

# Endocrine Changes Associated with Overripening of Ovulated Eggs in Goldfish, *Carassius auratus* L.

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## Abstract

Changes in steroid hormone levels in the serum and ovarian fluid were studied during overripening in goldfish. Ovulated eggs retained in the ovarian cavity become overripe at around 12 h after ovulation and completely overripe 24 h after. Blood and ovarian fluid were taken at 0, 3, 6, 12, 18, and 24 h after ovulation. Estradiol-17 $\beta$  ( $E_2$ ), testosterone (T), progesterone (P) and 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\beta$ -P) in the serum were extracted directly with a solvent while those in the ovarian fluid were separated by HPLC before radioimmunoassay. Both serum and ovarian fluid P showed a highly significant decline at 18 h with a further decline at 24 h; P levels were higher in the ovarian fluid. Serum 17 $\alpha$ ,20 $\beta$ -P showed a progressive and more rapid decline, decreasing significantly at 12 h with further decreases at 18 h and 24 h; the level was five-fold lower at 24 h compared to the 0 h level. Serum T increased significantly at 3 h which was maintained until 18 h, when it declined to 0 h level. No significant changes in  $E_2$  were observed in the serum, except for a significant difference between 6 and 24 h. There were no significant changes in  $E_2$ , T and 17 $\alpha$ ,20 $\beta$ -P in the ovarian fluid. Of the four steroids measured, only 17 $\alpha$ ,20 $\beta$ -P and P showed changes which bear some correlation with the time course of overripening. The declines in the mean ratios of 17 $\alpha$ ,20 $\beta$ -P/ $E_2$  in the serum and P/ $E_2$  in the ovarian fluid also appeared to have a good correlation with the time course of overripening. The postovulatory follicles (POFs) showed degenerative features which likewise correspond to the decline in P and 17 $\alpha$ ,20 $\beta$ -P.

## Introduction

Ovulated eggs become overripe if retained in the ovarian cavity for extended period. The overripe eggs are characterized by semi-transparent yolk, aggregated cytoplasm and oil droplets at the animal pole and hydration (Nomura et al. 1974,

Hirose et al. 1977, 1979, Lam et al. 1978, McEvoy 1984). More recently, histological changes in eggs and the ultrastructural changes of the chorion during overripening were studied in goldfish (Formacion et al. 1993). There are no external manifestations of overripening in fishes except in the three spined stickleback (Lam et al. 1978). Therefore, in fish hatcheries, overripening of eggs poses a problem when ovulated eggs are not stripped on time, especially for fishes whose eggs can only be expressed by manual stripping and fertilized artificially, such as the turbot and Asian catfish (McEvoy 1984, Tan-Fermin and Emata 1993).

The underlying causes of overripening are still not understood. Lam et al. (1978, 1979) have shown a relationship between overripening and the degeneration of postovulatory follicle cells (POFs) in the three spined stickleback, and postulated that steroids (e.g. progesterone) secreted by the POFs stimulate the secretion of ovarian fluid which help maintain ovulated eggs in the ovarian cavity. In goldfish, Nagahama et al. (1976) have shown that POFs degenerate rapidly (within 30 h) based on ultrastructural and histochemical evidence. Therefore, to further examine the hypothesis of Lam et al. (1978, 1979), changes in the serum and ovarian fluid steroid hormones as well as the histology of the POFs during overripening were studied in goldfish.

### **Materials And Methods**

The selection and care of spawners as well as the induction of ovulation have been previously described (Formacion et al. 1993).

Both blood and ovarian fluid samples were collected at 0, 3, 6, 12, 18, and 24 h after ovulation in individual females. Serum  $17\alpha,20\beta$ -hydroxy-4-pregnen-3-one ( $17\alpha,20\beta$ -P), progesterone (P), estradiol ( $E_2$ ) and testosterone (T) were extracted directly with redistilled diethylether. The serum RIA procedure was modified from the WHO Special Program of Research, Development and Research Training for Human Reproduction Methods Manual (Sufi et al. 1993). The four steroids assayed in the serum were separated from the ovarian fluid sample by reverse phase high performance liquid chromatography (HPLC) with Nova Pak C18 column, followed by RIA, as described and validated by Venkatesh et al. (1989). The steroid levels in both serum and ovarian fluid were determined using the software program developed by Dr. P.R. Edwards for WHO.

