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Possible roles for prostaglandins E_2 and $F_{2\alpha}$ in seasonal changes in ovarian steroidogenesis in the frog (*Rana esculenta*)

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Concentrations of PGE_2, $PGF_{2\alpha}$, androgens and oestradiol in plasma, and ovary weights were measured in the female frog, *Rana esculenta*, during the annual breeding cycle. Experiments were carried out *in vivo* to study the effects of PGE_2 and $PGF_{2\alpha}$ on plasma sex steroids during the following stages: pre-reproduction (April), reproduction (May), post-reproduction (June) and recovery (October). Experiments were performed *in vitro* during these stages to evaluate the effects of these two prostaglandins on the secretion of ovarian steroids. Concentrations of PGE_2 were low in plasma during winter hibernation, the reproduction and post-reproduction stages, whereas they were high during the pre-reproduction and recovery stages. PGE_2 treatment *in vivo* increased androgen secretion in April, whereas $PGF_{2\alpha}$ treatment increased oestradiol secretion in June and October. In experiments *in vitro*, PGE_2 increased androgen secretion and decreased oestradiol secretion from ovaries collected in April, whereas $PGF_{2\alpha}$ increased oestradiol secretion from ovaries collected in October. These results suggest that a seasonal increase in plasma PGE_2 may inhibit breeding activity, probably by stimulating ovarian androgen secretion, whereas, as previously reported, a seasonal increase in plasma $PGF_{2\alpha}$ may inhibit breeding, by stimulating ovarian oestradiol secretion.

Introduction

In mammals, ovarian steroidogenesis (Espey *et al.*, 1986; Sedrani and El-Banna, 1987; Dharmarajan *et al.*, 1989; Greenhalgh, 1990) is regulated by PGs of both F and E series. Prostaglandins are also implicated in the control of ovulation in the chicken (Shimada *et al.*, 1984) and of reproductive function in reptiles (Guillette *et al.*, 1984, 1990, 1991; Jones *et al.*, 1990).

Little is known, however, about the role of PGs in the reproductive processes of amphibians. In the anuran frog, *Rana esculenta* (Gobbetti *et al.*, 1990, 1991a; Gobbetti and Zerani, 1991), and in the urodele crested newt, *Triturus carnifex* (Gobbetti *et al.*, 1991b, c), seasonal changes in concentrations of $PGF_{2\alpha}$ in plasma have been observed to be related to the stages of the annual breeding cycle. The possibility that these seasonal changes in concentrations of the PGs may be related to reproductive function has not been investigated. A role for PGE_2 is suggested by a study *in vitro* on the male *Triturus carnifex* abdominal gland, where it was found to induce pheromonal activity (Gobbetti and Zerani, 1992a).

The objective of this study was to establish whether in the female frog *Rana esculenta* there are seasonal changes in the concentration of PGE_2 in plasma and how this relates to seasonal changes in $PGF_{2\alpha}$. A further objective was to determine *in vivo* and *in vitro* the effects of PGE_2 and $PGF_{2\alpha}$ on the secretion of ovarian steroids at different stages of the annual breeding cycle.

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Materials and Methods

Animals

Adult female frogs were collected in Umbria, Italy, from a pond 870 m above sea level. The frogs breed in May (reproductive stage), when the temperature increases and enter a post-reproductive phase in the summer. Gonad recrudescence is initiated in mid-summer, and continues into the autumn (recovery stage). The animals hibernate during the cold months of winter to emerge when the temperature increases in the next spring. At the beginning of spring, the frogs return to the pond (pre-reproductive stage).

