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Chicken GnRH-II and salmon GnRH effects on plasma and testicular androgen concentrations in the male frog, *Rana esculenta*, during the annual reproductive cycle

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In the frog, *Rana esculenta*, two molecular forms of GnRH, coeluting with chicken (c) GnRH-II and salmon (s) GnRH, have been detected using HPLC and radioimmunoassay. Mammalian (m) GnRH seems to be also present. In amphibians the role of cGnRH-II seems to be primarily the involvement in the regulation of neuroendocrine processes and, while the mGnRH has been postulated to act as a neurotransmitter and/or neuromodulator, the activity of sGnRH-like material has not been investigated. Therefore, we have treated the frogs with single or multiple injections of cGnRH-II or sGnRH (6 μg) or both peptides (6 μg of each) to detect differences in the response measured as testicular or plasma androgen (testosterone plus 5α-dihydrotestosterone) concentration during the annual reproductive cycle. The basal profile of testicular and plasma androgen shows that the spring peak disappeared in control animals given multiple injections and kept in short-term captivity. We show in the treatment with cGnRH-II and/or sGnRH that the effects of the peptides depend on the season, the experimental design, and the tissue in which androgen levels were measured. In particular, both peptides strongly stimulate androgen production during the autumn-winter period, the time of the greater response to the GnRHs when basal levels of steroids are highest.

Key words: GnRH; Brain; Pituitary; Testis; Androgens; Reproductive cycle; Amphibians; Frogs.

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Introduction

Gonadotropin-releasing hormone (GnRH) regulates reproductive activity in vertebrates through gonadotropin discharge from the pituitary. In addition, GnRH-like material may act directly at the gonadal level (Chieffi *et al.*, 1991; Pierantoni and Fasano, 1991).

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In the vertebrate brain several GnRH forms have been isolated so far and two or more forms appear to be contemporaneously present (Chieffi *et al.*, 1991; King and Millar, 1992; Currie *et al.*, 1992). In amphibians, studies based on HPLC and radioimmunoassay suggest that chicken (c) GnRH-II, salmon (s) GnRH-like material and mammalian (m) GnRH are present in salamander (*Taricha granulosa*), newt (*Ambystoma gracile*), clawed toad (*Xenopus laevis*) and frogs (*Rana catesbeiana*, *Rana esculenta*, *Rana pipiens*, *Rana ridibunda* and *Hyla regilla*) (Chieffi

et al., 1991; Conlon *et al.*, 1993). In *Rana catesbeiana*, metamorphic tadpoles are characterized by a GnRH form that is different from mGnRH which predominates in adult frogs (Branton *et al.*, 1982). In *Rana esculenta*, the retention time of immunoreactive peaks after HPLC purification indicates the presence of cGnRH-II and sGnRH-like peptides in the brain of adult animals (Cariello *et al.*, 1989). Their concentrations show parallel fluctuations in relation to the season and reproductive physiological stage. Chicken GnRH-II, but not sGnRH binding sites have been evidenced in the pituitary (Fasano *et al.*, 1993). In *Rana ridibunda* (Conlon *et al.*, 1993), a species closely related to *Rana esculenta*, cGnRH-II and mGnRH have been characterized. Chicken GnRH-II has been supposed to be primarily involved in the regulation of neuroendocrine processes while mGnRH has been postulated to act as a neurotransmitter and/or neuromodulator. Immunocytochemical evidence supporting the presence of mGnRH in *Rana esculenta* has also been detected (Licht *et al.*, 1994; Di Matteo *et al.*, 1996).

Studies on the seasonal sex steroid hormone profile in the male frog, *Rana esculenta*, indicate that treatments with a mGnRH agonist are ineffective in inducing changes of androgen concentrations both *in vivo* and *in vitro* (Pierantoni *et al.*, 1984a; 1984b; Di Matteo *et al.*, 1992) during summer. This was carried out at a selected time of the year but without consideration that other two GnRH molecular forms exist in the brain of this frog species. In the present study we have treated the male frog, *Rana esculenta*, with the two GnRH forms (e.g., cGnRH-II and/or sGnRH). The sGnRH has been used because its properties seem to be similar to those of the third not yet fully characterized peptide present in the amphibian brain (King and Millar, 1992). Single or multiple injections were given during the annual reproductive cycle to evaluate changes in the testicular and plasma androgen concentration.

