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Seasonal Changes in Plasma Levels of Steroid Hormones in an Asynchronous Fish the Gudgeon *Gobio gobio* L. (Teleostei, Cyprinidae)

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Annual changes in plasma of estradiol-17β, testosterone, and 17,20β-dihydroxy-4-pregnen-3-one concentrations were measured, by radioimmunoassay, in female gudgeon *Gobio gobio* a fish which has an asynchronous-type ovary containing oocytes at various stages of development and spawns several times during the reproductive period. The gonadosomatic index and the relation between stages of maturity and steroid concentrations were also followed during the reproductive cycle. Plasma levels of estradiol-17β, testosterone, and 17,20β-dihydroxy-4-pregnen-3-one were low from October to April and increased rapidly in May to reach 0.61 ± 0.31; 2.3 ± 0.42; and 3.17 ± 0.68 ng/ml, respectively. Elevated levels were maintained during spawning when vitellogenic oocytes are present alongside oocytes in final maturation. Histological analysis of the ovary indicated that an important number of spawnings has occurred since the proportion of oocytes in final maturation stage was very low (less than 1%). Fish in the regressive phase also presented high steroid levels. The vitellogenic oocytes in preovulatory atresia and the postovulatory follicles may be responsible for these events.

Seasonal and annual changes in the plasma steroid levels of teleost fish have been extensively investigated (Scott *et al.*, 1980, 1983; Breton *et al.*, 1983; Fostier *et al.*, 1983; Kagawa *et al.*, 1983; Kadmon *et al.*, 1985; Fostier and Jalabert, 1986; Kobayashi *et al.*, 1986; Pankhurst and Conroy, 1987; Rosenblum *et al.*, 1987; Galas and Bieniarz, 1989; Kobayashi *et al.*, 1989; Berlinsky and Specker, 1991; Matsuyama *et al.*, 1991; Tamaru *et al.*, 1991). These studies were carried out mostly on species which present a synchronous or a group-synchronous oocyte development and spawned only once per year or once during their life. Fish with asynchronous oocyte development have received little attention. This type of oocyte development implies that during reproductive period, ovary contains oocytes at all stages of development and since yolky oocytes still remain in the ovary after ovulation, these species are able to spawn several times during a spawning period.

The purpose of the present study was to follow seasonal changes in the serum concentrations of several gonadal steroids in the gudgeon *Gobio gobio*, one of the cyprinid fish, which spawns several times during the reproductive season. Correlations between these changes and the stages of gonadal development were also examined.

MATERIALS AND METHODS

The study was conducted between October, 1990 and July, 1991. In October, an outdoor pond (area: 112 m^2, maximum depth:90 cm) was stocked with 504 gudgeons and maintained under these seminatural conditions until the end of the experiment. Fish were sampled by electrofishing during the whole reproductive cycle at 11:00 AM.

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Sampling Procedures

The fish were anesthetized with ethylene glycol monophenyl ether (2–5 ml/10 liter H_2O). Total body length (±0.1 cm) and weight (±0.1 g) were measured. Blood samples were taken from the caudal vessel into a heparinized syringe, centrifuged for 15 min at 10,000*g*, and the plasma was stored at −20° until radioimmunoassay. The ovaries were removed, weighed (±0.001 g), and fixed in Bouin's solution for histological examination. Tissues embedded in paraffin were prepared into 6-μm sections and stained with trichrome:hemaluin, phloxine, and light green (Langeron, 1942).

Morphological Parameter

Gonadosomatic index (GSI) = $100 \times W_o/W_f$, where W_f = weight of the entire fish (g), and W_o = weight of the ovary (g).

Radioimmunoassay

Estradiol-17β (E2), testosterone (T), and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) were measured by radioimmunoassay (Gamma Master Counter 1277 for E2 and T and Miniaxi Tricarb 4000 Series United Technologies Packard for 17,20βP) in plasma following cyclohexane/ethyl acetate (v/v) extraction. Samples of 50 or 75 μl of plasma were extracted with 800 or 1200 μl cyclohexane/ethyl acetate, respectively, dried down, and redissolved in 40 μl 0.01 phosphate buffer.

