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Teleost maturation-inducing hormone, 17,20β-dihydroxypregn-4-en-3-one, peaks after spawning in *Tinca tinca*

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ABSTRACT

During an eight month study of the reproductive cycle in two age groups, and in both sexes, of tench (*Tinca tinca* L.), it was found that plasma concentrations of the presumptive 'maturation inducing hormone (MIH)' 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) did not reach a peak during the spawning season, but as much as two months after spawning had ceased. The cessation of the spawning season was confirmed by histological examination of the gonads and by measurement of 11-ketotestosterone and 17β-estradiol in the plasma of males and females, respectively. Measurements were also made of the 'alternative MIH' 17,20β,21-trihydroxypregn-4-en-3-one in the older fish. However, this steroid did not show the same pattern as 17,20β-P. An assessment was made of the prevalence of primary spermatocytes in the testes of post-spawned fish – to test an alternative hypothesis that 17,20β-P might be involved in the stimulation of meiosis. However, there was no evidence for any increase in testis differentiation post-spawning. In fact the testes became increasingly undifferentiated as the autumn progressed. The role, if any, of this 'unseasonal' peak of 17,20β-P production remains to be determined.

1. Introduction

In a previous study of sex steroid changes during the reproductive season of tench, *Tinca tinca* L., it was noted that 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) levels did not peak during the spawning season, but at least one month afterwards, when ovaries and testes appeared to be devoid of advanced-stage gametes [30]. This was a very unexpected finding, and one that runs contrary to the dogma that 17,20β-P is the major 'maturation-inducing hormone' (MIH) – with responsibility for initiating germinal vesicle breakdown (just prior to ovulation) in females [27] and for inducing 'spermiation' in males [41]. In all other studies on fish that have been published to date, peak 17,20β-P production has been shown to occur at and about the time of ovulation in females [29] and at the time of peak sperm production (spermiation) in males [41].

A plausible hypothesis for an alternative role for 17,20β-P (that might explain the post-spawning peak in tench) comes from studies on Japanese eel, *Anguilla japonica*. When 17,20β-P was added to eel testis cells *in vitro*, it caused a significant increase in the mRNA of two protein markers that are associated with early meiotic division [26]. This finding was confirmed in Japanese huchen, *Hucho perryi* Brevoort, 1856 [15] and ovaries of the common carp, *Cyprinus carpio* L. [25]. The results led the authors to hypothesize that 17,20β-P is responsible for initiating meiosis in both ovary and testis.

In order to, firstly, confirm the earlier observation that peak 17,20β-P production occurs in the post-spawning period in *T. tinca*, and, secondly, to establish whether this peak possibly coincides with the timing of the first meiotic division, males and females of two age groups of *T. tinca* were sampled regularly over an eight month period. Measurements were made of not just 17,20β-P, but of several other sex steroids including 11-ketotestosterone (11-KT), testosterone (T), 17β-estradiol (E_2) and the 'alternative MIH', 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P). As fresh gonadal tissue was not collected in the present study, it was not possible to test for the presence of meiotic markers proteins. Instead, histological sections of testes were examined for the prevalence of primary spermatocytes, which are the accepted stage at which chromosome replication occurs according to Schulz et al. [36]. These are relatively easy to identify (see study by Geraudie et al. [13] on another cyprinid, the roach, *Rutilus rutilus* L.) because they form in discrete clusters and have nuclei that are distinctly darker and more evenly stained than the nuclei of

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the preceding stage (spermatogonia B) and do not yet show signs of nuclear condensation of the succeeding 'secondary' spermatocyte stages. Ovaries were also examined in the present study, for the appearance of primary oocytes. However, these were present at all times and were difficult to quantify.

2. Materials and methods

2.1. Animals

Tench, *T. tinca*, of two age categories (three-year-old virgin fish and seven-year-old adult fish) were reared separately at the University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology in Vodnany (49°09′ N, 14°10′ E), Czech Republic. During the experimental period, the virgin fish were kept in 200 m^2 and the adult fish in 1200 m^2 outdoor earthen ponds with an average water depth of 90 cm. Stocking density of fish was approximately 3 individuals m^{-3} for virgins and 0.5 individuals m^{-3} for adults. The ponds were supplied with through-flowing river water. Tench were cultured in earthen ponds under natural photoperiod, ambient temperature and semi-intensive management as followed: fish fed on natural food (plankton, benthos), and during growing season (from April to September) wheat, barley grain and/or carp pellets (KP 1, GRANA a.s., Czech Republic) were distributed to ponds three times a week as additional feed at an average daily feeding ratio of 2% of total fish mass. The mean (+ S.E.) total length (mm) and weight (g), respectively of adults, were: 29.1 + 0.2 cm and 319.6 + 9.8 g in April and 31.5 + 0.5 cm and 496 + 26.0 g in November. The equivalent measurements for virgin fish were 19.2 + 0.4 cm and 87.9 + 5.9 g in April and 20.3 + 0.6 cm and 125.9 + 10.2 g in November. Water temperature in both ponds (Fig. 1) was recorded by data logger RT-F52 (Qi Analytical, Czech Republic) at four-hour intervals.