Material and Methods

Male frogs, *Rana esculenta*, (n = 80), weight ranging between 25–30 *g*, were collected each month, the same day, during 1991 in the vicinity of Napoli. Forty animals per month were divided into 4 experimental groups (n = 10) and treated (24 hr after the capture) with a single injection (in the dorsal sac) of medium alone (amphibian Krebs Ringer bicarbonate buffer, KRB, pH 7.4), or with 6 μg of cGnRH-II or sGnRH. Each peptide (6 μg) was given in the combined treatment. The remaining 40 animals were divided into experimental groups as above described, but they received three injections subcutaneously (6 μg of each peptide or their combination), in the dorsal sac, on alternate days. Experiments were performed under controlled conditions of temperature and light typical of the month in which the treatments were carried out. After 2.5 hr from the last injection, frogs were anesthetized (MS222, Sigma) and blood was collected through a heparinized capillary inserted into the conus arteriosus. Plasma and testes were stored at −80°C until analysed for androgen concentration. Previous data (Pierantoni *et al.*, 1984a; 1984b; D'Antonio *et al.*, 1992) and preliminary experiments indicate that the maximal androgen response is achieved after 2.5 hr using GnRH doses between 1 and 20 μg (Fig. 1a, b).

Hormone assay

Testicular and plasma androgens were measured by radioimmunoassay as described elsewhere (Pierantoni *et al.*, 1984a; 1984b). Intra- and interassay coefficients of variations were 5% and 8%, respectively, and the sensitivity, calculated at ED_{90}, was 2 pg/tube. Since the antiserum (Dr. G. F. Bolelli, Bologna, Italy) raised against testosterone-3-CMO-BSA, crossreacts fully with testosterone and 5α-dihydrotestosterone, results are expressed as "androgens" (Fasano *et al.*, 1989, for further details).

Data presentation and statistics

Data have been calculated as mean ± SD and expressed in terms of weight of androgen/ml plasma or mg testis. Identical data were obtained if results were expressed as total androgen content in the testes. Significance of differences was evaluated by using one-way ANOVA followed by Duncan's test for multigroup comparisons and by two-way ANOVA with interaction when appropriate.

Results

Single-injection treatment

Testes (Fig. 2a). Control values showed that testicular androgens changed cyclically reaching the highest values during January ($p < 0.01$ vs December and February). A peak of androgens has been detected also during April ($p < 0.01$ vs May and March), while the lowest androgen concentrations have been detected during May–June period ($p < 0.01$).

Maximal values after GnRH treatments were detected during October–February period ($p < 0.01$ vs spring–summer). From