Test kits for the direct radioimmunological determination of E2 and T were purchased from Mallinckrodt Laboratories Diagnostica (France) and Biotecx Laboratories Inc. (U.S.A.), respectively. Dilution of plasma showed parallelism with standard curves for these hormones. Testosterone was assayed in extracted samples using the procedure described by Lamba *et al.* (1982). A sensitivity of 0.312 ng/ml was obtained and the inter- and intraassay coefficients of variation were 11.5 and 6.0%, respectively.

Plasma concentrations of 17,20βP were measured according to the methods of Fostier *et al.* (1981). Cross-reactivities of various steroids with the antisera used in the radioimmunoassays have been described (Fostier and Jalabert, 1986).

Histological Analysis

Each gonad was classified according to the most advanced type of oocyte present (Table 1). Kestemont (1987) showed that one section of gonad and one field per section are representative of the whole ovary. Ovary development was examined by a histomorphometric approach similar to that described by Kestemont (1987). Two parameters were examined: (1) distribution of oocyte size, evaluated by measuring 50 profiles for stage 1 to 3 or 25 profiles for stage 4 to 8; (2) relative proportion of each stage, i.e., counting 300 cells per ovary and then dividing the percentage of a defined stage by the corresponding oocyte mean diameter.

Data Analysis

All data are expressed as the mean ± SEM. Data were statistically analyzed by an analysis of variance (ANOVA 1) followed by comparison of means using Scheffe *F*'s test. A Hartley test was carried out to verify the homogeneity of variance. The data of E2 were log-transformed before statistical analysis.

RESULTS

Seasonal changes in pond temperature and daylength during the study period are shown in Fig. 1. Pond temperature varied from a minimum value of 2° during February to a maximum of 19° in summer. During January a layer of ice formed on the pond. Daylength was longest in July (16 hr/30 min) and shortest (8 hr/30 min) in December.

Gonadosomatic Index (Fig. 1)

From October to March the gonadosomatic index of *G. gobio* increased slowly from 3.10 ± 0.17% to 4.59 ± 0.26% and then rapidly to reach a peak in May–June (16.53 ± 1.53%). In mid-June, the GSI decreased greatly, indicating the onset of the gonadal regression.

Plasma Sex Steroid Levels

Profiles of plasma levels of E2, T, and 17,20βP in females are shown in Fig. 2.

Plasma E2 levels were low (<0.16 ng/ml) from October to April and rapidly increased to 0.61 ± 0.36 ng/ml in early May. Levels then declined to 0.19 ± 0.05 ng/ml in late May but in June, there was a new peak of E2 (1.60 ±0.35 ng/ml).

Plasma T levels were low from October to April but during the reproductive period, three peaks were observed, reaching 1.63 ±

