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Seasonal-dependent effect of temperature on the response of adenylate cyclase to FSH stimulation in the oviparous lizard, *Podarcis sicula*

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Abstract

The study of environmental factors affecting vertebrate reproduction has long interested both developmental and evolutionary biologists. Although photoperiod has been considered to be an important environmental parameter for vertebrates such as birds, temperature is probably a primary external factor responsible for reproductive cyclicity in reptiles. In spite of the progress made in the understanding of reptilian reproductive strategies and adaptations, much remains to be learned about the interplay between endocrine physiological factors, such as hormones, and environmental parameters.

In this report, we have examined the effects of *in vivo* administered FSH on oocyte recruitment during the most significant periods of the reproductive cycle of the lizard, *Podarcis sicula*. The results show that when FSH is administered in proximity to the reproductive period, it stimulates oocyte growth and ovulation; when the hormone is administered at the beginning of the winter stasis it affects ovarian activity without inducing ovulation. Ovarian adenylate cyclase activity is moderately sensitive to *in vitro* FSH stimulation during the pre- and post-reproductive periods. The sensitivity to hormone stimulation increases significantly during the reproductive period and winter stasis.

We have also tested the hypothesis that environmental temperature affects the responsiveness of ovarian adenylate cyclase to FSH stimulation. For such a purpose, we exposed animals to 28 °C or 4 °C in different periods of the ovarian cycle. The results show that, whenever the temperature applied mimics the thermal regime of the coming season, adenylate cyclase sensitivity to FSH shifts towards levels that anticipate the natural responsiveness.

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Introduction

To gain reproductive success, organisms have adopted a bewildering array of adaptive mechanisms. Among them, seasonal reproduction is an important strategy that, by restricting breeding to periods characterised by favourable climatic conditions, ensures a high rate of offspring survival (Crews 1979). A fundamental role in this strategy is played by environmental parameters, such as photoperiod and temperature, that may act as ultimate factors capable of modulating reproductive functions (Randall *et al.* 1998). Local environmental parameters have particular effects on the reproduction of poikilotherm vertebrates, and especially on the species living at high latitudes or altitudes that are exposed to dramatic changes in temperature and photoperiod. In lower vertebrates, photoperiod may control rhythmic activities by acting as a 'zeitgeber' of melatonin synthesis in the pineal gland (Randall *et al.* 1998). Temperature, on the other hand, has been postulated to be the main non-photic factor capable of driving a number of seasonal events, including reproduction (Firth *et al.* 1989).

Although the correlation between endogenous control mechanisms and environmental parameters has been investigated to some extent (Bronson 1985, Hastings *et al.* 1985), little information is available on how gonadal activities are influenced by environmental factors. For this reason, we carried out a study using *Podarcis sicula*, the wall lizard living in the temperate lowlands of southern Europe, as the experimental model. In this species, reproduction occurs with a single ovulatory wave in spring-summer and is preceded by typical morpho-physiological modifications of the ovary. These include an increase in the number and size of follicles, and the onset of vitellogenesis. At the end of the ovulatory period, the ovary enters a fall-winter stasis that lasts until the next spring (Filosa 1973).

In mammals, ovarian activity is under the control of hypophyseal gonadotrophins (Pierce & Parson 1981). In

reptiles, studies on the effect of gonadotrophins are usually performed with heterologous hormone preparations, because no reptilian gonadotrophin is available at the present time. However, in a previous study on the ovary of *P. sicula*, we demonstrated the presence in the lizard genome of nucleotide sequences homologous to mammalian follicle-stimulating hormone (FSH) (Borrelli *et al.* 1997). As in other reptiles, heterologous FSH is capable of inducing oogonial proliferation, oocyte growth and differentiation, vitellogenesis (Limatola & Filosa 1989), estrogen production (Callard *et al.* 1976, Lance & Callard 1978) and ovulation (L Borrelli, R De Stasio, CM Motta, E Parisi & S Filosa, unpublished observation). We have previously demonstrated that the hormone acts by stimulating adenylate cyclase activity present in the membranes of follicular cells (Borrelli *et al.* 1997).