Observations on free-living and captive animals

Fifteen adult female frogs, *Rana esculenta*, were collected every month during 1991. Immediately after capture, they were anaesthetized with 3-aminobenzoic acid ethyl ester (Tricaine: Sigma Chemical Co., St Louis, MO), weighed, and a blood sample was taken through a heparinized microtube inserted into the heart. Individual blood samples (600 μl) were collected into chilled tubes containing acetylsalicylic acid (Aspirin; Sigma) and EDTA (5 μg and 7 μg ml^{-1} of blood, respectively) (Gobbetti *et al.*, 1990). After centrifugation (1500 ***g*** for 10 min), plasma samples were stored at −20°C. Ovaries were removed and weighed. In April, May, June and October, frogs were captured and blood samples were taken in the field (control 1), while a further 15 animals were transferred to laboratory aquaria, kept under natural lighting and temperatures and fed *ad libitum* to

study the effects of captivity on concentrations of hormones in plasma. A week later, blood samples were taken from the animals in captivity, and at the same time blood samples were taken from 15 animals in the field (control 2).

Experiments in vivo

In April, May, June and October, 84 female frogs were captured each month, transferred to laboratory aquaria and maintained under natural lighting and temperature. The frogs collected in May had not ovulated. One week later, the animals were assigned to four groups: (a) 21 frogs received a single s.c. injection of 20 ng PGE_2 (Sigma) g^{-1} body weight dissolved in 100 µl amphibian saline (0.64% w/v NaCl solution); (b) 21 frogs received a single s.c. injection of 15 ng $PGF_{2\alpha}$ (Sigma) g^{-1} body weight dissolved in 100 µl amphibian saline; (c) 21 frogs received a single s.c. injection of 100 µl amphibian saline only; (d) 21 untreated control animals. Blood samples were taken from seven of the 21 frogs in each group at 6, 18 and 36 h after treatment. After centrifugation (1500 *g* for 10 min), plasma samples were stored at −20°C. Blood samples were taken from a further batch of seven untreated animals at the beginning of the experiments (controls, time 0). The times of treatment and the minimum effective doses of PGE_2 and $PGF_{2\alpha}$ were chosen after preliminary experiments (data not shown).

Experiments in vitro

In April, May, June and October, seven female frogs were captured each month and transferred to laboratory aquaria. The frogs collected in May had not ovulated. One week later, the animals were decapitated, the ovaries rapidly removed and placed in cold Dulbecco's modified Eagle medium (DME; Sigma) containing 10 mmol Hepes l^{-1}, 1 mg Penicillin G ml^{-1}, and 2 mg streptomycin ml^{-1}. Each month, the ovary from one animal was divided into equal sized fragments, pooled and equally distributed over 12 incubation wells. Multiwell tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ) were used. Each set of wells was divided into three experimental groups, each consisting of four wells. The experimental groups were: (a) ovarian tissue incubated with DME alone; (b) ovarian tissue incubated with DME plus PGE_2 (75 ng); (c) ovarian tissue incubated with DME plus $PGF_{2\alpha}$ (75 ng). The final volume of each well was 1 ml. Culture plates were wrapped in aluminium foil and incubated in a shaking water bath (19°C), at 30 revolutions min^{-1}. Incubation medium was removed from separate wells after 25, 50, 100 and 200 min of incubation. The incubation medium samples were stored at −20°C until hormone assays were performed; ovarian tissues were homogenized in amphibian saline, and protein contents were determined using a commercial kit (Bio-Rad, Richmond, CA). The experiment was repeated with incubation media without ovarian tissue. The experiment was repeated seven times for each month. The incubation conditions and the minimum effective doses of PGE_2 and $PGF_{2\alpha}$ were determined in preliminary experiments (data not shown).

Determination of PGE_2, $PGF_{2\alpha}$, androgens and oestradiol

Concentrations of PGE_2 and $PGF_{2\alpha}$ were measured by radioimmunoassay in plasma and incubation media as described by Gobbetti *et al.* (1990) and Gobbetti and Zerani (1992a), respectively. Determinations were carried out on duplicate plasma samples (100 µl) and in incubation media (500 µl) extracted with 10 volumes of diethyl ether for 5 min. After centrifugation (1000 *g* for 5 min), organic fractions were transferred into glass tubes and evaporated to dryness under nitrogen. Extracts were resuspended in 100 µl assay buffer before assay. The recoveries of added labelled PGE_2 and $PGF_{2\alpha}$ were 86.9 ± 0.81% and 85.3 ± 0.71%, respectively (means of 10 determinations ± SD). Standard curves, for the two prostaglandins in buffer and extracted incubation medium were parallel.