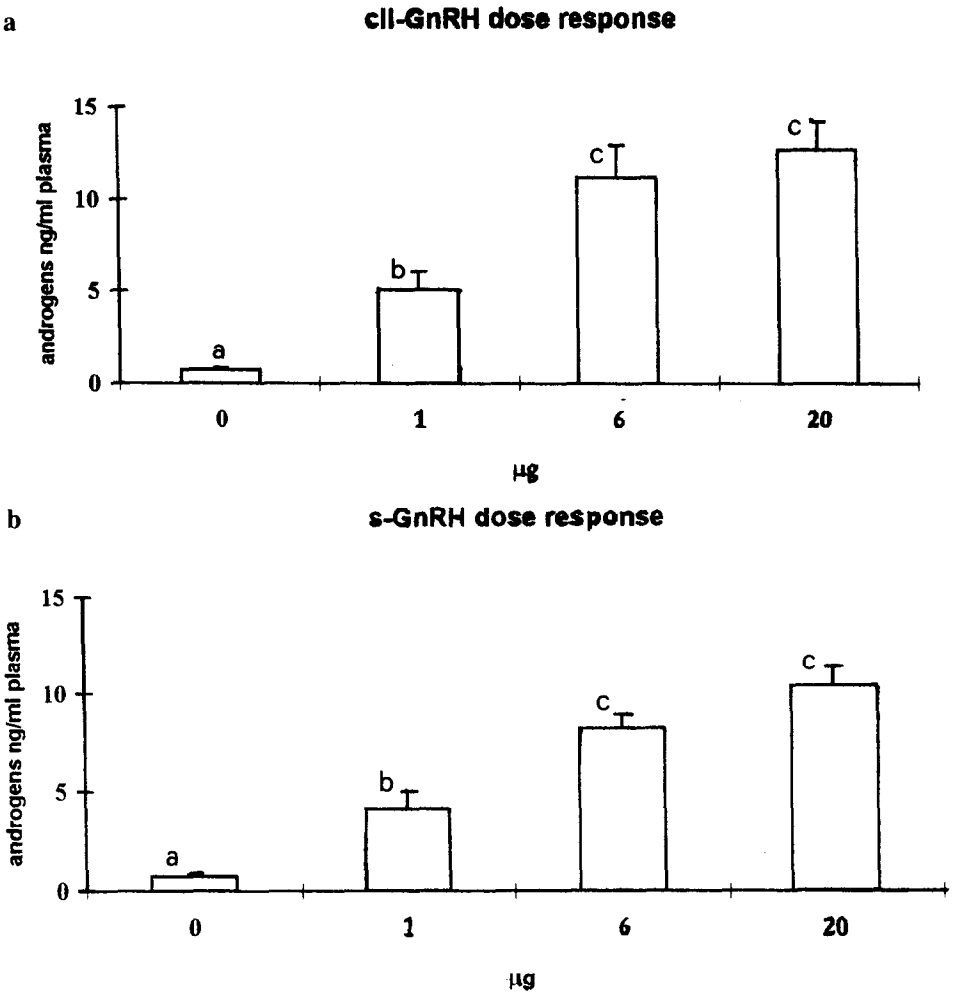

Fig. 1. Plasma androgen concentration in the frog, *Rana esculenta*, after chicken GnRH-II (a) or Salmon GnRH (b). Each observation represents the mean of 5 animals. Vertical bars indicate the standard deviations (SD). a versus b $p < 0.05$; a versus c $p < 0.01$.

October until May, no statistically evaluable differences were observed among the experimental groups, whether cGnRH-II or sGnRH or both were used.

It is interesting to note that the androgen response (e.g., the difference between treated and control groups) was reduced in January and April. During June sGnRH + cGnRH-II induced higher androgen values than those detected after cGnRH-II alone ($p < 0.05$). During the July and September period, different androgen values were observed following different treatments. Indeed, cGnRH-II was more potent than sGnRH. Finally, during September the combined treatment resulted in a significantly negative interaction ($p < 0.01$).

Plasma (Fig. 3a). Androgens increased in the plasma during December–January ($p < 0.01$), declining afterwards until May. During the spring–summer period, androgen levels remained at base-line values.

Maximal values after GnRH treatments (cGnRH-II or sGnRH) were detected during November–February period ($p < 0.01$) and maximal response (68 fold) was detected during November. Differential effects exerted by sGnRH, cGnRH-II or their combination were detected during January, May, July and September. Indeed, a positive interaction ($p < 0.05$) due to the two treatments was detected in January, and sGnRH induced a lower androgen increase than that elicited by the combined treatment in May and July ($p < 0.05$ at least). In September cGnRH-II induced higher androgen levels as compared with sGnRH or with the combined treatment ($p < 0.05$).

Multiple-injection treatment

Testes (Fig. 2b). Androgen values of control animals showed a general picture similar to control animals of the single-injection experiment. In fact, during winter a peak occurred in January ($p < 0.01$) and low androgen levels occurred during the spring–summer pe-