TABLE I
THE MATURITY STAGES OF THE OVARY OF THE GUDGEON *Gobio gobio*

Ovarian stage	Oocyte stages present in the ovary	Description of the most advanced oocytes
(1) Immature	Previtellogenic oocytes	Oocytes with vacuole free cytoplasm (without yolk substance) (previtellogenesis)
(2) Onset of endogenous vitellogenesis	Previtellogenic oocytes and oocytes in endogenous vitellogenesis	Oocytes at primary yolk vesicle stage, glycoprotein appear and occupy two or three rings in the cytoplasm periphery (early endogenous vitellogenesis)
(3) Complete of endogenous vitellogenesis	Previtellogenic oocytes and oocytes at different stages of endogenous vitellogenesis	Oocytes are full of glycoprotein inclusions. Follicular and cellular layers are differentiated (late endogenous vitellogenesis)
(4) Onset of exogenous vitellogenesis	Previtellogenic oocytes, oocytes at different stages of endogenous vitellogenesis, and oocytes in exogenous vitellogenesis	Oocytes at primary yolk globule stage with small lipoprotein inclusions in periphery of the cytoplasm (early exogenous vitellogenesis)
(5) Progress of exogenous vitellogenesis	Previtellogenic oocytes and oocytes at different stages of endogenous and exogenous vitellogenesis	Oocytes are full of yolk globules and the yolk vesicles are in periphery of the cytoplasm (progress exogenous vitellogenesis)
(6) Complete of exogenous vitellogenesis	Previtellogenic oocytes and oocytes at different stages of endogenous and exogenous vitellogenesis	Appearance of the micropyle in yolk oocytes (late exogenous vitellogenesis)
(7) Onset of final maturation	Previtellogenic oocytes, oocytes at different stages of endogenous and exogenous vitellogenesis, and oocytes in final maturation	Migration of the germinal vesicle (G.V.) to the micropyle (mig. G.V.)
(8) Progress of final maturation	Previtellogenic oocytes, oocytes at different stages of endogenous and exogenous vitellogenesis, and oocytes in final maturation	Germinal vesicle couple with the micropyle (G.V. couple)
(9) Preparation to a new spawning	Previtellogenic oocytes, oocytes at different stages of endogenous and exogenous vitellogenesis, and postovulatory follicles	The follicle cells in the postovulatory follicles, hypertrophy and multiply, showing phagocytosis
(10) Postspawning	Previtellogenic oocytes and pre- and postovulatory follicles	The granulosa cells of preovulatory follicles hypertrophy and the yolk substance degenerates

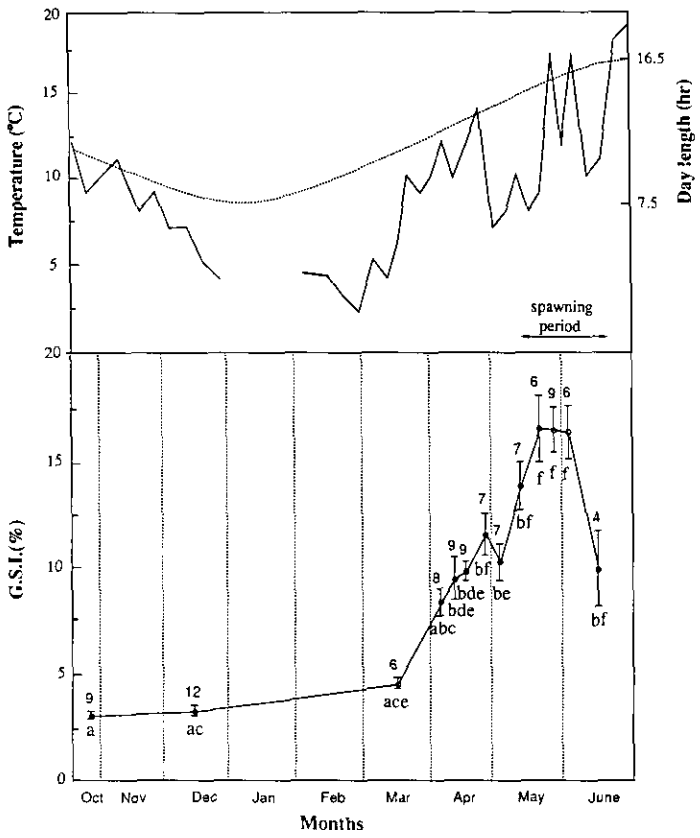

FIG. 1. Seasonal changes in the gonadosomatic index (GSI) of female gudgeon *G. gobio* with changes in water temperature (—) and daylength (---). Each value represents the mean ± SEM. The numbers above the data points indicate the number of fish sampled. The data points showing the same letter are not significantly different ($P < 0.05$).

0.38; 2.30 ± 0.42; 1.86 ± 0.69 ng/ml, respectively.

Plasma 17,20βP levels increased significantly in May to peak at 2.81 ± 0.49 ng/ml and then fall to 2.04 ± 0.53 ng/ml in early June. A significant amount of 17,20βP was found in the last sample (2.46 ± 0.51 ng/ml). Although these peaks are not statistically significant, probably because of a lack of synchrony between individuals, they do indicate a cyclic pattern of changes in these batch spawners.