Central parts of the ovaries from ovulated fish were collected at 0, 3, 6, 12, 18, and 24 h ovulation and fixed in formol-calcium. Paraffin sections (7-9  $\mu$ m) were stained in hematoxylin-eosin. The POFs were examined in the prepared slides, and representative sections at 0, 12, and 24 h representing the Stages 1, 2, and 3 of overripening, respectively (Formacion et al. 1993), were photographed.

Data on steroid levels at various time intervals after ovulation were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) multiple range test, using the SPSSPC+ Statistical Package.

## Results

Ovulated eggs retained in the ovarian cavity become overripe at around 12 h and completely overripe 24 h after ovulation (Formacion et al. 1993). Both serum and ovarian fluid P showed a highly significant decline at 18 h with a further decline at 24 h; P levels were higher in the ovarian fluid (Fig. 1a). Serum  $17\alpha,20\beta$ -P showed a progressive and more rapid decline, decreasing significantly at 12 h with further decreases at 18 h and 24 h; the level was five-fold lower at 24 h compared to the 0-h level (Fig. 1b). Serum (T) increased significantly at 3 h which was maintained until 18 h after which it declined to 0-h level (Fig. 1d). No significant changes in  $E_2$  were observed in the serum, except for a significant difference between 6 and 24 h (Fig. 1c). There were no significant changes in  $17\alpha,20\beta$ -P,  $E_2$  and T in the ovarian fluid (Fig. 1b, c, and d). Of the four steroids measured, only  $17\alpha,20\beta$ -P and P showed changes which bear some correlation with the time course of overripening. The declines in the mean ratios of  $17\alpha,20\beta$ -P/ $E_2$  in the serum and P/ $E_2$  in the ovarian fluid also appeared to have a good correlation with the time course of overripening (Figs. 2a and 2b).

In the ovaries containing newly ovulated eggs in the ovarian cavity, the outer thecal layer (TC) and inner granulosa layer (GC) of the POFs were hypertrophied (Fig. 3a). The GC were columnar in shape and has prominent nuclei. The TC were composed of spindle-shaped cells. During overripening of the retained eggs in the ovarian cavity, many cells of the POFs had assumed irregular shapes and possessed vacuoles and pycnotic nuclei (Fig. 3b). Finally, when the retained eggs had become fully overripe, irregularly-shaped masses of cells were observed in the POFs, suggestive of the degeneration of TC and GC (Fig. 3c).

## Discussion

Of the steroids studied, only the levels of P and  $17\alpha,20\beta$ -P showed changes whose time course bore some correlation with that of overripening. Also, of the steroids measured in the ovarian fluid, only P showed a progressive decline, with significant changes after 12 h (at 18 and 24 h). However, changes in serum  $17\alpha,20\beta$ -P were better correlated with overripening than the changes in serum P. Serum P did not decline significantly until after 12 h, whereas serum  $17\alpha,20\beta$ -P was already significantly reduced at 12 h with further significant decreases at 18 h and 24 h. The

decline of serum  $17\alpha,20\beta$ -P to low levels at 24 h agrees well with the results obtained by Kagawa et al. (1983) and Kobayashi et al. (1987) in goldfish.