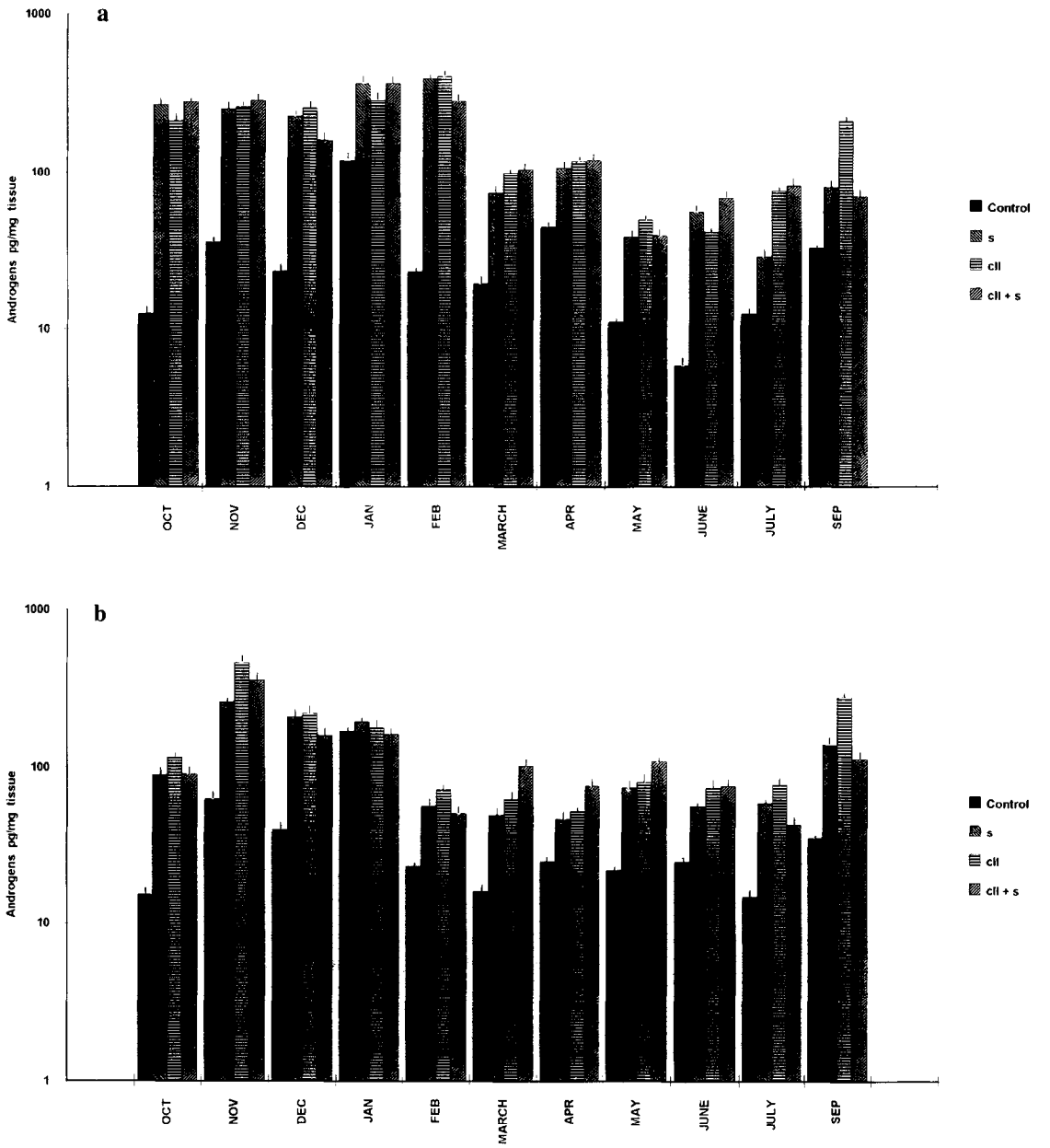

Fig. 2. Testicular androgen concentration in the frog, *Rana esculenta*, after a single (a) or multiple (b) injections of chicken GnRH-II and/or salmon GnRH. Vertical bars indicate standard deviations (SD).

riod. Conversely, April values did not indicate the presence of a spring peak. In general, both peptides or their combination were equally effective ($p < 0.05$ at least) in eliciting an androgen concentration increase, except in March, April, May, July, September and December. In March, April and May the combined treatment was more effective as compared with single peptide treatments, while it was less effective in July ($p < 0.05$). In September, cGnRH-II was the most active form ($p < 0.05$) and in December the combined treatment resulted in a negative interaction ($p < 0.01$). Interestingly, during January androgen levels did not show different values either using separately sGnRH or cGnRH-II or a combination as compared with the controls.

Plasma (Fig. 3b). Androgen values of control animals showed an increase ($p < 0.01$) during November, which was not evidenced in the controls of single injections treated animals. As for the results obtained in the other periods, they are in general similar to those measured in single-injection animals. Indeed, a peak occurred in January ($p < 0.01$) and low androgen levels occurred during spring–summer period. Highly variable effects were exerted by the two peptides or their combina-

