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Effect of 17β-Estradiol and Testosterone Treatment on Sex Steroid Binding Proteins in the Female of the Green Frog *Rana esculenta.*

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ABSTRACT—In this paper we report the effect of gonadectomy and/or long-term sex steroid (17β-estradiol and testosterone) treatment on binding activity of 17β-estradiol and testosterone binding proteins (EBP and TBP, respectively) in the plasma of the female of the green frog *Rana esculenta*. Experiments were carried out during different periods of the reproductive cycle when circulating levels of 17β-estradiol and androgens were : 1) low, 2) medium; 3) medium-high; 4) high. This study shows that EBP, but not TBP activity were affected by 17β-estradiol and testosterone treatment. The effect of the hormonal treatment changed according to the period of the reproductive cycle when it was carried out. Both 17β-estradiol and testosterone were ineffective when the circulating levels of 17β-estradiol and androgens were medium-high and high. On the contrary, the maximum effect was registered when circulating 17β-estradiol and androgens were at their minimum levels. Thus, our data indicate that binding activity of EBP apparent changes in response to 17β-estradiol and testosterone treatment varied according to the period of the reproductive cycle, an indication that studies on sex steroid binding proteins regulation should take into consideration the internal endocrine condition before drawing any final conclusion especially in species with a seasonal mode of reproduction.

INTRODUCTION

In vertebrates sex steroid binding proteins (SSBPs) are homodimeric glycoproteins with a single steroid-binding site. They modulate the gene regulatory actions of nuclear sex steroid receptors by controlling the availability of sex steroids. In plasma, SSBPs control the metabolic clearance rate of sex steroids (Martin, 1980; Petra *et al.*, 1986; Hammond and Bocchinfuso, 1996; Lee *et al.*, 1997). In addition, the identification of plasma membrane receptors in target tissues supports functions other than regulate free steroid hormone levels (Hryb *et al.*, 1985, 1989; Petra, 1991). Among vertebrates SSBPs bind two or three classes of sex steroids with similar affinity. In amphibians, SSBPs preferentially bind 17β-estradiol and testosterone (see Callard and Callard, 1987 for review).

Despite the importance of SSBPs in modulating hormone access to target cells, little is known about their regulation. Gene sequencing experiments indicate that the regulation of the SSBP gene is very complex (Joseph *et al.*, 1991; Joseph, 1994). In amphibians data on SSBP regulation are scarce and fragmentary. In *Pleurodeles waltlii* gonadectomy causes a decrease in 17β-estradiol binding capacity of SSBP which is restored by 17β-estradiol treatment (Martin and Ozon, 1975). Androgen treatment fails to restore the androgen binding capacity of SSBP in gonadectomized *Taricha granulosa* (Moore *et al.*, 1983).

We have previously reported the presence of SSBPs in the plasma of the green frog *Rana esculenta*. In both sexes SSBPs bind 17β-estradiol and testosterone. SSBP binding capacity for 17β-estradiol (estradiol binding protein - EBP) in the female and SSBP binding capacity for testosterone (testosterone binding protein - TBP) in the male vary in relation to the reproductive cycle. In the female EBP binding capacity in the plasma paralleled the 17β-estradiol fluctuations throughout the reproductive cycle, although it kept high when 17β-estradiol titer dropped after the first ovulatory wave, most likely a way to assure an adequate delivery of 17β-estradiol to target organs when this is decreasing in the blood (Paolucci and Di Fiore, 1994). The reproductive cycle of the female of *Rana esculenta* at this latitude, can be divided into different periods: recovery (September-November), when vitellogenesis starts and a sharp increase in the ovary weight takes place, due to the incorporation of vitellogenin into the growing oocytes; winter stasis (December-January), when frogs hibernate and the metabolism is reduced at its low; pre-breeding

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(February-early March), when the hibernating frogs awake and eat vigorously prior to spawning; breeding (late March-June), when egg laying occurs and there may be up to three main waves of egg deposition, although the number of clutches laid by individual females in a season cannot be ascertained with certainty and differences may take place from year to year, based on variations in temperature, food availability, and rain fall; post breeding (July-August), when frogs again feed vigorously in order to replenish their fat bodies in preparation for the next winter (Rastogi *et al.*, 1983). It is worth noting that differences in the beginning and duration of the periods of the reproductive cycle, as well as in the profile of sex steroid fluctuations in the plasma which, in turn, affect the ovarian cycle, may take place from year to year, associated with variations in the environmental temperature and humidity.