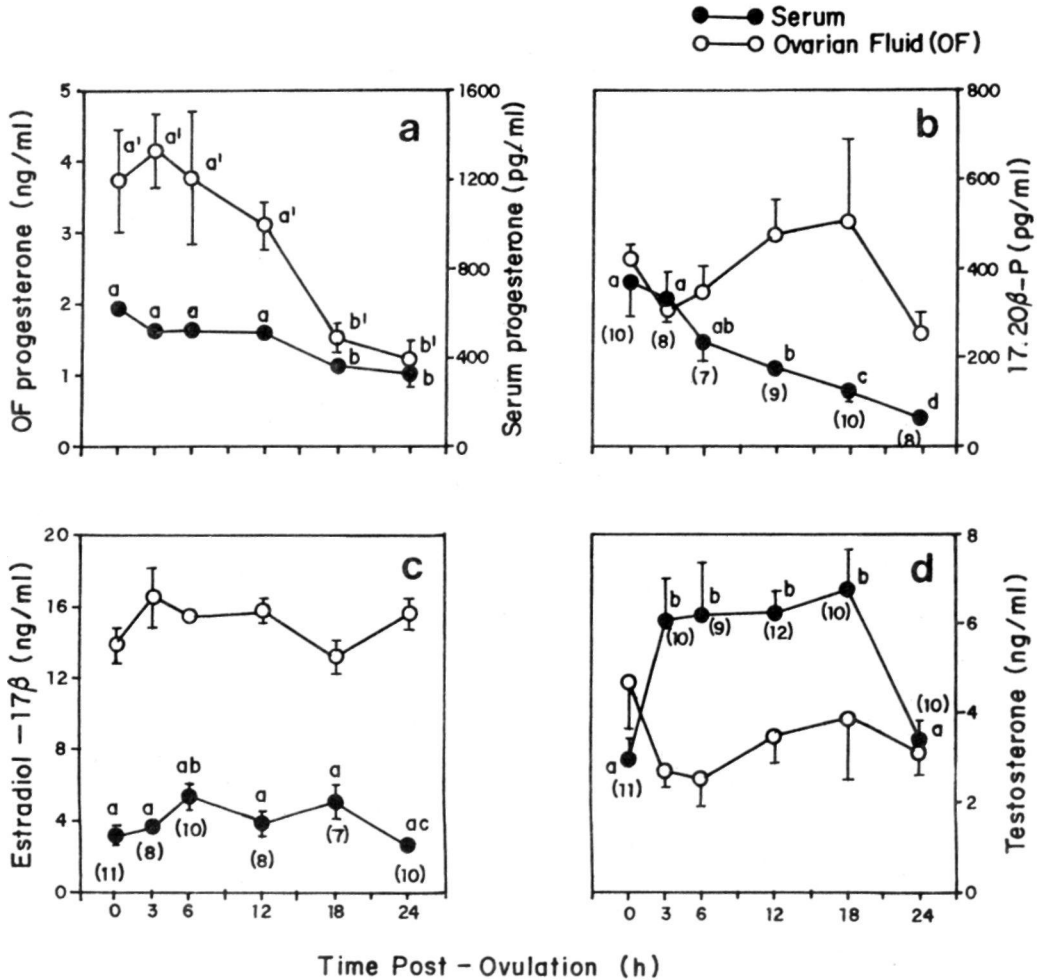


Fig. 1. Changes in steroid levels of serum and ovarian fluid during egg overripening in goldfish. a) Progesterone (P) b)  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha,20\beta$ -P) c) Estradiol-17 $\beta$  ( $E_2$ ) d) Testosterone (T). Each point shows the mean  $\pm$  SE. Numbers in parenthesis refer to sample size in serum, except progesterone (a) where  $n=5$ , for all groups. For ovarian fluid,  $n=6, 5, 5, 7, 4,$  and  $7$  at 0 h, 3 h, 6 h, 12 h, 18 h, and 24 h, respectively, for each steroid. Means with the same letter(s) are not significantly different from each other ( $P>0.05$ ).

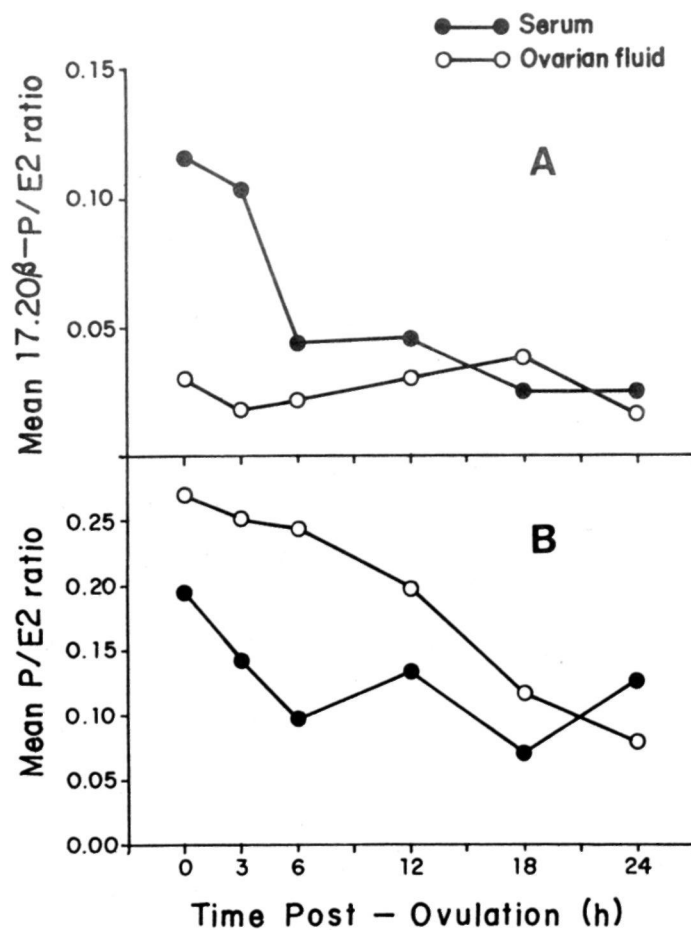


Fig. 2. Plots of the ratios of the mean 17 $\alpha$ ,20 $\beta$ -P/E<sub>2</sub> (A) P/E<sub>2</sub> (B) in the serum and ovarian fluid. (17 $\alpha$ ,20 $\beta$ -P=17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one; P=progesterone; E<sub>2</sub>=estradiol-17 $\beta$ ).