Concentrations of androgens, oestradiol in plasma and incubation media were measured by radioimmunoassay as described by d'Istria *et al.* (1974).

Intra- and interassay coefficients of variation and minimum detectable doses were PGE_2, 6.5%, 12%, 18 pg; $PGF_{2\alpha}$, 5%, 11.5%, 17 pg; androgens, 6%, 9%, 9.0 pg; oestradiol, 4%, 9%, 8.5 pg, respectively. The $PGF_{2\alpha}$, testosterone and oestradiol antisera were provided by G. F. Bolelli and F. Franceschetti (CNR-Physiopathology of Reproduction Service, University of Bologna) and the PGE_2 antiserum was purchased from Cayman Chemical (Ann Arbor, MI). Testosterone was not separated from 5α-dihydrotestosterone and, therefore, as the antiserum used is not specific, the data are expressed as total androgens. Tritiated PGE_2, $PGF_{2\alpha}$, testosterone and oestradiol were purchased from Amersham International (Buckinghamshire), non-radioactive PGE_2, $PGF_{2\alpha}$, testosterone and oestradiol from Sigma.

Statistical analysis

An analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan, 1955; Sokal and Rohlf, 1981) was used to analyse the data. Correlation coefficients were calculated as described by Scossiroli and Palenzona (1979). Data on ovary weights are shown as the gonadosomatic index (GSI: gonad weight:body weight).

Results

Seasonal changes in concentrations of plasma prostaglandins, gonadal steroids and GSI

The concentration of plasma PGE_2 was low in November, December and January, increased in February ($P < 0.01$) and remained high until April; it then fell in May ($P < 0.01$), increasing again in July until October ($P < 0.01$) (Fig. 1). The concentration of plasma $PGF_{2\alpha}$ was high in January and February ($P < 0.01$), decreased from March to May ($P < 0.01$), then increased from June to October ($P < 0.01$), and decreased in November and December ($P < 0.01$) (Fig. 1). Androgen concentrations were highest from January to April ($P < 0.01$), decreased in May ($P < 0.01$), remained low until August and increased from September to December (Fig. 1). Oestradiol concentrations in plasma showed a peak in June ($P < 0.01$) (Fig. 1). GSI decreased between May and June ($P < 0.01$), and then increased until October, and decreased between November and January. GSI began to increase again in February, reaching its