In this study we evaluated the effect of gonadectomy and/or long-term sex steroid (17β-estradiol and testosterone) treatment on EBP and TBP levels during different periods of the reproductive cycle of the female of *Rana esculenta*. The period of the reproductive cycle was ascertained on the grounds of the presence and percentage of follicles of different diameter and circulating levels of sex steroids (17β-estradiol and androgens). We decided to follow this strategy considering that, although animals were ovariectomized, it was hard to evaluate the "memory effect" of having been exposed to a certain internal hormonal milieu. Experiments were started either when levels of circulating sex steroids were low, medium, medium-high, or high.

MATERIALS AND METHODS

Animals

Ten adult females of *Rana esculenta* were captured in the outskirts of Naples monthly, from September 1996 to August 1997. Soon after capture, the animals were anesthetized by immersion in water containing 1% MS-222 Sandoz (3-aminobenzoic acid ethyl ester). Blood was collected through a heparinized glass capillary inserted into the heart and centrifuged at 800 for 10 min. at 4°C to generate plasma, which was stored at –80°C until use. Ovaries were removed and fixed in saline solution for amphibians (Ringer solution) added with 10% formalin.

In addition to ten females captured monthly in order to assess the period of the reproductive cycle, adult females were collected during the following periods of the reproductive cycle: recovery (medium levels of circulating sex steroids), pre-breeding (medium-high levels of sex steroids), at the beginning and at the end of the breeding period, since circulating sex steroid levels were respectively high and low. These two periods will be defined as early-breeding (high levels of sex steroids) and late-breeding (low levels of sex steroids), respectively. Animals were transferred into laboratory aquariums with a photoperiod corresponding to the season. Meal worms were furnished *ad libitum*.

Experimental design

Experiment 1: 70 females collected during the recovery period (medium levels of circulating sex steroids). Animals were separated into seven groups (10 animals/each group) and underwent to the following experimental design:
group 1 = frogs sacrificed soon after the arrival. These animals are referred to as control group (con);
group 2 = intact untreated frogs kept in captivity for the whole duration of the experiment (four weeks) (capt);
group 3 = frogs sham operated soon after the arrival and kept in captivity for four weeks (sham).
The following 40 females were ovariectomized soon after the arrival (ovx) and allowed to recover for two weeks. Afterwards, they were gathered into four groups:
group 4 = ovariectomized untreated (ovx).
The following groups were treated, every other day, for two weeks as follows:
group 5 = frogs injected with 0.1 ml of saline solution (NaCl 0.64%) (ovx+SS);
group 5 = frogs injected with 17β-estradiol (0.1 μg/injection) in 0.1 ml of saline solution (ovx+E);
group 6 = frogs injected with testosterone (0.1 μg/injection) in 0.1 ml of saline solution (ovx+T);
Animals were sacrificed the day after the last injection.

Experiment 2 70 females captured during the pre-breeding period (medium-high levels of circulating sex steroids) and treated as in the experiment 1.

Experiment 3 70 females captured at the early-breeding period (high levels ofcirculating sex steroids) and treated as in the experiment 1.

Experiment 4 75 females captured at the late-breeding period (low levels of circulating sex steroids) and gathered into five groups (15 animals/each group). They underwent to the following experimental design:
group 1 = frogs sacrificed soon after the arrival (con);
group 2 = untreated frogs kept in captivity for the whole duration of the experiment (two weeks) (capt);
The following 45 females were treated, every other day, for two weeks as follows:
group 3 = frogs injected with 0.1 ml of saline solution (NaCl 0.64%) (SS);
group 4 = frogs injected with 17β-estradiol (0.1 μg/injection) in 0.1 ml of saline solution (E);
group 5 = frogs injected with testosterone (0.1 μg/injection) in 0.1 ml of saline solution (T).
The animals were sacrificed the day after the last injection.

