

## Fat Body Involvement in the Plasma Vitellogenin Changes During Recovery Phase in *Rana esculenta*

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**ABSTRACT**—The role of fat body in the reproductive physiology of *Rana esculenta* was investigated during recovery phase. Changes in the ovarian weight, plasma vitellogenin (VTG) and estradiol-17 $\beta$  levels were analyzed in intact frogs, in those without fat bodies (FBD) and in males and females injected with estradiol-17 $\beta$ . The ovarian weight decreased by day 28 in intact females but not in FBD animals. At the same time plasma VTG titers sharply decreased on day 21 in intact animals, but no significant changes were found in FBD females. Male frogs never synthesize VTG; they do so, however, after injection of estradiol-17 $\beta$ , FBD males showed higher VTG titers than intact males. The results obtained *in vitro* showed a sharp decrease of vitellogenin added in the medium of cultured fat body. Thus, the *in vivo* and *in vitro* results allow us to suggest that fat bodies metabolize plasma vitellogenin, at least during recovery phase; therefore ovarian growth may depend on an interplay among hepatic vitellogenin synthesis, fat body metabolic activity and ovarian uptake.

### INTRODUCTION

A peculiarity, at least so it seems, of the reproductive physiology of amphibians is the insertion of the fat body (FB) in the hypothalamo-hypophysial-gonadal axis [1]. The importance of the fat body in the regulation of testicular activity in *Rana esculenta* has been widely demonstrated by Chieffi *et al.* [2]. These authors found that the fat bodies are the main deposit for lipids in the body; the lipids, synthesized by the liver are released in the plasma and subsequently incorporated in the fat body and testis; the annual changes in these parameters correlate with gametogenetic activity. The relationship between fat body and ovarian cycle have been ascertained by Pierantoni *et al.* [3] in the same species. Fat body removal impairs the ovarian growth, during the recovery period, resulting in a lack of lipid uptake by the gonad and in a reduction in the protein synthesis. Recently Variale *et al.* [4] investigated the annual variations of VTG in the FB, liver, plasma and ovaries of adult female, *Rana esculenta* frogs, these variations have

been demonstrated to be correlated with the plasma estradiol-17 $\beta$  (E<sub>2</sub>) profile. These authors found that fat body does contain vitellogenin, at least during the pre-spawning and spawning periods, but does not synthesize it.

In order to clarify the involvement of fat body in the regulation of *Rana esculenta* plasma vitellogenin, this paper describes: i) the effects of FB removal during the recovery phase on ovarian weight and in the plasma levels of VTG and E<sub>2</sub> in female frogs; ii) the variations of VTG and E<sub>2</sub> plasma titers in estrogenized intact and fat body deprived males and females; iii) whether female fat body can release VTG using an *in vitro* culture system.

### MATERIALS AND METHODS

#### Animals

Adult male (15–20 g) and female (20–25 g) frogs were collected in the mountain pond (Colfiorito, Umbria) in November and FB removal was immediately performed. The experiments began on the 1st November: the animals were maintained in tanks with deep water at 18°C of temperature



and fed on bovine liver and fly larvae.

### *Experimental design*

Five days after fat body removal (day 0), experimental groups were composed of intact frogs in captivity, intact frogs injected with 100  $\mu$ l of E<sub>2</sub> (7  $\mu$ g/ml, Sigma), fat bodies deprived frogs (FBD) and FBD frogs treated with 100  $\mu$ l of E<sub>2</sub> (7  $\mu$ g/ml). Ten animals, for each experimental group, were weighed and killed at weekly intervals until day 28. The ovaries were then removed and weighed to the nearest milligram and blood samples were taken by capillary inserted in the "conus arteriosus"; after centrifugation at 2500 g the supernatants were frozen at  $-80^{\circ}\text{C}$  until assayed.

### *Culture Media and Preparation of Fat Body Cultures*

Ten adult female frogs were anesthetized in ice; the abdomen was cleaned with 5% chlorhexadine gluconate in 70% methanol and was then opened taking care not to cut the abdominal vein. Fat bodies were removed into sterile petri dishes containing Eagle-Earle's salts (Gibco Bio-Cult Glasgow Scotland) prepared as described by Wang and Knowland [5] and containing a final concentration of 20 mM HEPES, 2 mM glutamine, 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin. All solutions and glassware were sterilized before use by filtration or autoclaving. Explanted FB were chopped with scissors and rinsed thoroughly and drained on autoclaved 3MM paper. Sixty mg (about 6–7 pieces) were then weighted and placed in each well of a Falcon culture dish, and covered with 1 ml of culture medium.