Histological Analysis

Based on histology, spawning first took place in early May. Indeed, at this time, ovary presented oocytes in late exogenous vitellogenesis (stage 6) with micropyle (stage 7). In the last sample, some fish always presented these types of oocytes and had probably not completed their spawning season.

To understand oocyte growth in an asynchronous ovary, the fact that oogenesis is a continuous process must be taken into account. At a given stage, the growth of oocytes increases the mean size of that stage, but growth of the largest oocytes into the next stage (e.g., by inclusion of yolk vesicles or yolk globules) or the entry of small oocytes from the lower stage produces a decrease in the mean diameter of this stage (Kestemont, 1987).

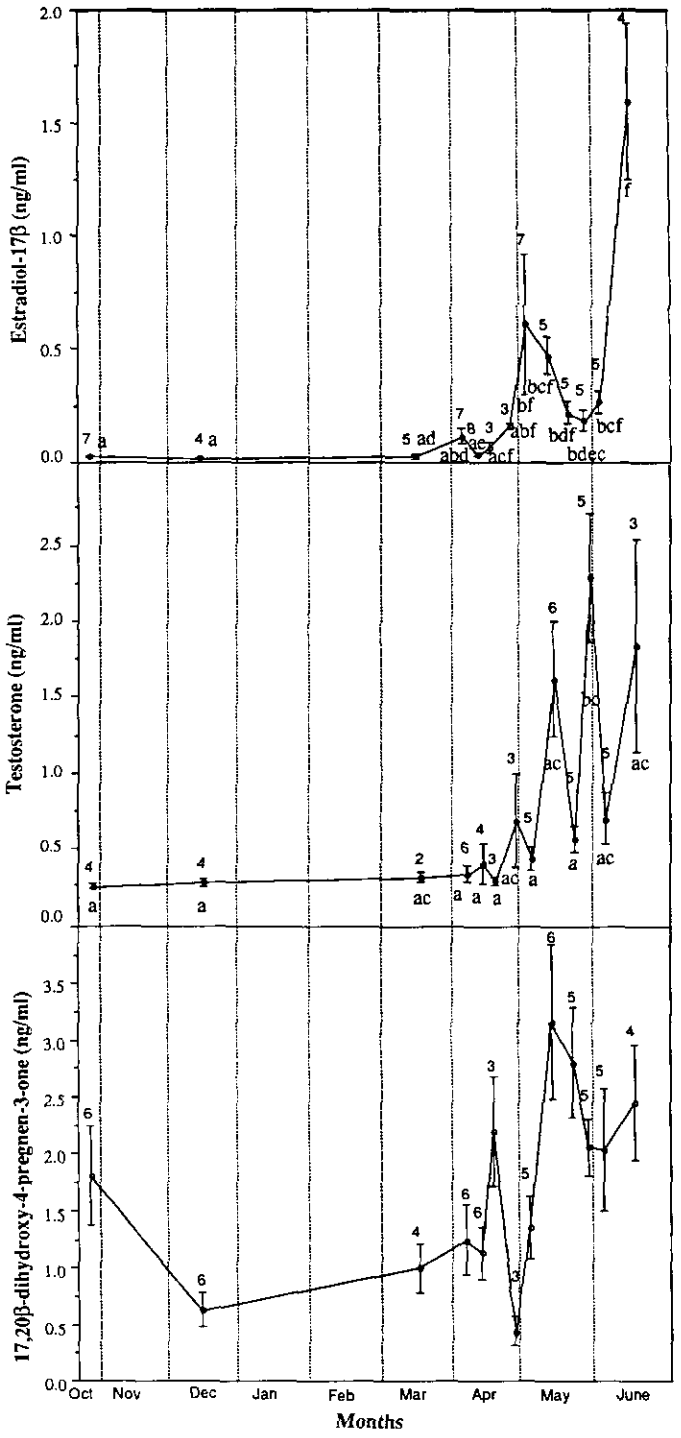

FIG. 2. Annual changes in steroid hormone levels in female gudgeon. Each value represents the mean ± SEM. The numbers above the data points indicate the number of fish sampled. The data points showing the same letter are not significantly different ($P < 0.05$). No significant differences are found for 17,20β-dihydroxy-4-pregnen-3-one.