Experiment 5 60 females captured during the recovery period and kept in captivity for two months (povx). After such a period in captivity circulating sex steroids were undetectable and animals can be considered physiologically gonadectomized. Animals were separated into four groups (15 animals/each group) and underwent the following experimental design:
group 1 = untreated frogs kept in captivity (capt);
The following females were treated, every other day, for two weeks as follows:
group 2 = frogs injected with 0.1 ml of saline solution (NaCl 0.64%) (povx+SS);
group 3 = frogs injected with 17β-estradiol (0.1 μg/injection) in 0.1 ml of saline solution (povx+E);
group 4 = frogs injected with testosterone (0.1 μg/injection) in 0.1 ml of saline solution (povx+T).
Animals were sacrificed the day after the last injection. The control group (con) of this experiment is the same control group of the experiment 1 (females captured during the recovery period).
Steroids were dissolved in a minimum amount of absolute ethyl ethanol prior to being diluted in saline solution at the required concentration (0.1 μg/injection). The average mortality rate was between 5% and 40%. The number of survived and analyzed specimens for each group (n) is reported in the legends.

17β-Estradiol binding proteins (EBP) and testosterone binding proteins (TBP) single point binding assay

Radioactive [2,4,6,7-^{3}H]17β-estradiol (^{3}H-E_2) (SA = 90–110 Ci/mmol) and [1,2,6,7-^{3}H]testosterone (^{3}H-T) (SA = 80–105 Ci/mmol) were purchased from Amersham Radiochemical Center (Amersham, Bucks, UK). Unlabeled steroids were obtained from Sigma (St. Louis, MO).

To remove endogenous steroids, plasma was charcoal stripped prior to analysis by incubation for 5 min at 4°C with dextran coated charcoal (0.5% charcoal, 0.05% Dextran T-70) pellet derived from a suspension equivalent to sample volume, followed by centrifugation (800 x g for 10 min at 4°C). Charcoal-stripped plasma was used diluted with TEMG (10 mM Tris-HCl, 1 mM 2-mercaptoethanol, 10% glycerol, v/v, pH 7.5) at a protein concentration of 1 mg/ml. For single point assay 200 µl of stripped plasma were incubated for 16 hr at 4°C with 20 nM of ^{3}H-E_2 and ^{3}H-T in absence or presence of 200-fold excess of unlabeled 17β-estradiol and testosterone respectively. Based on previous experiments, the concentration of 20 nM of labeled steroid was proved to be the concentration of saturation when the total protein concentration in the sample was 1mg/ml. After incubation 0.6 ml of dextran coated charcoal suspension was added. The mixture was vortexed and kept in ice for 5 min, followed by centrifugation at 800 x g for 10 min. The supernatant was decanted in counting vials with 5.0 ml Maxifluor scintillation fluid. Radioactivity was measured in a liquid scintillation counter (Packard 1600-CA) at 45% counting efficiency.

Sex Steroid Radioimmunoassay

A RIA method, modified for this species, was utilized to measure 17β-estradiol and androgens in plasma samples (d'Istria *et al.*, 1974). The following limits of detection were observed: 17β-estradiol, 3 pg (intraassay variation, 5%; interassay, 9%); androgens, 3 pg (Intraassay variation, 7%; interassay, 12%). Since the antiserum did not discriminate between testosterone and DHT, we used the term androgens. Antisera to sex steroids were kindly supplied by G.F. Bolelli of the Physiopathology of Reproduction Service, University of Bologna, Italy.

Follicle diameter measurement

Fixed ovaries were dissected under a microscope, and follicles were separated avoiding ruptures. Follicles were then gathered into classes of different diameter, using sieves of different size. Sieves were submerged into Ringer solution and subjected to gentle vibration. Follicles were sieved and counted. Atresic follicles, recognizable by their dark yellow-brown color, were first separated and counted. The number of follicles was expressed as percentage and the mean percentage was evaluated on ten ovaries for each month. By this method follide deformation, so frequent in classic histological sections, was avoided.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), using BSA as a standard.

Statistical analysis

Numerical data were analyzed by a one-way ANOVA method, followed by Duncan's multiple range test. Values were expressed as means ± SD.