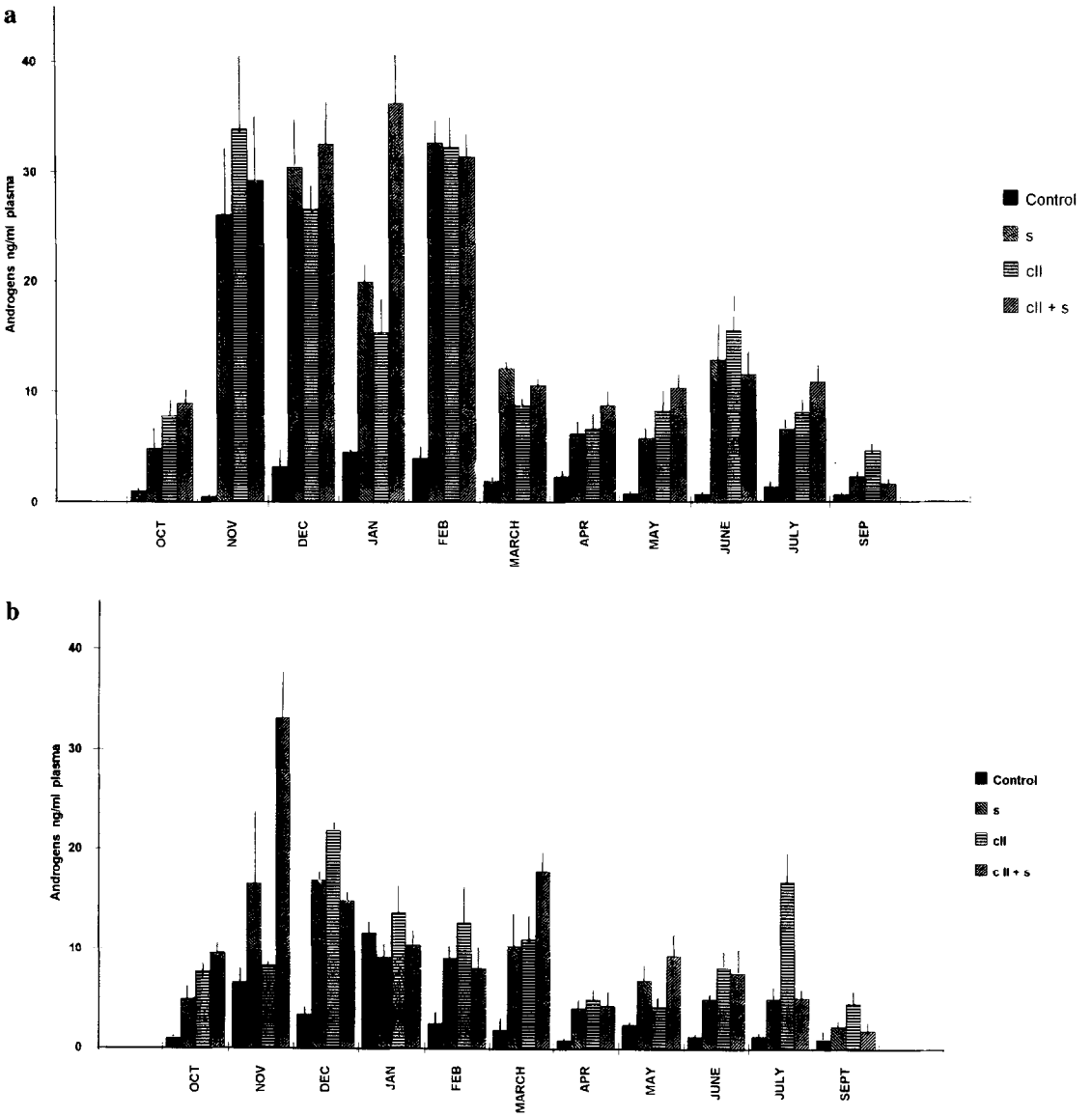

Fig. 3. Plasma androgen concentration in the frog, *Rana esculenta*, after a single (a) or multiple (b) injections of chicken GnRH-II and/or salmon GnRH. Vertical bars indicate standard deviations (SD).

tion in the annual reproductive cycle. During October, November and March the combined treatment exerted the major effect ($p < 0.05$ at least) whereas during December a negative interaction ($p < 0.05$) was evidenced. During July and September, the major effect was elicited by cGnRH-II ($p < 0.05$ at least). No significant differences between treated groups and control animals were detected in January. Conversely, cGnRH-II or sGnRH did not elicit an androgen increase during November.

Discussion

An interesting observation to emerge from the present data is that, in the annual profile of androgen variations, some differences exist between animals maintained in laboratory conditions and freshly captured animals. These differences are mainly concerned with the detection of a spring peak of androgens (Fasano *et al.*, 1989; 1992; Paolucci *et al.*, 1992) in testes of freshly captured animals compared with captive frogs. In captive animals, the disappearance of the spring peak may account for divergent results previously published (d'Istria *et al.*, 1974; Pierantoni *et al.*, 1984; Varriale *et al.*, 1986) which failed to find such a peak of the hormone. Previous data on the effects of capture or captivity (Rastogi and Chieffi, 1970; Minucci *et al.*, 1990) on reproductive activity of frogs demonstrated the inhibition of gonadal function in

such conditions. Present results support the above findings and suggest that short-term captivity mainly acts by depressing the androgen peak during spring.