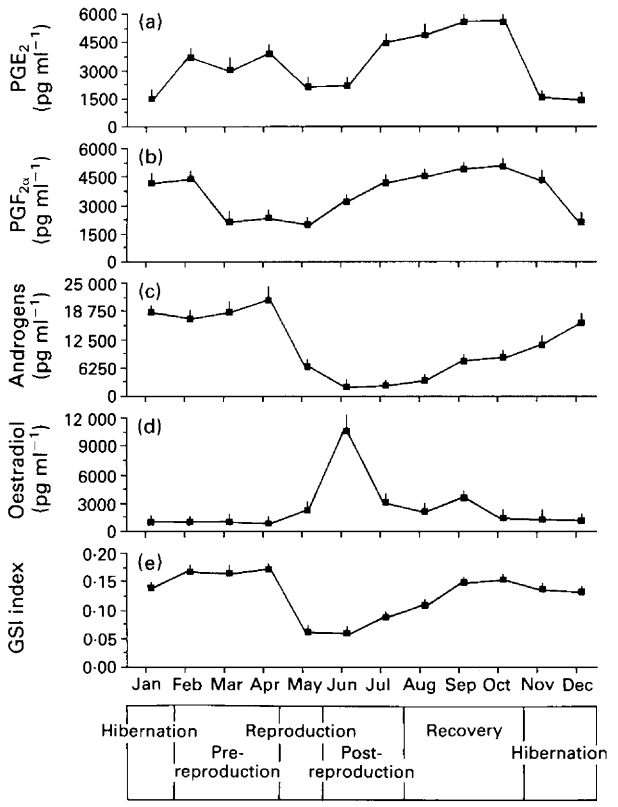

Fig. 1. Concentrations of plasma (a) PGE_2, (b) $PGF_{2\alpha}$, (c) androgens and (d) oestradiol, and (e) gonadosomatic index (GSI: ovary weight:body weight) during the annual breeding cycle in female frog, *Rana esculenta*. Each mean refers to 15 determinations ± SD.

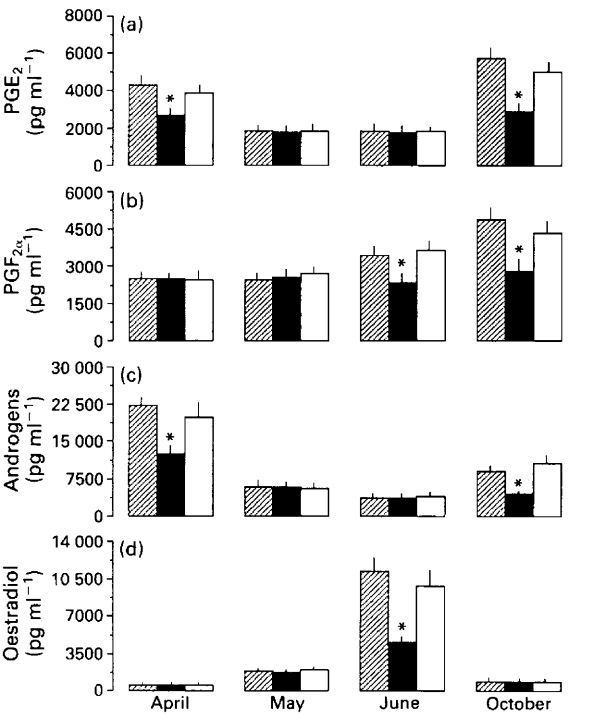

Fig. 2. Effects of one week in captivity on the concentrations of plasma (a) PGE_2, (b) $PGF_{2\alpha}$, (c) androgens and (d) oestradiol in female frog, *Rana esculenta*, during April (pre-reproduction), May (reproduction), June (post-reproduction), and October (recovery). Experimental groups: (▨) frogs captured in the field and blood samples taken at once (control 1); (■) blood samples taken from frogs after one week in captivity in laboratory; (□) frogs captured and blood samples taken at once in the field, one week after control 1 (control 2). Each mean refers to 15 determinations ± SD. *$P < 0.01$ versus control 1 and 2 (Duncan's multiple range test).

maximum value in spring ($P < 0.01$) (Fig. 1). Values of plasma PGE_2 were positively correlated with those of GSI ($r = 0.253$, df = 178, $P < 0.01$).

Effects of captivity on plasma prostaglandins and gonadal steroids

After one week in captivity, concentrations of plasma PGE_2 decreased in April and October ($P < 0.01$), concentrations of plasma $PGF_{2\alpha}$ decreased in June and October ($P < 0.01$), concentrations of plasma androgens decreased in April and October ($P < 0.01$), and concentrations of plasma oestradiol decreased in June ($P < 0.01$) (Fig. 2).

Experiments in vivo

Treatment with PGE_2 increased androgen concentrations in April, 6, 18 and 36 h after injection ($P < 0.01$) (Fig. 3). Treatment with $PGF_{2\alpha}$ increased oestradiol concentrations in June and October, 18 and 36 h after injection ($P < 0.01$) (Fig. 4).