RESULTS

Ovarian follicle classes frequency distribution

Follicles were gathered into the following classes according to the diameter: follicles with diameter < 0.4 mm, follicles with diameter comprised between 0.4–0.6, 0.6–1.0, 1.0–1.2, 1.2–1.4, 1.4–1.6, 1.6–1.8 mm, and follicles with diameter >1.8 mm. Follicle classes distribution throughout the year is shown in Fig. 1. Follicles with diameter less than 0.4 mm were previtellogenic and were abundant throughout the year, attaining the highest concentration in March. Follicles with diameter comprised between 0.4–0.6, 0.6–1.0, and 1.0–1.2 mm started increasing in March-April and reached the highest percentage in July, September and October, respectively. Follicles with diameter comprised between 1.2–1.4 mm reached the highest percentage in November. Follicles with diameter of 1.4–1.6 and 1.6–1.8 mm were most abundant in January and follicles with diameter > 1.8mm were most abundant in February. Atresic follicles were present throughout the year, and their percentage ranged between 0.7% in April and 23% in August (data not show).

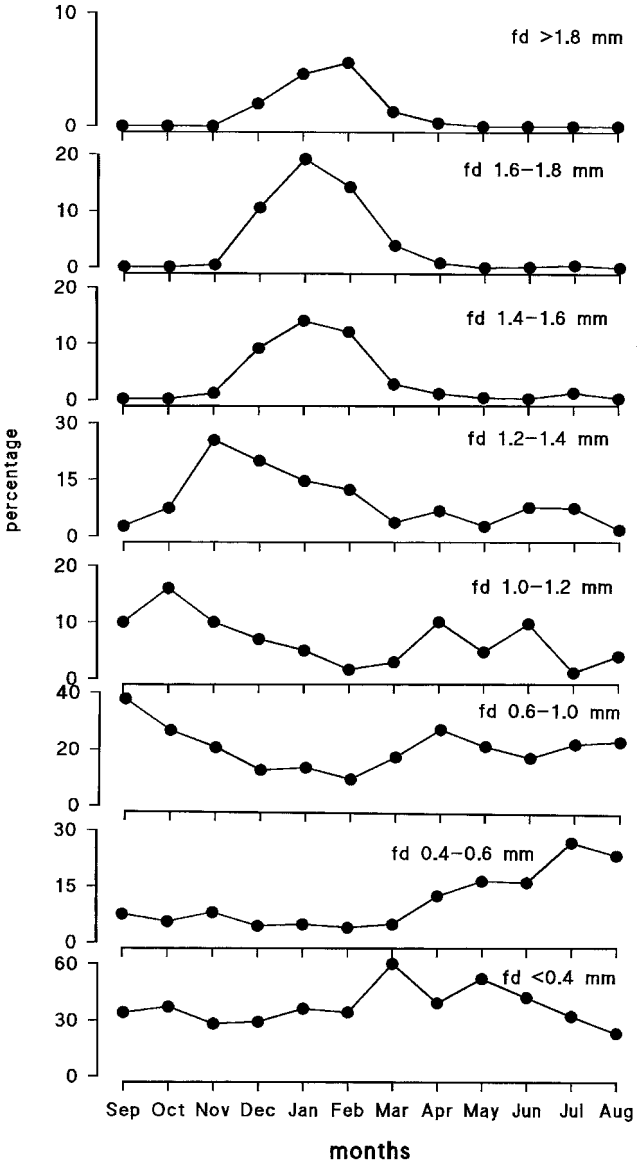

Fig. 1. Ovarian follicle distribution, according to their diameter, throughout the year. Values are reported as percentage calculated on ten ovaries each month. *F*d = follicle diameter.

17β-estradiol and androgen plasma levels

17β-estradiol and androgen plasma levels fluctuated throughout the reproductive cycle showing medium levels during the recovery period and the winter stasis, then increased during the pre-breeding period, attained the highest values during the early-breeding period, and dropped during the late-breeding period (Fig. 2). 17β-estradiol and androgen plasma levels were also measured two weeks after ovariectomy (experiments 1, 2, 3), and after two-months captivity (experiment 4), and their levels were undetectable.