Culture were incubated at room temperature ( $20^{\circ}\text{C}$ ) in air on a rocking table (4 cycles/min). The medium was daily changed for up to five days. After incubation for two days in culture medium alone, estradiol-17 $\beta$  ( $2 \times 10^{-6}$  M), was added to the wells. Finally 60 mg/well incubated for 24 hr with 10 mg/ml of VTG.

### *Purification of VTG*

VTG was purified from the plasma of estrogenized males by  $\text{MgCl}_2$ /EDTA precipitation according to Wiley *et al.* [6]. The concentration of purified VTG was determined by a procedure

which uses bovine serum albumin (BSA) as standard following Lowry [7].

### *Measurement of VTG concentrations by ELISA*

Purified VTG was used to raise antibodies by a protocol developed in our laboratory [8] and the polyclonal antibody raised in the rabbit was used to determine VTG titer in the medium by enzyme-linked-immuno-sorbent-assay (ELISA).

Buffer and reagents for ELISA (i) Coating buffer: phosphate buffered saline (PBS) 0.15 M pH 7.2 plus 0.02% sodium azide. (ii) Washing buffer: PBS plus 0.05% Tween 20. (iii) Rabbit anti VTG antiserum. (iv) Dilution buffer per conjugate: PBS plus 0.05 Tween 20, 0.1% BSA and 5% pig serum. (v) Conjugate: alkaline phosphatase-conjugated goat anti rabbit immunoglobulin antiserum (Sigma). (vi) Substrate buffer: diethanolamine buffer 1 M, pH 9.8, containing 0.5 mM  $\text{MgCl}_2$ . (vii) Substrate: p-nitrophenylphosphate 1 mg/ml in substrate buffer.

### *Assay procedure*

Flat bottomed microtiter plates (Greiner, medium affinity; SIAL, Rome, Italy) were coated overnight with 100  $\mu$ l of culture media from incubations. Standard curve was carried out with gradual dilution (10–1280 ng) of purified VTG. Plates were washed 4 times, post-coated overnight with 10  $\mu$ g/ml BSA in PBS, washed with PBS and distilled water and dried under vacuum in a desiccator. Rabbit anti VTG antiserum was diluted 1:1000 in buffer containing 5% pig serum to reduce non specific binding.

Plates were washed after 1 h and incubated with substrate. The reaction was stopped with 3 M NaOH, 25  $\mu$ l/well after 30–60 min.

The optical density was read at a wavelength of 405 nm. All steps were performed at room temperature. Post coating was done with 150  $\mu$ l/well, antigen (VTG), samples, rabbit anti-VTG antiserum, conjugate and substrate were added in volumes of 100  $\mu$ l/well.

A reliable calibration curve enables the antigen titer (VTG) to be measured in all cultured media and in the plasma.



*Estradiol-17 $\beta$*  assay

Plasma samples, were extracted with ether; sub-

sequently, radioimmunological analysis (RIA) of estradiol-17 $\beta$  (E<sub>2</sub>) was carried out as previously described by Polzonetti-Magni *et al.* [8]. A sensi-