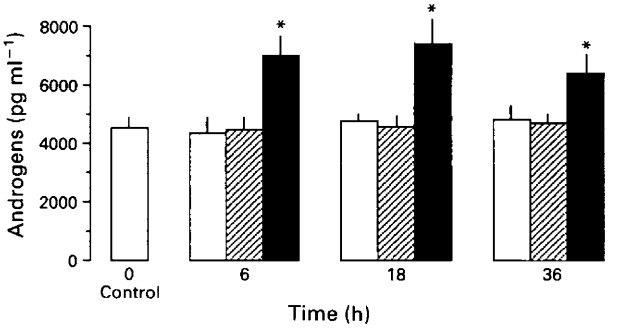

Fig. 3. Effects *in vivo* of 20 ng PGE_2 g^{-1} body weight on concentrations of plasma androgens in female frog, *Rana esculenta*, during April (pre-reproduction). Experimental groups: (□) untreated frogs; (▨) amphibian-saline-only injected frogs; (■) PGE_2 injected frogs. Each mean refers to seven determinations ± SD. *$P < 0.01$ versus untreated and amphibian-saline-only injected frogs (Duncan's multiple range test).

Experiments in vitro

The basal release of PGE_2 from the ovary was higher in April and October ($P < 0.01$) than in May and June. Values in October were higher ($P < 0.05$) than those of April (Fig. 5). The basal release of $PGF_{2\alpha}$ in June was lower ($P < 0.01$) than in other months (Fig. 5). The basal release of androgens was higher in April ($P < 0.01$) than in other months (Fig. 6). Values in October were higher ($P < 0.01$) than in May or June (Fig. 6).

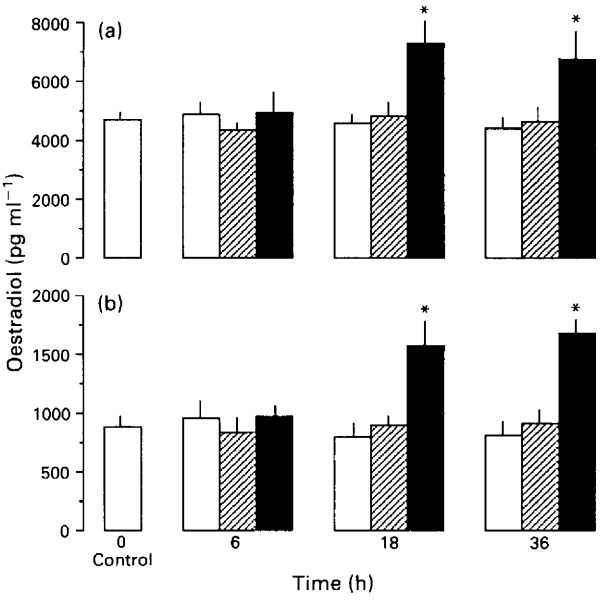

Fig. 4. Effects *in vivo* of 15 ng $PGF_{2\alpha}$ g^{-1} body weight on concentrations of plasma oestradiol in female frog, *Rana esculenta*, during (a) June (post-reproduction) and (b) October (recovery). Experimental groups: (□) untreated frogs; (▨) amphibian-saline-only injected frogs; (■) $PGF_{2\alpha}$ injected frogs. Each mean refers to seven determinations ± SD. *$P < 0.01$ versus untreated and amphibian-saline-only injected frogs (Duncan's multiple range test).