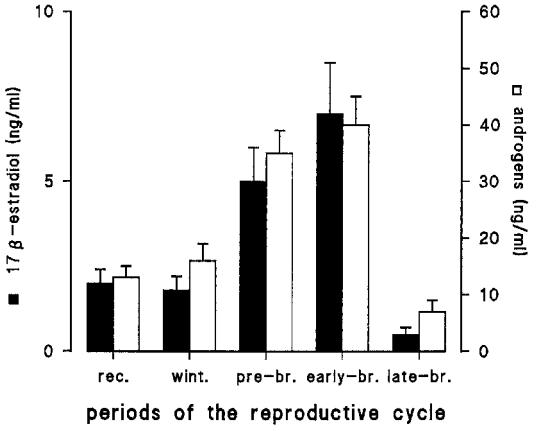

Fig. 2. 17β-Estradiol (■) and androgen (□) plasma levels in the female of the green frog *Rana esculenta* during the reproductive cycle. Values are means ± SD. rec.=recovery period; wint.=winter stasis; pre-br.=pre-breeding; early-br. = early-breeding; late-br.=late breeding.

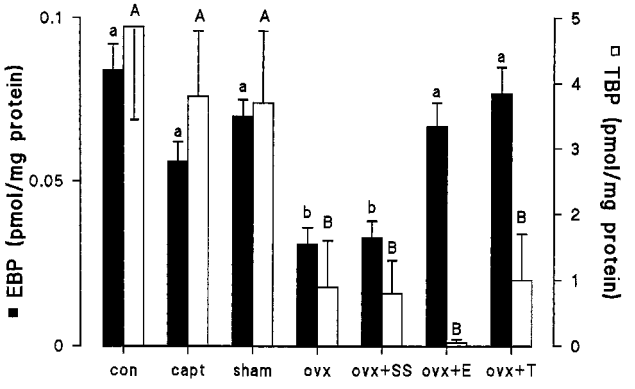

Fig. 3. (Experiment 1) EBP (■) and TBP (□) levels in the plasma of the female of *Rana esculenta* during the recovery period (November). con = control group (n=10); capt = untreated frogs kept in captivity (n=8); sham = sham operated (n=8); ovx = ovariectomized (n=7); ovx+SS = ovariectomized injected with saline solution (n=7); ovx+E= ovariectomized injected with 17β-estradiol (n=7); ovx+T =ovariectomized injected with testosterone (n=7). For details see Materials and Methods. Data for EBP and TBP were analyzed separately with Duncan's test and identified with capital and italic letters, respectively. Different letters over bars indicate significant differences at $P<0.05$. ANOVA analysis for EBP gave a "F" of 5,21 (significant at 5%) with 53 and 6 FD. For TBP the value of "F" was 6,13 (significant at 5%) with FD=53 and 6. Values are means ± SD.

Experiment 1

Females were ovariectomized during the recovery period, when 17β-estradiol and androgen plasma levels were medium (Fig.2). Ovariectomy brought about a significant decrease in both EBP and TBP. Treatment with 17β-estradiol and testosterone restored the EBP levels, but had no effect on TBP levels. Both captivity and sham operation did not have any effect on EBP and TBP, whose levels were only slightly

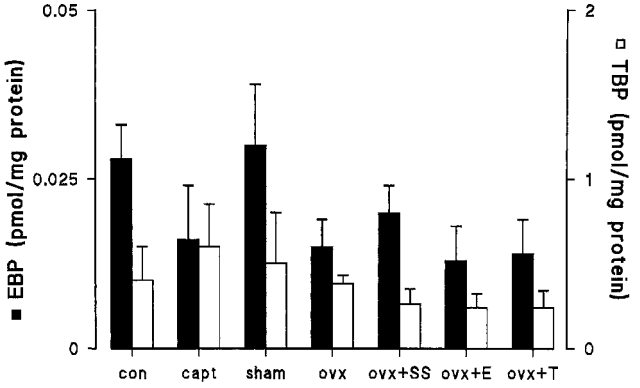

Fig. 4. (Experiment 2) EBP (■) and TBP (□) levels in the plasma of the female of *Rana esculenta* during the pre breeding period (February). con=control group (n=10); capt=untreated frogs kept in captivity (n=9); sham = sham operated (n=7); ovx = ovariectomized (n =8); ovx+SS=ovariectomized injected with saline solution (n=7); ovx+E=ovariectomized injected with 17β-estradiol (n=6); ovx+T =ovariectomized injected with testosterone (n=6). For details see Materials and Methods. Data for EBP and TBP were analyzed separately with Duncan's test. Values were not significantly different. ANOVA analysis for EBP gave a "F" of 1,51 (not significant) with 59 and 6 FD. For TBP the value of "F" was 2,68 (not significant) with 59 and 6 FD. Values are means ± SD.