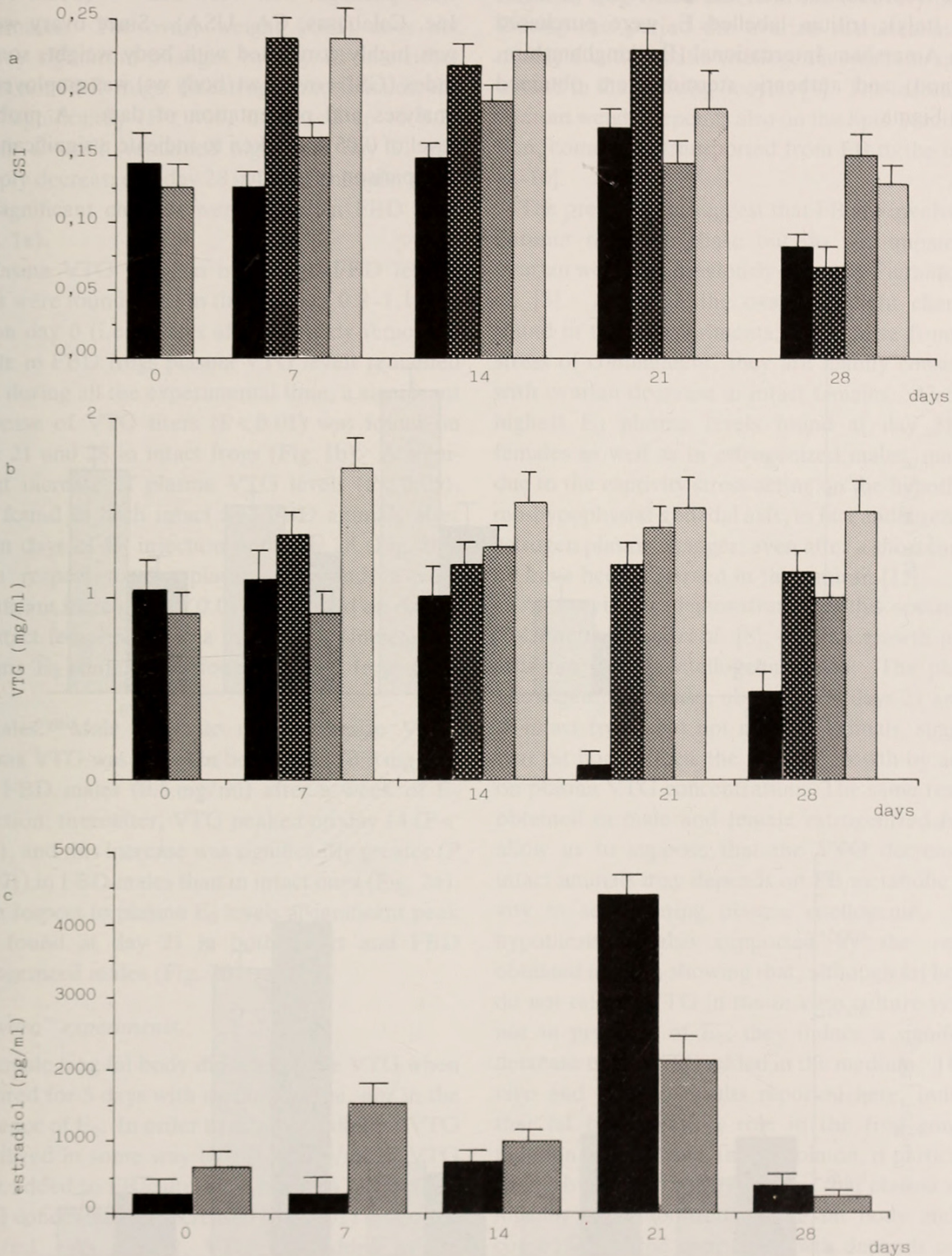


FIG. 1. a) Ovarian weight (gonadosomatic index, GSI), b) plasma vitellogenin and c) estradiol-17 $\beta$  titers in the intact (■) and estrogenized (▨) female frogs, in those deprived of their fat bodies (▧) and those without fat bodies and injected with estradiol-17 $\beta$  (▩). Vertical lines indicate SD.



tivity of 7 pg (intraassay variation, 4.5%; interassay variation 7.5%) was observed. Steroid antiserum was provided by G. Bolelli (Physiopathology of Reproduction Service, University of Bologna, Italy); tritium labelled  $E_2$  were purchased from Amersham International (Buckinghamshire, England) and authentic steroids were obtained from Sigma.

#### Statistical analysis

Results were analyzed using a statistical software package, Stat View 512<sup>+</sup>™ (Brain Power Inc. Calabasas, CA, USA). Since ovary weight was highly correlated with body weight, somatic index ( $GSI = \text{ovary wt/body wt}$ ) was employed for analyses and presentation of data. A probable level of 0.05 was taken to indicate a significance in comparison of means.