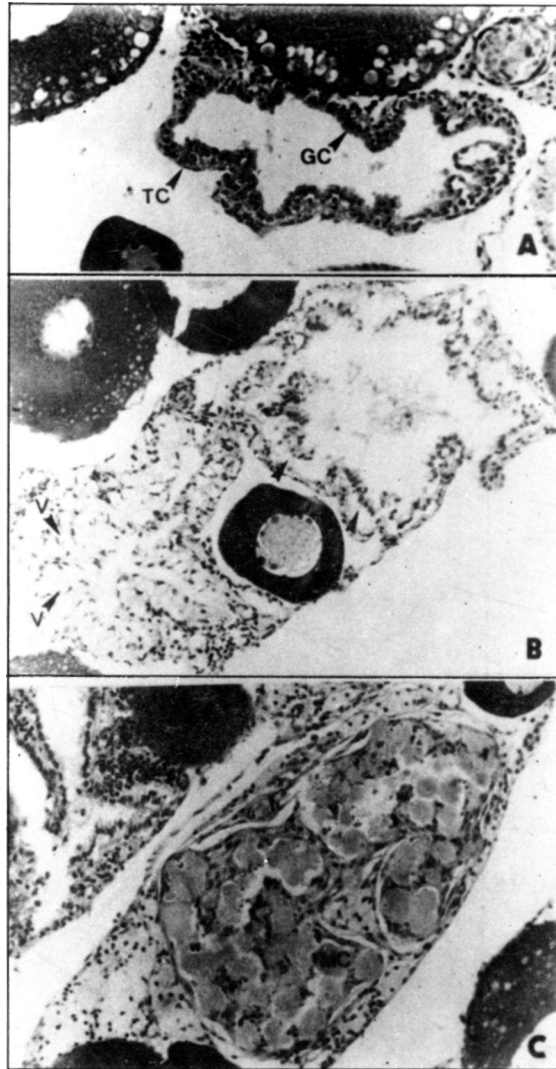


Fig. 3. Photomicrographs of goldfish postovulatory follicle (POFs) during egg overripening in goldfish. (A) 0 h showing convoluted POF with hypertrophied inner granulosa cells (GC) and outer thecal cells (TC). Note columnar GC and spindle-shaped TC with their prominent nuclei (x 60). (B) 12 h, POF becoming irregularly-shaped; vacuoles (V) appear and nuclei become pycnotic; GC starting to separate from TC (arrow) (x 60). (C). 4 h, irregularly-shaped mass of cells (MC) filling the lumen of POF (x 60).

The POFs immediately following ovulation showed hypertrophied GC. This is in agreement with the observations of Nagahama et al. (1976) on POFs 6-10 h after ovulation which were characterized by a highly vascular thecal layer and hypertrophied GC, both of which showed a weak but evident 3 $\beta$ -HSD activity. An active steroidogenic appearance of special thecal cells of POFs shortly after ovulation in the white-spotted char, in parallel with high levels of P, was reported by Kagawa et al. (1981), suggesting that these thecal cells are major sites of P production during the postovulatory period. Young POFs in amago salmon were also reported to be able to produce other steroids, mainly progestogens (17 $\alpha$ ,20 $\beta$ -P and 17- $\alpha$  hydroxyprogesterone) in response to gonadotropins (Nagahama 1983).

By 24 h postovulation, when the eggs retained became fully overripe, the POFs showed degenerative TC and GC which correspond to the decline in P and 17 $\alpha$ ,20 $\beta$ -P. Electron microscopic data of Nagahama et al. (1976) on the POFs 30 h after ovulation likewise revealed advanced degeneration of the TC and GC, with hardly any steroidogenic enzyme activity. Thus, the present results supports the suggestion of Lam et al. (1978, 1979) that a correlation between overripening and the degeneration of POFs, and also their hypothesis that steroids secreted by the POFs may be involved in the maintenance of ovulated eggs in the ovarian cavity.

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