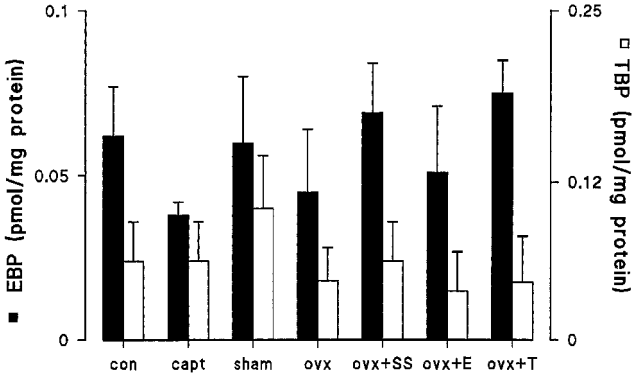

Fig. 5. (Experiment 3) EBP (■) and TBP (□) levels in the plasma of the female of *Rana esculenta* at the early breeding period (March). con=control group (n=9); capt = untreated frogs kept in captivity (n=8); sham=sham operated (n=6); ovx = ovariectomized (n=7); ovx+SS= ovariectomized injected with saline solution (n=6); ovx+E= ovariectomized injected with 17β-estradiol (n=6); ovx+T=ovariectomized injected with testosterone (n=6). For details see Materials and Methods. Values are means ± SD. Data for EBP and TBP were analyzed separately with Duncan's test. Values were not significantly different. ANOVA analysis for EBP gave a "F" of 2,25 (not significant) with 47 and 6 FD. For TBP the value of "F" was 2,91 (not significant) with 47 and 6 FD. Values are means ± SD.

lower than the control group. Such a decrease was not statistically significant (Fig.3).

Experiment 2

Females were ovariectomized during the pre-breeding period, when the levels of circulating 17β-estradiol and androgens were medium-high (Fig.2). Ovariectomy had no significant effect on EBP and TBP levels. Neither 17β-estradiol nor testosterone treatment caused any significant change in EBP and TBP levels. EBP and TBP levels in captivity and sham operated females were similar to the control group (Fig.4).

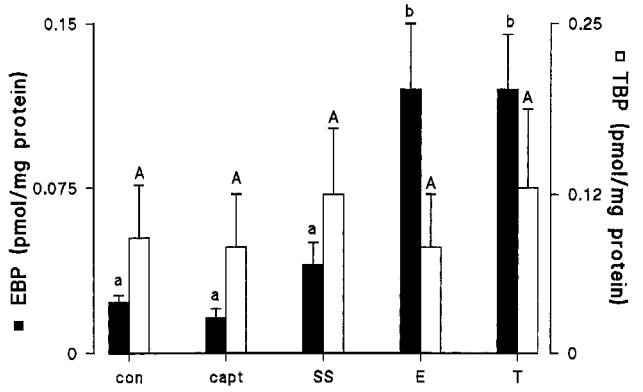

Fig. 6. (Experiment 4) EBP (■) and TBP (□) levels in the plasma of the female of *Rana esculenta* at the late breeding period (May). con=control group (n=15); capt=untreated frogs kept in captivity (n=12); SS = frogs injected with saline solution (n=10); E=frogs injected with 17β-estradiol (n=10); T=frogs injected with testosterone (n=10). For details see Materials and Methods. Data for EBP and TBP were analyzed separately with Duncan's test and identified with capital and italic letters, respectively. Different letters over bars indicate significant differences at P<0.05. ANOVA analysis for EBP gave a "F" of 5,73 (significant at 5%) with 56 and 4 FD For TBP the value of "F" was 1,38 (not significant) with 56 and 4 FD. Values are means ± SD.