The change in mean percentages of different oocyte stages and in mean size (diameter) of oocyte are shown in Figs. 3 and 4, respectively.

From October to March, only stages 1, 2, and 3 were present in each sample in percentages of 66.5 to 85%, 6.5 to 19%, and 2 to 6.5%, respectively.

In April, with the onset of exogenous vitellogenesis, the diameter of the oocytes increased (up to 772 μm at the end of exogenous vitellogenesis). Enlargement of the oocytes by accumulation of yolk globules caused a marked increase in GSI.

In May and June, oocyte maturation occurred. However, the percentages of ovary presented oocytes in maturation are as follows: stages 6, 7, and 8 were very low, 0.4, 0.1, and 0.06%, respectively. The mean diameter of the most advanced oocyte increased and reached 840 μm for stage 6, 878 μm for stage 7, and 917 μm for stage 8. If these three stages are grouped, less than 1% of oocytes was laid in each spawning.

For individual fish, there was no relation either between E2 levels and the proportion of oocytes in exogenous vitellogenesis (stages 4 and 5) or between 17,20βP levels and the proportion of oocytes in final maturation (stages 6, 7, and 8).

Plasma Hormone Levels in Relation to Oocyte Stages

Changes in plasma E2, T, and 17,20βP levels at various oocyte stages are shown in Fig. 5.

The level of E2 was low during endogenous vitellogenesis, increased significantly at the beginning of the yolk accumulation, and remained at high levels during exogenous vitellogenesis. In stage 8, just before germinal vesicle breakdown, the level of E2 decreased. In stage 9, containing both oocytes in complete vitellogenesis and postovulatory follicles, the levels of E2 were very high (0.61 ± 0.32 ng/ml). Concentrations remained high in stage 10, with the ovary entering the regressive phase (0.61 ± 0.33 ng/ml). In these stages, there were large individual variations.

The level of T was low during the endog-

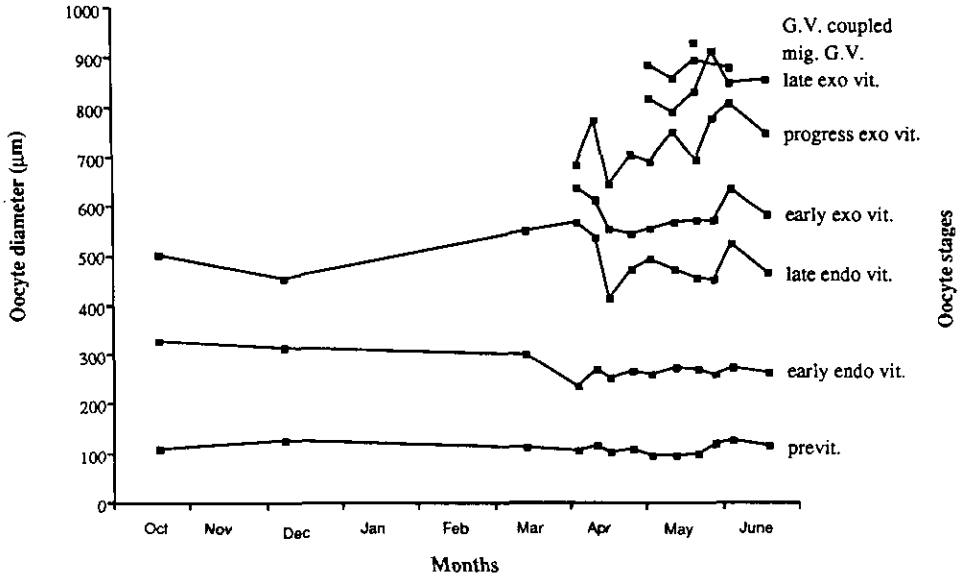

FIG. 3. Annual changes of mean diameter of oocyte stages in female gudgeon (see Table 1 for the descriptions of oocyte stages).