Fish were sampled monthly from April 2005 prior to spawning season till November 2005 after the termination of spawning season, by netting at full water. For each sampling, eight females and eight males from the catch were taken randomly. Despite the fact that tench presents external sexual dimorphism (females' pelvic fins do not reach the anus unlike males; and males pelvic fins are also slightly curled with noticeable thicker fin rays) there was the occasional misattribution in the virgin fish (leading to sample sizes of nine males and seven females). Fish were brought to the lab and 1 ml blood samples were taken from the caudal vein using heparinized syringes according to Pravda and Svobodová [33], then transferred to 1.5 ml tubes and placed on ice. Blood was centrifuged, the plasma drawn off, frozen and stored at −70 °C until steroid determination. Before dissection, the fish were killed and the total length (L, mm) and the body weight (W, g) of the fish were recorded. Following measurement, the gonads were excised and weighed (nearest 0.01 g) and the gonadosomatic index (I_g) was calculated as: $I_g = 100 \times$ (gonad weight/total body weight). The mid-section of the gonads was fixed in Bouin's solution for 24 h, then dehydrated in a graded series of ethanol, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E) fluid for future histological assessment.

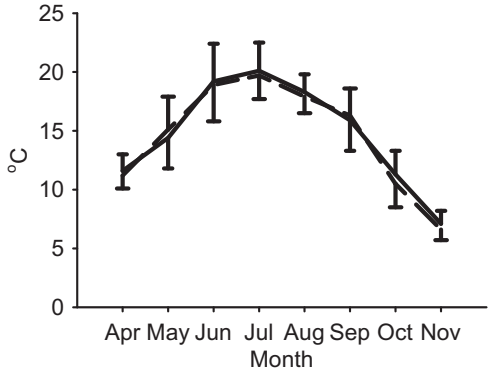

Fig. 1. Mean monthly water temperatures (± Standard Deviation) in ponds holding adult (continuous line) or virgin (discontinuous line) tench, *T. tinca*.

All procedures on fish were performed in compliance with relevant laws (mainly Act No. 246/1992 of the Czech Republic against animal cruelty in the valid statutory text) and inferential institutional guidelines with approval of the institutional committee and official state body.

2.2. Histological assessment

All ovary sections were examined with a light microscope under relatively low power (10× objective) and testis sections at high power (40× objective).

Testes were examined specifically for the presence of cysts containing primary spermatocytes, cysts containing secondary spermatocytes (inclusive of spermatids) and spermatozoa. If a stage was relatively dominant (i.e. it was spread over most of the field of view), the section was given a score of 3. If the stage was sparser (less than five cysts in the field of view), the section was given a score of 2. If it was very sparse (only a few in the whole testis section), it was given a score of 1. If it was entirely absent from the section, it was given a score of 0. In order to calculate an average 'maturity index' for each month, the scores for each fish were adjusted to reflect the relative abundance of the stages (3 → 3; 2 → 0.75; 1 → 0.1; 0 → 0). The weightings were chosen by comparing (and subsequently deriving a straight line relationship between) the average scores for all three developmental stages (from both adults and virgins) and plasma concentrations of 11-KT (see Section 3). Although no photographs of spermatocyte stages are included in the present paper, readers are referred to those in a paper on the roach [13]. The primary spermatocytes in both species have slightly oval-shaped nuclei that are distinctively stained by hemotoxylin.

The ovaries were examined specifically for the presence of oocytes containing yolk (i.e. vitellin). If the yolk globules had not coalesced, they were termed 'early stage vitellogenic oocytes'. If the yolk filled most of the oocyte, they were termed late stage vitellogenic oocytes. If an oocyte stage was relatively dominant (i.e. the oocytes were spread over most of the field of view), the section was given a score of 3. If the stage was sparser (only two to five in view), the section was given a score of 2. If it was very sparse (two to five only in the whole section), it was given a score of 1. If it was absent, it was given a score of zero. The scores were weighted in the same way as for males in order to calculate an average 'maturity index'. The relative abundance of primary oocytes and yolk vesicle stages was also noted. However, these were relatively abundant at all times in most ovaries and the data were not used. Any ovaries containing atretic oocytes (evidenced by disintegration of the *zona radiata*) were also noted.

2.3. Radioimmunoassay of steroids

The procedure for the extraction and subsequent measurement of 17,20β-P, 17,20β,21-P, E_2, T and 11-KT in tench plasma was the same as that used in the previous study by Pinillos et al. [30]. The same reagents were used. Moreover, a pool of mixed male and female plasma (*ca.* 500 μl) from fish sampled in August and September was passed through a primed solid phase extraction cartridge (SepPak C18, Waters Corp.). The cartridge was washed with distilled water and eluted with 5 ml methanol from which