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Research paper

Hormonal changes over the spawning cycle in the female three-spined stickleback, *Gasterosteus aculeatus*

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ABSTRACT

Female three-spined sticklebacks are batch spawners laying eggs in a nest built by the male. We sampled female sticklebacks at different time points, when they were ready to spawn and 6, 24, 48 and 72 h post-spawning (hps) with a male. Following spawning, almost all females (15 out of 19) had ovulated eggs again at Day 3 post-spawning (72 hps). At sampling, plasma, brain and pituitaries were collected, and the ovary and liver were weighed. Testosterone (T) and estradiol (E2) were measured by radioimmunoassay. Moreover, the mRNA levels of follicle-stimulating hormone (*fsh-β*) and luteinizing hormone (*lh-β*) in the pituitary, and of the gonadotropin-releasing hormones (GnRHs: *gnrh2*, *gnrh3*) and kisspeptin (*kiss2*) and its G protein-coupled receptor (*gpr54*) in the brain were measured by real-time qPCR.

Ovarian weights peaked in "ready to spawn" females, dropped after spawning, before again progressively increasing from 6 to 72 hps. Plasma T levels showed peaks at 24 and 48 hps and decreased at 72 hps, while E2 levels increased already at 6 hps and remained at high levels up to 48 hps. There was a strong positive correlation between T and E2 levels over the spawning cycle. Pituitary *lh-β* mRNA levels showed a peak at 48 hps, while *fsh-β* did not change. The neuropeptides and *gpr54* did not show any changes. The changes in T and E2 over the stickleback spawning cycle were largely consistent with those found in other multiple-spawning fishes whereas the marked correlation between T and E2 does not support T having other major roles over the cycle than being a precursor for E2.

1. Introduction

Reproduction in teleost fishes, as in other vertebrates, is regulated by the brain-pituitary-gonad (BPG) axis. The gonads are controlled by two gonadotropins secreted from the pituitary; follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kawauchi et al., 1989). The secretion of gonadotropins is stimulated by gonadotropin-releasing hormones (GnRHs) from the hypothalamic region in the brain (Zohar et al., 2010), but also controlled by other hypothalamic substances such as dopamine and kisspeptin.

In teleosts, oogenesis is mainly under the control of gonadotropins, where FSH regulates the earlier stages and vitellogenesis, while LH is more prominent in the later stages leading to oocyte maturation and ovulation (Lubzens et al., 2010). While gonadotropins regulate the timing of the events during oogenesis, their actions are exerted via sex steroid hormones: androgens, estrogens and progestogens (Pankhurst, 2008). The androgen testosterone (T) can be converted to the estrogen estradiol (E2) and although T is present at high levels in females of many teleosts, including the breeding female three-spined sticklebacks, *Gasterosteus aculeatus* (Borg, 1994; Borg and Mayer, 1995), the biological role of unconverted T acting as an androgen, is still not well known in fish (Borg, 1994). In male teleosts, 11-ketotestosterone (11KT) is usually the dominating androgen. Levels of 11KT in the plasma of three-spined stickleback females in the breeding season have been studied by Borg and Mayer (1995) and were found to be very low (c. 1 ng/mL) as in most studied teleost females. E2 is responsible for stimulating vitellogenesis, while the progestogens, such as 17,20*β*-dihydroxypregn-4-en-3-one (17,20*β*-P), are mainly responsible for inducing final oocyte maturation and ovulation (Nagahama and Yamashita, 2008).

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Many studies have been performed on the endocrine control of reproduction in female teleosts (reviewed by Patiño and Sullivan, 2002; Nagahama and Yamashita, 2008). In teleosts, three patterns of ovarian oocyte development have been observed: synchronous, group-synchronous and asynchronous (Wallace and Selman, 1981). Salmonids are usually annual spawners and the oocytes develop in the ovaries synchronously since they only develop one batch of eggs per season (synchronous spawners). Ovulation takes place only once during the spawning period. In multiple spawners, such as goldfish, *Carassius auratus*, and three-spined stickleback, a number of distinct cohorts of oocytes are present the same time in the ovary, each at different developmental stage (group-synchronous spawners). In this case, ovulation can occur several times over a spawning period. Female sticklebacks can produce and spawn clutches of eggs at intervals of a few days (Baggerman, 1957; Wootton, 1973, 1974, 1977). Baggerman (1957) observed a mean of 10.8 and a maximum of 15 spawnings over the four months breeding period in three-spined sticklebacks in the Netherlands, though this is longer than in our area, Southern Sweden (c. two months). Sticklebacks ovulate and spawn at any time of the day, whereas in many other fishes, this is confined to certain times of the day. In cyprinids, ovulation occurs from midnight to early morning (kanehira bitterling, *Acheilognathus rhombea*, Shimizu et al., 1985; goldfish, Kobayashi et al., 1987, 1988; common carp, *Cyprinus carpio*, Santos et al., 1986). In other multiple spawners, such as killifish mummichog, *Fundulus heteroclitus*, and zebrafish, *Danio rerio*, the oocytes develop asynchronously and are present at all developmental stages in the ovaries (asynchronous spawners). Ovulation and spawning can happen at short intervals, even daily, over a spawning period (Wallace and Selman, 1981). The endocrine control of oocyte development may be more complicated in group-synchronous and asynchronous spawners compared to synchronous spawners. In the latter, vitellogenesis and final maturation take place at different times, whereas in the former several phases of oogenesis can occur simultaneously (reviewed by Wootton and Smith, 2015).