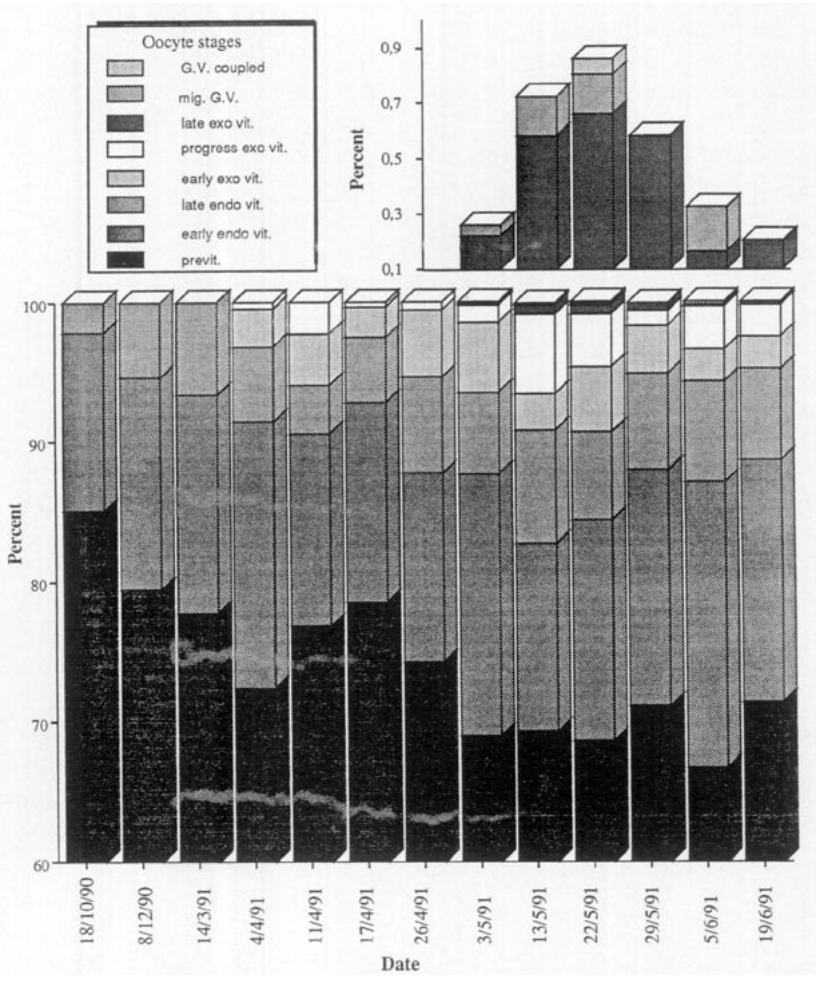

FIG. 4. The percentages of the different oocyte stages in female gudgeon during the sexual cycle (see Table 1 for the descriptions of oocyte stages).

enous vitellogenesis and the beginning of the exogenous vitellogenesis. It increased at the end of exogenous vitellogenesis (stage 6). Just before ovulation (stage 8) the concentration of T decreased to 0.42 ng/ml. As with E2, the levels of T were high during stages 9 and 10 at 1.32 ± 0.63 and 0.94 ± 0.28 ng/ml, respectively.

The levels of 17,20βP were low during vitellogenesis but in the late exogenous vitellogenesis the levels increased to 2.87 ± 0.30 ng/ml and remained high during the final maturation. In stages 9 and 10, the concentrations of this steroid as for the other steroids remained high.