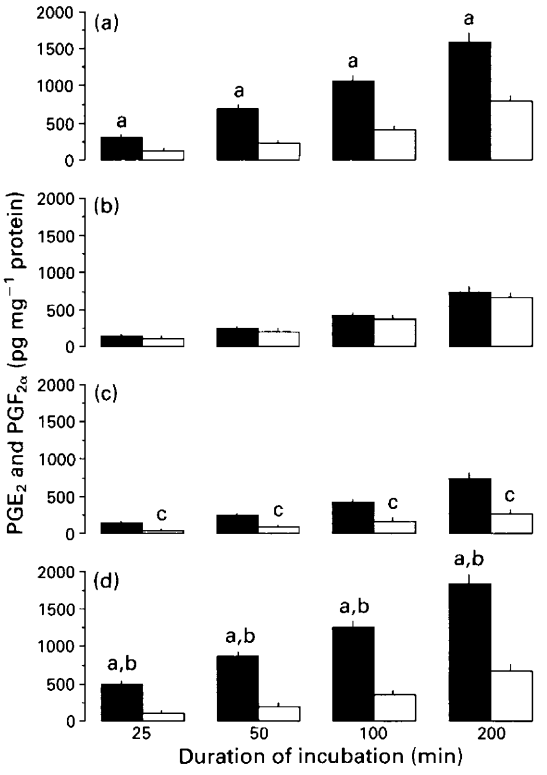

Fig. 5. Basal release *in vitro* of PGE_2 (■) and $PGF_{2\alpha}$ (□) from ovary of female frog, *Rana esculenta*, incubated (a) in April (pre-reproduction), (b) May (reproduction), (c) June (post-reproduction) and (d) October (recovery). Each mean refers to seven determinations ± SD. a, $P < 0.01$ versus same time in May and June; b, $P < 0.05$ versus same time April; c, $P < 0.01$ versus same time April, May and June (Duncan's multiple range test).

The basal concentration of oestradiol was lower in June ($P < 0.01$), than in other months (Fig. 7).

Treatment with PGE_2 increased the secretion of androgens in April after 50, 100 and 200 min ($P < 0.01$) (Fig. 6), and decreased oestradiol concentrations in April after 50, 100 and 200 min ($P < 0.01$) (Fig. 7). Treatment with $PGF_{2\alpha}$ increased oestradiol concentrations in October after 100 and 200 min ($P < 0.01$) (Fig. 7).

Hormones were not detected in media incubated without ovarian tissue (data not shown).

Discussion

This work reports, for the first time, the presence of plasma PGE_2 in the female frog, *Rana esculenta*, and showed that the concentrations of this prostaglandin are related to the annual reproductive cycle. Concentrations of PGE_2 were high in plasma during the pre-reproductive and recovery stages and low during the breeding season and winter hibernation. The seasonal changes in plasma $PGF_{2\alpha}$, androgens, oestradiol and ovarian weight confirm the findings of Gobbetti *et al.* (1990) and Zerani *et al.* (1991). However, Gobbetti *et al.* (1990) did not observe a peak in plasma oestradiol in June; this may be because this hormone peak is transient. Concentrations of plasma $PGF_{2\alpha}$ were high during the recovery stage of the breeding cycle; concentrations of androgens were high during the pre-reproduction stage, whereas concentration of oestradiol peaked during the post-reproductive stage.

The data *in vitro* on the basal release of PGE_2 and androgens from the ovary correlated with seasonal changes in plasma concentrations of these hormones. The highest amounts of PGE_2 and androgens released in ovarian incubations were observed in April and October. In contrast, the lowest release of $PGF_{2\alpha}$ and oestradiol from ovarian tissues was found in June, despite the fact that concentrations of plasma $PGF_{2\alpha}$ and oestradiol are high during this month. In previous studies it was suggested that the major source of these two hormones, during June, may be the interrenal gland (Gobbetti and Zerani, 1991).

The high concentrations of plasma PGE_2 and androgens in frogs, during the pre-reproductive phase of the breeding cycle, suggest a causal relationship between these two hormones. The ability of PGE_2 to stimulate androgen synthesis *in vivo* and *in vitro* during the pre-reproductive phase of the breeding cycle supports this hypothesis. On the contrary, in May, this phenomenon was not observed in experiments *in vivo* and *in vitro* with frogs that had not ovulated. It is suggested that PGE_2, by stimulating androgen synthesis, inhibits breeding during the pre-reproductive phase, when the environmental conditions are unfavourable for the survival of young. Androgens could act at ovarian, pituitary or hypothalamic levels, as has been proposed for oestradiol (Pavgi and Licht, 1989; Fasano *et al.*, 1991). In this context, it should be noted that during the breeding and post-reproductive phases of the annual cycle, concentrations of plasma PGE_2 and androgens were low, in agreement with the low basal release of androgens from the ovaries *in vitro*, and with the observations that treatment with PGE_2 failed to