In *Rana catesbeiana*, where gonadotropin evaluation has been carried out (Licht *et al.*, 1983), cGnRH-II and mGnRH were equipotent in stimulating FSH and LH release (Licht *et al.*, 1984). In *Rana esculenta* (D'Antonio *et al.*, 1992) we showed that cGnRH-II and mGnRH were equipotent in stimulating androgen production while cGnRH-II was more potent than sGnRH when used in a multiple-injection regimen. The combined treatment resulted in a negative interaction during the late autumn and early spring periods. This result is confirmed in the present study in December experiments. Moreover, we show that the effects of peptides are dependent on the period of the year in which the experiment was carried out and on the experimental design. Our results indicate that multiple GnRH injections during January did not induce an increase of androgen levels either in the testes or plasma. This was limited to a period of the year concomitant with the winter peak of androgens and it was not present after a single injection. It is not clear if this is a consequence of pituitary desensitization (a lack of pituitary response to GnRH due to the down regulation of receptor sites), which is proposed to be absent in frogs (Porter and Licht, 1985). As for the other periods investigated after GnRH stimulation, during the spring–summer period, androgen levels were lower than those detected during the autumn–winter period.

Previous results indicated the absence of response during late June–early July after stimulation with a mGnRH analog or with a homologous pituitary homogenate prepared from summer animals or ovine LH. Conversely, in *in vitro* experiments, February pituitary preparations gave maximal stimulation of androgen production in winter testes; significantly higher hormone levels, compared with controls, were obtained if pituitary preparations of other months were used (Pierantoni *et al.*, 1984b; Fasano *et al.*, 1988; Di Matteo *et al.*, 1992). It is important to note that results obtained using heterologous gonadotropin may be misleading and the crude pituitary homogenate may contain several inhibiting substances, as for example opioid peptides (Facchinetti *et al.*, 1993; Vallarino *et al.*, 1993).

The present experiments have been carried out all year around using cGnRH-II and sGnRH, both immunoreactive forms appear to be present in the median eminence in *Rana esculenta* (Di Meglio *et al.*, 1991; Di Matteo *et al.*, 1996). Therefore, it is possible to evaluate more accurately the testicular response after a GnRH challenge. Unfortunately, the direct measurement of circulating gonadotropins in *Rana esculenta* cannot be carried out because standards and specific antisera are not available. As a consequence, the evaluation of androgen levels is the only way to show changes of the hypothalamus-pituitary-gonadal activity. It is necessary to note that in a recent paper (D'Antonio *et al.*, 1992) we have shown that sGnRH does not affect directly androgen production by *Rana esculenta* testes, whereas cGnRH-II does. In this light, the interpretation of the present results related to cGnRH-II (but not to sGnRH) treatments may be complicated by the direct activity of cGnRH-II on the testis. However, the present results show that both peptides have a strong stimulating activity on androgen production during the autumn–winter period, the time of the greater response to the GnRHs when basal levels of the steroids are highest. The effect is also similar to that found using the mGnRH analog, both *in vivo* and *in vitro* (Pierantoni *et al.*, 1984a; 1984b; Di Matteo *et al.*, 1992). We conclude that seasonal changes of androgen production after GnRH treatments are due to the combination of several possibilities related to the fluctuations of gonadotropin content within the pituitary (Itoh *et al.*, 1990), changes of pituitary sensitivity to GnRH as a consequence of circulating estradiol (Polzonetti-Magni *et al.*, 1984; Varriale *et al.*, 1986; Pavgi and Licht, 1993) and/or brain-pituitary opioids (Facchinetti *et al.*, 1993; Vallarino *et al.*, 1993). A seasonal change of testicular sensitivity to gonadotropins (Pierantoni *et al.*, 1984a; 1984b) due to the presence of locally acting estradiol (Pierantoni *et al.*, 1986; Fasano *et al.*, 1991) and/or opioids should also be considered (Facchinetti *et al.*, 1993; Vallarino *et al.*, 1993). It is interesting to note that in reptiles (Tsai and Licht, 1993) the desensitizing effects of various forms of GnRH may be related to their clearance.

Finally, examining the effects detected in testes and in plasma after the three treatments, we show some differences in the efficacy of the two molecular forms used alone or in combination. Whether accumulation and release of androgens depends on the molecular variant used (D'Antonio *et al.*, 1992) and whether sGnRH and cGnRH-II act with different efficacy during the annual reproductive cycle need to be further elucidated.

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