DISCUSSION

The occurrence of seasonally phased reproduction in gudgeon with spawning occurring in spring, is consistent with earlier observations made over several years (Kestemont, 1987, 1988, 1990). This small fish, presenting an asynchronous oocyte development as defined by Wallace and Selman (1981), can ovulate several batches of eggs within a single reproductive season. The hypothesis that gudgeon females ovulated at least four times during the reproductive season with a 7- or 15-day interval between two successive spawnings was suggested

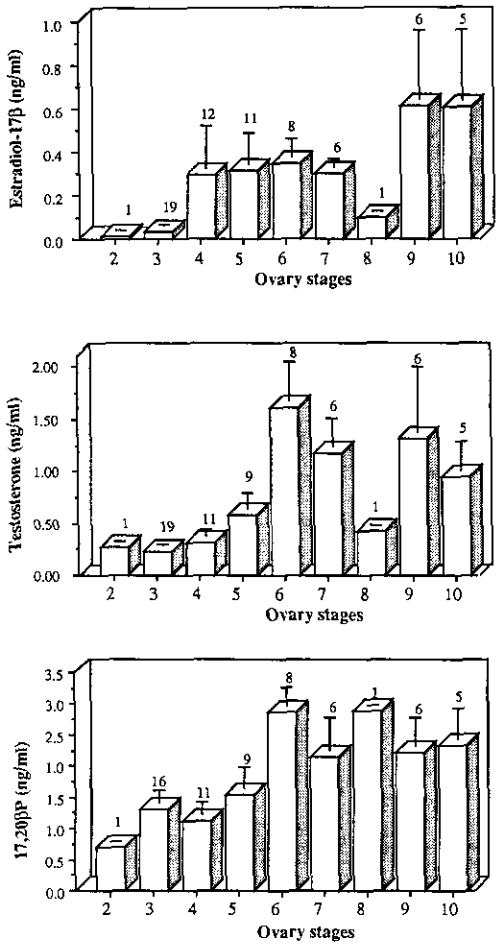

FIG. 5. Comparison of levels of steroid hormones in female gudgeon during ovarian development. Each value represents the mean ± SEM. The numbers above the data points indicate the number of fish sampled.

by Penaz and Prokes (1978) who studied the ontogenesis in gudgeon. In this study, it is not possible to determine the periodicity of spawning and the number of eggs released during a spawning event. However, histological analysis of ovary indicates that an important number of spawnings have taken place since the proportion of oocytes in final maturation stage was very low (less than 1%) during the spawning season.

This study describes for the first time the changes in plasma steroid hormones (E2, T, 17,20βP) during the annual reproductive cycle of the gudgeon. Oocyte development is relatively slow from October to March and the ovaries contained only previtellogenic and different stages of endogenous vitellogenesis oocytes. During this period, blood concentration of estrogen and androgen are both low, associated with a low GSI. This is in agreement with findings in other teleosts (Pankhurst and Conroy, 1987; Berlinsky and Specker, 1991). The level of serum E2 began to increase in April coincident with the appearance of yolk globule in the oocytes. This is consistent with the role of estrogen in promoting hepatic synthesis of the yolk precursor. As observed in other species (Kagawa *et al.*, 1983; Breton *et al.*, 1983; Galas and Bieniarz, 1989), the females have an increased GSI and oocyte diameter (mean diameter of oocytes in exogenous vitellogenesis: 726 μm) correlated with increase of E2. The highest level of E2 was observed during the spawning period (May–June). Similar patterns have also been observed in other multiple spawning fishes as *Carassius auratus* (Kagawa *et al.*, 1983), *Sparus aurata* (Kadmon *et al.*, 1985), and *Dicentrarchus labrax* (Prat *et al.*, 1990). These teleosts have an asynchronous ovarian development and after ovulation, their ovaries contain both protoplasmic oocytes and oocytes at various stages of vitellogenesis; these remaining vitellogenic oocytes were able to produce this steroid after ovulation (Kagawa *et al.*, 1984).

Although a significant increase of E2 occurred during the season of reproduction, the absolute level of this steroid was low. Low absolute levels of plasma E2 also occur among species with either asynchronous (Pankhurst and Conroy, 1987) or synchronous ovarian development (Pankhurst and Conroy, 1988) except in salmonid, which may be as high as 50 ng/ml (Scott and Sumpter, 1983). It is clear that the large interspecific differences in absolute levels of plasma E2 that occur in teleosts during recrudescence are not a function of mode of

ovarian development (Pankhurst and Conroy, 1988). On the other hand, Berlinsky and Specker (1991) suggested that low levels of steroids may be due in part to differences in sampling procedure. Indeed, stress of capture can have profound effects upon the endocrine profiles of teleosts. But it is not known how long it takes for the effects of stress to be observed in terms of changes in plasma levels of gonadal steroids (Pankhurst and Conroy, 1988).