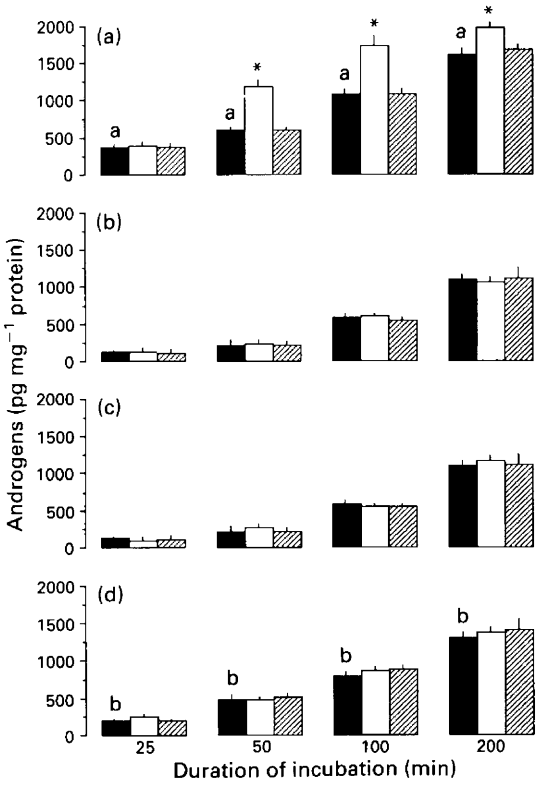

Fig. 6. Effects *in vitro* of 75 ng PGE_2 or 75 ng $PGF_{2\alpha}$ on androgen release from ovary of female frog, *Rana esculenta*, incubated in (a) April (pre-reproduction), (b) May (reproduction), (c) June (post-reproduction) and (d) October (recovery). Experimental groups: (■) ovary incubated with medium alone; (□) ovary incubated with PGE_2; (▨) ovary incubated with $PGF_{2\alpha}$. Each mean refers to seven determinations ± SD. a, $P < 0.01$ versus same time in May, June and October; b, $P < 0.01$ versus same time in May and June; *$P < 0.01$ versus same time with medium-alone (Duncan's multiple range test).