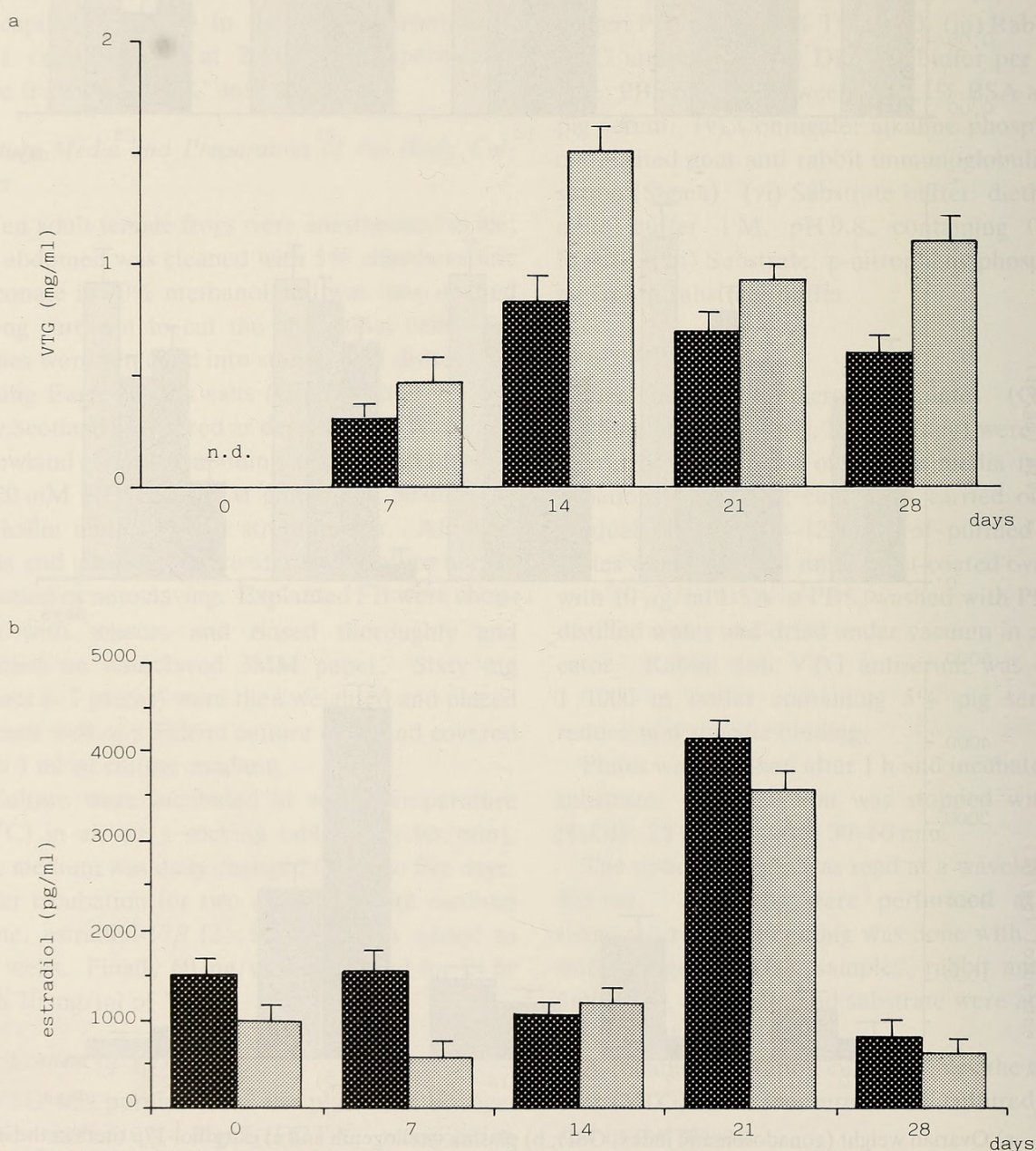


FIG. 2. a) Plasma vitellogenin and b) plasma estradiol-17 $\beta$  titers in estrogenized intact (■) and fat bodies-deprived (▨) male frogs. Vertical lines indicate SD.



## RESULTS

### *In Vivo* experiments

**Females:** The ovary weight (GSI) does not display significant changes by day 21 in neither intact nor FBD frogs. In estrogenized females, the GSI significantly ( $P < 0.01$ ) increased up to 0.25 within a week, remained high until day 21 and sharply decreased at day 28 in intact animals, while no significant changes were found in FBD ones (Fig. 1a).

