficult to interpret, as the biological role of testosterone (apart from being a precursor for estradiol) is poorly understood in fishes.

The most clearly understood action of estradiol in fish is to stimulate vitellogenesis in the liver (for a review, see Nagahama and Yamashita, 2008). As with testosterone, estradiol levels were lower in overripe than in non-overripe sticklebacks (Fig. 3B). Estradiol was also lower in overripe than non-overripe ovulated females, although they were too few in number for statistical analysis (Table 3). A decline in estradiol at ovulation and in the period following ovulation is also seen in rainbow trout (Scott *et al.,* 1983; Chyb *et al.,* 1999). A decline in estradiol would be expected to result in a decrease in vitellogenesis.

The final steps of egg maturation in female fish are stimulated by a peak in luteinizing hormone (LH) leading to synthesis of a maturation-inducing hormone (MIH), responsible for oocyte maturation and egg ovulation (Nagahama, 1997; for a review, see Nagahama and Yamashita, 2008). In many teleost fishes, such as salmonids, 17,20*β*-P is a potent MIH, whereas in others such as striped bass, *Morone saxatilis* (Mylonas *et al.*, 1997), 17,20*β*,21-P may be the MIH (for reviews, see Nagahama and Yamashita, 2008; Scott *et al.*, 2010). In all female plasma samples in the present study, 17,20*β*,21-P was undetectable, suggesting that 17,20*β*-P is the more likely MIH in the three-spine stickleback, which is also supported by the fact that the highest individual levels were found in females judged close to ovulation. The 17,20*β*-P plasma levels were also relatively low and often non-detectable, although several samples contained quantifiable amounts. The 17,20*β*-P levels were lower in overripe than non-overripe females from Skåre (Fig. 4A). In the fish from Askö, overripe females had lower 17,20*β*-P levels than non-overripe, non-ovulated females, but the non-overripe, ovulated ones had equally low levels (Fig. 4B). In rainbow trout, 17,20*β*-P levels show a gradual decrease following ovulation (Scott *et al.*, 1983; Chyb *et al.*, 1999). Thus, there is an overall decrease of sex steroids hormones after ovulation in both rainbow trout and stickleback.

A reduction of sex steroid hormones after ovulation may be related to the loss of egg viability. Stickleback females with overripe eggs have less ovarian fluid in the ovarian lumen than normal non-overripe, ovulated females, which is associated with degeneration of post-ovulatory follicles (POFs, or corpora lutea) 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 progesterone (P4) or 1 ppm estradiol or solvent alone in the water. The ovaries of the P4-treated fish contained more ovarian fluid than the controls; in overripe fish, P4 also increased the GSI. Lam *et al.* (1979) proposed that steroid secretion of POFs may be crucial to maintain viability of the eggs in the ovarian cavity after ovulation, through stimulation of fluid secretion by the ovarian epithelium.

In sticklebacks, *lh-β* mRNA levels in the pituitary (Fig. 5B) decreased in overripe females, which could be responsible for the low levels of plasma 17,20*β*-P in that group. On the other hand, *fsh-β* mRNA levels did not differ between groups (Fig. 5A).

Sticklebacks have two types of GnRHs, which appear to have roles in the control of seasonal reproduction (Shao *et al.*, 2015). Brain *gnrh2* and *gnrh3* expression was not different between groups in the present study, although the latter showed a tendency to decline in overripe females (Fig. 6A, B). In the stickleback, there is only one kisspeptin form, the kisspeptin 2 (*kiss2*) and its G protein-coupled receptor (*gpr54*) (Pasquier *et al.*, 2012). Both *kiss2* and its receptor (*gpr54*) mRNA levels were much lower in overripe than non-overripe females (Fig. 6C, D). Kisspeptins and their receptors are known to be important factors

triggering reproduction in fishes and in controlling the action of GnRHs (for a review, see Zohar *et al.*, 2010). Thus their decline at overripening could be a factor suppressing the BPG axis.

Many factors have been suggested to be associated with the failure to spawn ovulated eggs by female fish, such as a lack of mates or spawning sites (e.g. male nests), a biased sex ratio, overcrowding, environmental factors (e.g. pollution, oxygen concentration, pH, salinity), and pheromones (for a review, see Rideout *et al.*, 2005). Although small female body size has been associated with egg retention in the stickleback (Lam *et al.*, 1978), we also found large overripe females. Observations on sticklebacks in nature have shown that it is often the females that court the males rather than the other way around, especially in the later parts of the breeding period (Kynard, 1978; Borg, 1985). Also at Askö, we often observed females courting males, which may be related to the fact that overripe females were common. This suggests that the brood-caring males could be the limiting sex and perhaps not all females get an opportunity to spawn. It has also been observed previously that many females caught in the field in the south of Sweden have hard overripe eggs in their ovaries at the end of the breeding season (Borg and van Veen, 1982).

In summary, we found that many components of the BPG axis showed a strong reduction in stickleback females with hardened overripe eggs, though we do not know which is cause and effect. If overripening is indeed due to less opportunity to spawn, the low hormone levels in the overripe fish are likely to be an effect. However, what does this really mean for the fish? It seems reasonable to assume that the condition may reduce further ovulations, though these still occurred. If an egg-bound female can no longer reproduce, it makes little difference from an evolutionary perspective if she dies due to her oversized ovaries, is taken by predators due to impaired swimming ability or if she survives. In this case, the endocrine state of the female would be of little importance. However, at least in southern Sweden, sticklebacks may survive the breeding season, since 'berried' females can be found in winter. Their chances of surviving, reabsorbing their overripe eggs, and spawning again the following season, ought to be better with lower levels of reproductive hormones and a reduced likelihood of further ovulations.

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