Testosterone has been reported in the blood of a number of female teleosts but the precise role of this steroid is unclear. T as a precursor for E2 synthesis is released into the plasma when no longer needed for aromatization. Fostier *et al.* (1983) suggested that at high concentrations T may have vitellogenic actions on the liver. Plasma T levels increase at the end of vitellogenesis. This acute rise in T indicates that oocytes are fully mature in the ovary and ready to ovulate (Kobayashi *et al.*, 1987). The functional significance of the increased T remains unknown. However, Nagler and Idler (1992) suggest a role for T during the final maturation–ovulation process in winter flounder, as highest levels of T occurred during the prespawning and spawning periods. After ovulation, high levels are maintained since the ovary contains oocytes at different stages of maturity.

17,20βP is one of the most potent steroids for inducing final oocyte maturation and high levels have been found in the plasma of mature species (Nagahama, 1987; Nagahama and Yamoshita, 1989). Since the gudgeon displays asynchronous oocyte development, high levels of plasma E2 and 17,20βP were maintained during the spawning season, as vitellogenic oocytes coexist with oocytes in final maturation. However, in the present study, E2 and the absolute concentration of 17,20βP observed are very low compared with those in salmonid fish. In these species, final maturation and ovulation occurred for all oocytes simultaneously and high 17,20βP levels mediated this event. In asynchronous fish, only a small proportion of the follicles will be producing maturational steroids at any time. In gudgeon, the proportion of oocytes in final maturation stage was very low: 0.4% for stage 6, 0.1% for stage 2, and 0.06% for stage 8. As reported in *C. auratus* (Kagawa *et al.*, 1983), the follicle may appear to have a lower capacity to produce this steroid compared to the follicles of rainbow trout *Oncorhynchus mykiss* (Fostier *et al.*, 1981). On the other hand, 17,20βP would be rapidly metabolized and present only for a brief period and rapidly deactivated (Kime, 1990). A peak is unlikely to be found during weekly sampling unless it coincides closely with spawning. In goldfish (Kobayashi *et al.*, 1987), low levels of plasma 17,20βP were due to short-term secretion and/or rapid plasma clearance of this steroid. Another suggestion to explain low levels of 17,20βP is that this progestagen is not the maturation induction steroid (MIS). In fact, as was previously indicated in some other species (Prat *et al.*, 1990), no measurable change in plasma levels of 17,20βP was found during the spawning period of gudgeon. *In vitro* experiments using gudgeon ovarian tissue are required to determine the potency of this or other steroids in oocyte final maturation.

During the postspawning, high levels of all steroids measured are found. At this time, the ovaries contain protoplasmic oocytes, postovulatory follicles, and many vitellogenic oocytes in preovulatory atresia. When yolky oocytes become atretic, the *granulosa* cells hypertrophy and serve a phagocytic function. In addition to the removal of atretic oocytes, the follicular cells are involved in the steroidogenesis. In goldfish, Khoo (1975) observed that the steroidogenic function is apparent only after complete removal of atretic oocytes and in the postovulatory follicles steroidogenesis is immediate after ovulation. In this species, *in vitro* experiments showed that postvitellogenic follicles pro-

duced T but not E2 (Kobayashi *et al.*, 1989). The high levels of E2 found in gudgeon during the gonadal regression are probably produced by preovulatory atretic follicles. Kagawa *et al.* (1983) suggested that the postovulatory follicle of goldfish produces 17,20βP some 6 to 10 hr after ovulation. In *Oncorhynchus rhodurus* this steroidogenic activity of the postovulatory follicles remains for 1 to 3 days after ovulation (Young *et al.*, 1983).

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