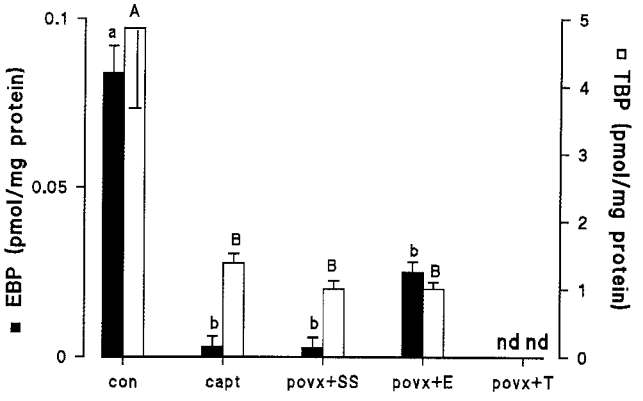

Fig. 7. (Experiment 5) EBP (■) and TBP (□) levels in the plasma of the female of *Rana esculenta* kept in captivity for two months. Con = control group (n=15); capt = untreated frogs kept in captivity (n=8); povx+SS=frogs injected with saline solution (n=8); povx+E=frogs injected with 17β-estradiol (n=6); povx+T=frogs injected with testosterone (n=7). For details see Materials and Methods. Data for EBP and TBP were analyzed separately with Duncan's test and identified with capital and italic letters, respectively. Different letters over bars indicate significant differences at P<0.05. ANOVA analysis for EBP gave a "F" of 4,15 (significant at 5%) with 43 and 4 FD. Values are means ± SD. For TBP the value of "F" was 5,63 (significant at 5%) with FD= 43 and 4. Values are means ± SD.

Experiment 3

Females were ovariectomized at the early-breeding period, when the circulating levels of 17β-estradiol and androgens were high (Fig.2). Neither ovariectomy, nor steroid treatment had any significant effect on EBP and TBP levels with respect to the control group. In ovariectomized and sham operated females EBP and TBP levels were not statistically different from the control group (Fig.5).

Experiment 4

Since circulating levels of 17β-estradiol and androgens were low in females captured during the late breeding period (Fig.2), steroid treatment was carried out on intact females. Captivity did not affect neither EBP nor TBP levels. The hormonal treatment increased EBP levels of 3-4 folds with respect to the control group. No effect was registered on TBP levels (Fig.6).

Experiment 5

Females were captured during the recovery period and kept in captivity for two months. After such a period in captivity plasma sex steroids were undetectable and animals could be considered physiologically castrated. Captivity caused a significant decrease of both EBP and TBP, whose levels could not be restored by hormonal treatment (Fig.7).

DISCUSSION

This study shows that, in *Rana esculenta*, both 17β-estradiol and testosterone affected plasma EBP binding capacity and that the effect of the hormonal treatment changed according to the period of the reproductive cycle it was carried out.

We have already reported the presence of SSBPs, binding 17β-estradiol and testosterone, EBP and TBP respectively, in the plasma of the female of *Rana esculenta*. EBP and TBP binding capacity changed throughout the year, although only EBP fluctuations were related to the reproductive cycle, an indication that EBP would play a physiological role in this species (Paolucci and Di Fiore, 1994). Data presented in this paper reinforce this view. Indeed, we have observed in this study that EBP, but not TBP were affected by hormonal treatment. Ovariectomy carried out during the recovery period, resulted in a decrease of both EBP and TBP. While TBP binding capacity could not be restored by hormonal treatment, EBP binding capacity increased almost to the level of the control group. These results are different from those obtained in mammals, where SSBPs are not affected by castration (von Shoultz and Carlstrom, 1989) and androgens seem to play an inhibitory action on SSBP levels in vivo (Toscano *et al*., 1992; Koritnik and Marschke, 1986; Kottler *et al*., 1988; 1990;). In the only other lower vertebrate studied so far, the trout, androgens do