Plasma VTG titers in intact and FBD female frogs were found to be in the range of 0.8–1.1 mg/ml on day 0 (i.e. 5 days after fat body removal). While in FBD frogs plasma VTG levels remained high during all the experimental time, a significant decrease of VTG titers ( $P < 0.01$ ) was found on days 21 and 28 in intact frogs (Fig. 1b). A significant increase of plasma VTG levels ( $P < 0.05$ ), was found in both intact and FBD animals after seven days of  $E_2$  injection until day 28 (Fig. 1b). With respect to the plasma  $E_2$  trend, a very significant increase ( $P < 0.01$ ) was found on day 21 in intact females, while a much lesser increase of plasma  $E_2$  content was found in FBD frogs (Fig. 1c).

**Males:** Male frogs do not synthesize VTG; plasma VTG was found in both intact (0.3 mg/ml) and FBD males (0.5 mg/ml) after a week of  $E_2$  injection; thereafter, VTG peaked on day 14 ( $P < 0.01$ ), and this increase was significantly greater ( $P < 0.01$ ) in FBD males than in intact ones (Fig. 2a). With respect to plasma  $E_2$  levels a significant peak was found at day 21 in both intact and FBD estrogenized males (Fig. 2b).

### *In vitro* experiments.

Female frog fat body did not release VTG when cultured for 5 days with medium alone, nor in the presence of  $E_2$ . In order to establish whether VTG is utilized in some way by FB, 10  $\mu$ g/ml of VTG were added to FB culture. After 24 h incubation, VTG concentration decreased ( $P < 0.01$ ) to around 5  $\mu$ g/ml. As control, VTG was added in the medium without FB and no changes of VTG concentration were observed (data not shown).

## DISCUSSION

Following a summer post-reproductive refractoriness, frog ovary enters in the recovery phase. During this period the ovarian recrudescence is mainly due to hepatic vitellogenin synthesis and its uptake in the growing oocytes [8]. The increase of ovarian weight depends also on the lipid accumulation, commonly transported from FB to the ovary [9–10].

The present data suggest that FB are involved in autumn recovery phase but do not impair the ovarian weight as previously found by Pierantoni *et al.* [3]. Although the ovarian weight changes, found in these experiments, could arise from the stress of confinement, they are mainly consistent with ovarian decrease in intact females. Also the highest  $E_2$  plasma levels found at day 21, in females as well as in estrogenized males, may be due to the captivity stress acting on the hypothalamo-hypophysial-gonadal axis; in fact androgen and estrogen plasma changes, even after a short captivity have been observed in this anuran [11].

As previously demonstrated in this species by Polzonetti-Magni *et al.* [8], ovarian growth parallels the plasma vitellogenin trend. The plasma vitellogenin decrease, observed on days 21 and 28 in intact frogs, but not in FBD animals, suggests that fat body affects the ovarian growth by acting on plasma VTG concentration. The same results, obtained in male and female estrogenized frogs, allow us to suppose that the VTG decrease in intact animals may depend on FB metabolic activity in sequestering plasma vitellogenin. This hypothesis is also supported by the results obtained *in vitro*, showing that, although fat bodies do not release VTG in the *in vitro* culture system nor in presence of  $E_2$ , they induce a significant decrease of the VTG added in the medium. The *in vivo* and *in vitro* results reported here, indicate that fat body plays a role in the frog gonadal function. Such a role, in our opinion, is particularly consistent with the hypothesis that plasma vitellogenin is metabolized by the fat body and, in consequence, the ovarian growth depends on an interplay among hepatic vitellogenin synthesis, fat body metabolic activity and ovarian uptake. More detailed studies are needed to investigate how FB



utilize this glycolipophosphoprotein in view of their involvement in the lipids storage as ascertained in this frog by Milone *et al.* [12].

The lower plasma E<sub>2</sub> levels found in FBD females, with respect to intact animals, agree with the results obtained by Chieffi *et al.* [1], showing FB involvement in the frog gonadal steroidogenesis; in addition, the plasma levels of this hormone in intact female frogs, reported here, confirm a multihormonal control of VTG synthesis, as suggested by Carnevali and Polzonetti [13], since the peak values of this hormone are concomitant with the lowest plasma VTG levels.

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