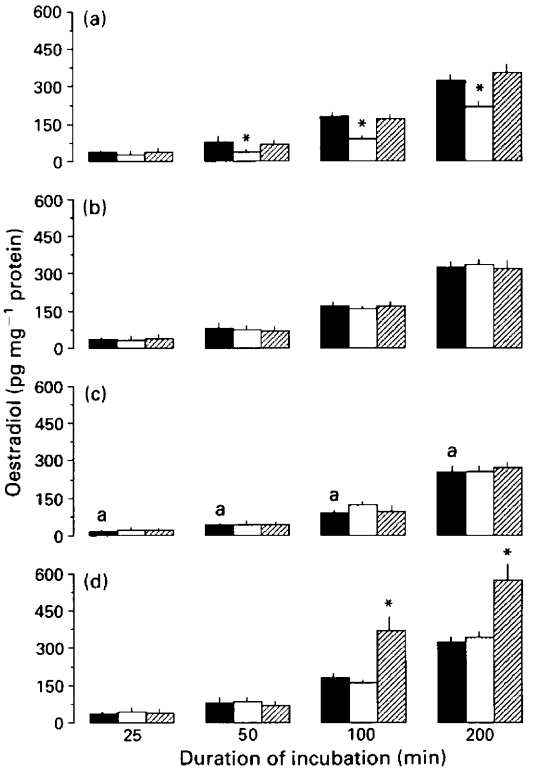

Fig. 7. Effects *in vitro* of 75 ng PGE_2 or 75 ng $PGF_{2\alpha}$ on oestradiol release from ovary of female frog, *Rana esculenta*, incubated in (a) April (pre-reproduction), (b) May (reproduction), (c) June (post-reproduction) and (d) October (recovery). Experimental groups: (■) ovary incubated with medium alone; (□) ovary incubated with PGE_2; (▨) ovary incubated with $PGF_{2\alpha}$. Each mean refers to seven determinations ± SD. a, $P < 0.01$ versus same time in April, May and October; *$P < 0.01$ versus same time with medium-alone (Duncan's multiple range test).

stimulate the release of androgens. The decrease in oestradiol secretion observed in April *in vitro*, after treatment with PGE_2, could be due to the enhanced synthesis of androgens.

The positive correlation between the seasonal change in concentration of plasma PGE_2 and ovarian weights is at present still unclear. In this context, it should be noted that the maturation of the amphibian oocytes is modulated by various hormones that control the amounts of 3',5'-cyclic monophosphate (cAMP) in the follicular cells. A receptor for PGE is present on the follicular cell membrane and mediates an increase in cAMP concentrations (Mori *et al.*, 1989).

$PGF_{2\alpha}$ injection *in vivo* in June and October increased the release of oestradiol, although experiments *in vitro* suggested that $PGF_{2\alpha}$ stimulated oestradiol secretion only from ovaries collected in October. The reason for this discrepancy may be that the target tissue for $PGF_{2\alpha}$ is the interrenal in June (Gobbetti and Zerani, 1991) and the ovary in October. During the post-reproductive phase the release of oestradiol from the interrenal glands by $PGF_{2\alpha}$ may be responsible for inhibiting ovarian function by exerting a negative feedback action at the ovarian, pituitary or hypothalamic levels (Pavgi and Licht, 1989; Fasano *et al.*, 1991). This phenomenon is an adaptive mechanism to prevent the presence of tadpoles in autumn, when the environmental conditions are unfavourable for their survival (Salthe and Mecham, 1974). The ability of $PGF_{2\alpha}$ to stimulate ovarian oestradiol synthesis in October is not adequately supported by evidence of increased circulating oestradiol concentrations at this time of year and the meaning of this phenomenon is unclear.

This study indicates that in *Rana esculenta* one week in captivity modified PGs and sex steroids. However, stress-related decreases in the secretion of these hormones were also reported in *Rana esculenta* (Paolucci *et al.*, 1991; Zerani *et al.*, 1991), in *Rana catesbeiana* (Licht *et al.*, 1983), in rough skinned newt, *Taricha granulosa* (Moore & Zoeller, 1985), and in crested newt, *Triturus carnifex* (Gobbetti *et al.*, 1991b, c). It is unlikely that the effects of PGs on steroidogenesis were merely a consequence of captivity as effects of PGs on steroidogenesis were observed irrespective of whether captivity affected steroidogenesis, in this and another amphibian species. Thus $PGF_{2\alpha}$ induced an increase in plasma oestradiol in October, when captivity did not affect the secretion of this steroid, in agreement with findings in male *Rana esculenta* (Gobbetti *et al.*, 1992a). In addition, in *Triturus carnifex*, treatment with PGE_2 decreased androgen secretion, which was modified by captivity (Gobbetti and Zerani, 1992b), whereas a progesterone secretion which was not modified by

captivity was increased after treatment with PGE_2 (Gobbetti *et al.*, 1992b).

In summary, this work indicates that seasonal changes in the concentration of these two PGs in blood may regulate the timing of breeding in *Rana esculenta* ensuring young are produced in the spring; PGE_2 inhibits reproductive function in April by stimulating androgen secretion, and $PGF_{2\alpha}$ may have a similar function in October by stimulating oestradiol secretion.

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