Hormonal changes during the final gonadal maturation and *peri*-ovulatory period have been studied in fishes such as in rainbow trout, *Oncorhynchus mykiss* (Gomez et al., 1999; Pavlidis et al., 1994; Scott et al., 1983), striped bass, *Morone saxatilis* (Mylonas et al., 1997), bambooleaf wrasse, *Pseudolabrus sieboldi* (Ohta et al., 2008), goldfish (Canosa et al., 2008; Kagawa et al., 1983; Kobayashi et al., 1987, Stacey et al., 1979), common carp (Santos et al., 1986). The hormonal changes over the spawning or ovulatory cycle in multiple spawners have been studied in only a few species, such as in kanehira bitterling (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).

The aim of the present study was therefore to describe the temporal changes in key reproductive hormones over the spawning period in the female stickleback. We predict that these changes will exhibit patterns similar to other multiple spawning fishes. In order to test that hypothesis, plasma levels of T and E2, as well as the mRNA levels of *fsh-β* and *lh-β* in the pituitary and of *gnrh2, gnrh3,* kisspeptin (*kiss2)* and its receptor *gpr54* in the brain were measured over the spawning cycle. Furthermore, the correlation between the levels of T and E2 were studied in order to test the hypothesis that T has important roles in the spawning cycle apart from being a precursor for E2.

2. Materials and methods

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

2.1. Experimental animals

Adult non-breeding three-spined sticklebacks were caught by drop nets in the southern Baltic at Skåre, southern Sweden (55°22′35″N 13°3′8″E) in December 2014. The fish were transported to the Stockholm University and kept in 700- or 1200-litre aquaria containing constantly filtered and aerated brackish water (0.5% salinity). Fine gravel covered the bottoms and clay pots provided hiding places. Algae were also present. Fish were fed daily with frozen red midge larvae or mysids. Temperature was gradually increased to 20 °C and the photoperiod set to long day (LD 16 h light: 8 h dark) to stimulate breeding. After about three weeks, sexually mature males showed breeding colouration and aggressive behaviour while females started to display swollen abdomens.

Breeding males were individually placed in 50-litre aquaria with constantly filtered water, where fine gravel and algae were provided for nest building. Females were then allowed to mate with the males. The maximum time for pair breeding was set at 30 min; if the female had not spawned by the end of this period, she was removed. If spawning took place, the time was noted. Then, each female was transferred to an individual 50-litre aquarium under similar conditions as for the males until sampling (see below). Moreover, for one of the groups we also used females that displayed courtship behaviour (e.g. head-up posture), but were not allowed to spawn. These females were sampled immediately after the behavioural test.

Mature females were sampled at different time points. Spawning could take place any time of the day since unlike many other fishes, sticklebacks do not a have a fixed time of the day for ovulation and spawning. The following five groups of females were used: A) females that were ovulated and showed a positive courtship response to a nesting male: "ready to spawn". After courtship the females were quickly removed from the aquarium before entering the nest (spawning) and sampled. The other four groups of females were all allowed to spawn and were sampled as follows: B) 6 ± 1 h 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 (15 out of 19) fish had ovulated again. The sampling took place a certain number of hours after spawning rather than at a specific time of day and the samplings were, like the spawnings, spread out in time (9.40–20.45).

2.2. Sampling procedures

Each female was netted out of the aquarium and, in order to avoid stress effects on the hormone levels, they were placed straight in 0.025% buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, MO, USA). After anesthesia and weighing, blood samples were collected in heparinized microhematocrit tubes (BRAND®, Wertheim, Germany) from the severed caudal peduncle. The tubes were sealed with hematocrit sealing compound (Vitrex Medical, Herlev, Denmark) and centrifuged (Haematokrit 20, Hettich Zentrifugen, Tuttlingen, Germany) at 13,000 rpm for 2 min. The plasma was transferred to pre-weighed (±0.01 mg) 0.5 ml Eppendorf tubes, weighed again in order to determine the amount of plasma, and then stored at −80 °C. Pituitaries and brains were excised following decapitation and fixed separately in RNA*later*® solution (Ambion, Austin, TX, USA), followed by overnight storage at 4 °C and then at −80 °C until RNA extraction.

Ovaries and livers were excised and weighed to the nearest mg. Gonadosomatic index (GSI) was calculated as: GSI = (gonad weight/total body weight) × 100. Hepatosomatic index (HSI) was calculated as: HSI = [liver weight/(total body weight-gonad weight)] × 100, to compensate for high GSI values at some sample

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