not affect SSBP plasma levels, a result also confirmed by in vitro observations (Foucher *et al.*, 1991). On the contrary, in *Rana esculenta* testosterone affected EBP binding capacity. Since our previous studies show that in *Rana esculenta* circulating sex steroid changes paralleled EBP fluctuations throughout the reproductive cycle, with the highest levels of EBP coinciding with the resumption of reproductive activity (Paolucci and Di Fiore, 1994), it is plausible that both 17β-estradiol and testosterone sustain EBP concentration in the female of this species, characterized by, as with other lower vertebrates, very high levels of circulating androgens (Delrio *et al.*, 1979; Paolucci *et al.*, 1990; Stuab and De Beer, 1997). Whether or not androgens act as estrogens, after their aromatization to 17β-estradiol we do not know at present. However, it is interesting to note that in the female of *Rana esculenta* both androgen binding molecules and aromatase activity are present in the liver and their concentrations fluctuate throughout the reproductive cycle, attaining the highest values shortly before the breeding period (Di Fiore *et al.*, 1998). Since SSBP are believed to be mainly synthesized in the liver (Mercier-Bodard *et al.*, 1989), we can hypothesize a testosterone effect through its aromatization to 17β-estradiol. Indeed, estrogens have been reported to increase SSBP in human, heifer and trout (Toscano *et al.*, 1992; Lermite and Terqui, 1991; Foucher *et al.*, 1991), although they have little or not effect in monkeys (Koritnk and Marschke, 1986; Kottler *et al.*, 1988).

Although our previous observations (Paolucci and Di Fiore, 1994) indicate that TBP binding capacity fluctuations in the female of *Rana esculenta* did not show any relationship with the reproductive cycle, we decided to monitor the possible changes in TBP binding capacity following the sex steroid treatment, since androgen plasma levels are, in the female of *Rana esculenta*, higher than in the male (Paolucci *et al.*, 1990). Up to now a role for such high levels of circulating androgens in the females has not been demonstrated with certainty (Del Rio *et al.*, 1979). The lack of a trend related to the reproductive cycle in TBP fluctuations throughout the year in the female of *Rana esculenta* (Paolucci and Di Fiore, 1994), along with the inefficacy of the hormonal treatment on TBP level (present data), reinforce the view that androgens may constitute a reservoir for 17β-estradiol synthesis.

As reported here, in *Rana esculenta* castration caused a decrease in EBP and TBP and both 17β-estradiol and testosterone treatment increased EBP binding capacity only when circulating 17β-estradiol and androgens showed medium levels. Similar experiments, repeated during the pre-breeding and at an early stage of the breeding period, when levels of circulating sex steroids were respectively medium-high and high, showed that neither ovariectomy nor hormonal treatment affected EBP binding capacity. These results might be interpreted in light of the "memory effect". We can hypothesize that castration had no effect as a consequence of having been exposed to medium-high, high levels of circulating 17β-estradiol and androgens, whose effects lingered over a period of time evidently longer than that one during which our experiments were carried out. In *Rana esculenta* the hormonal treatment resulted in no effect when carried out during the pre-breeding and the early-breeding period. This behavior could be the consequence of having been exposed to medium-high, high levels of steroids, which would be responsible for an insensitivity to further hormonal exposure. Another reason to ascribe the ineffectiveness of the hormonal treatment of gonadectomized females might be the need of ovarian factor(s), which would work in synergy with steroids in eliciting the physiological response. This possibility seems to be supported by the observation that the hormonal treatment carried out during the recovery period on ovariectomized animals, resulted in a complete restoration of EBP binding capacity, while the hormonal treatment on non gonadectomized females, carried out at the end of the breeding period, brought about a 4 to 5 fold increase in EBP with respect to the control group, suggesting that the presence of the ovary enhances the physiological response.

In summary, we have shown that both 17β-estradiol and testosterone affect EBP but not TBP binding capacity in the female of *Rana esculenta*. However, the regulation of EBP changed according to the period of the reproductive cycle. Both 17β-estradiol and testosterone were ineffective when the circulating levels of 17β-estradiol and androgens were medium-high and high. On the contrary, the maximum effect was registered during the late-breeding period, when circulating 17β-estradiol and androgens were at their lowest point. Thus, our data indicate that 17β-estradiol and testosterone are involved in EBP regulation and the statement that they only play a marginal role must be carefully reviewed, especially for those species with a seasonal mode of reproduction.

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