The aim of this report is to clarify the effects of endogenous and exogenous factors on gonadal activity in *Podarcis sicula*, during the most significant periods of the reproductive cycle. For such a purpose, we have studied, first, the effect of FSH on both oocyte recruitment and ovarian adenylate cyclase activity, and secondly, the influence of temperature on the responsiveness of follicular adenylate cyclase to FSH. The results obtained show that the ovarian responsiveness to FSH and the oogonial recruitment are high at the onset of reproduction, decline soon afterwards, and are resumed in late fall. In animals exposed to a temperature close to that of the forthcoming season, the sensitivity of the ovarian adenylate cyclase to FSH stimulation varies in a way that always anticipates the natural responsiveness.

Materials and Methods

Chemicals

ATP (specific activity 1·11 Tbq/mmol) and [2·8-^{3}H]cAMP (1·2 Tbq/mmol) were obtained from Dupont-NEN (Wilmington, DE, USA). Porcine FSH was from Sigma Chemicals (Poole, Dorset, UK). All other reagents were of the highest purity and were purchased from standard commercial sources.

Animals

Sexually mature female specimens of *Podarcis sicula* (6–8 cm, snout-vent length) collected by hand in the outskirts of Naples, were housed in groups of 6–15 animals in cages (60 × 40 × 35 cm) and were fed mealworms and water available *ad libitum*. Experiments were carried out for three consecutive years in March, June, September and December, i.e. in coincidence with the pre-reproductive, reproductive, and post-reproductive periods and with winter stasis (Filosa 1973). At sampling, the animals were anaesthetised with ether and killed by decapitation. Whole ovaries were processed for adenylate cyclase assay (see below) or fixed in ethanol:acetic acid (3:1 v/v) and processed for wax embedding. Prefollicular (preleptotene-early diplotene stages) and growing oocytes (diplotene stage surrounded by a follicular epithelium) were counted in serial sections.

Treatment of animals with FSH

Two doses of 125 μg/animal of porcine FSH (Sigma Chemicals), dissolved in saline, were administered i.p. every other day. The animals were killed five days after the beginning of the treatment. The hormonal dosage and time of treatment were chosen according to a previously published work (Limatola & Filosa 1989).

Exposure of animals to different temperatures

In order to realise conditions of photoperiod typical of each season, groups of six animals were housed in artificially lit rooms; the lights were controlled by a timer. We used photoperiods of 13 h light:11 h darkness in September, 8 h light:16 h darkness in December, 11 h light:13 h darkness in March and 16 h light:8 h darkness in June. The animals were exposed for seven days at 28 °C in a room equipped with a thermostat-regulated heater, and at 4 °C in a refrigerated room. These temperatures were chosen because they correspond to the maximum and minimum average temperature registered in the area of capture. The exposure time to the experimental temperatures was chosen after a number of trials demonstrating that maximal ovarian activity response could be obtained after 7 days.

Adenylate cyclase assay

Ovarian follicles, deprived of the external connectival theca under a dissection microscope, were homogenised in 5×10^{-3} M Tris–HCl buffer, pH 7·4, containing 0·25 M sucrose, using a Dounce homogeniser. The homogenate was centrifuged at 12 000 ***g*** for 15 min at 4 °C and the resulting pellet, containing the membrane fraction, was resuspended in the homogenisation buffer.

Adenylate cyclase activity was measured from the conversion of [α-^{32}P]ATP into cyclic AMP according to the method of Salomon *et al.* (1974). The reaction mixture contained: 0·03 M Tris–HCl, pH 7·4; $7{\cdot}5 \times 10^{-4}$ M EDTA; 0·01 M caffeine; 4×10^{-3} M $MgCl_2$; 5×10^{-4} M dithiothreitol; 0·02 M creatine phosphate; 1×10^{-4} M cAMP; 2 units creatine phosphokinase; 1×10^{-4} M ATP; 92·5–45·0 KBq [α -^{32}P]ATP (Dupont NEN). The reaction was carried out at 30 °C for 60 min with or without 10^{-6} M porcine FSH. Radioactivity was measured by liquid scintillation counting. Each data point was determined in duplicate and expressed as the

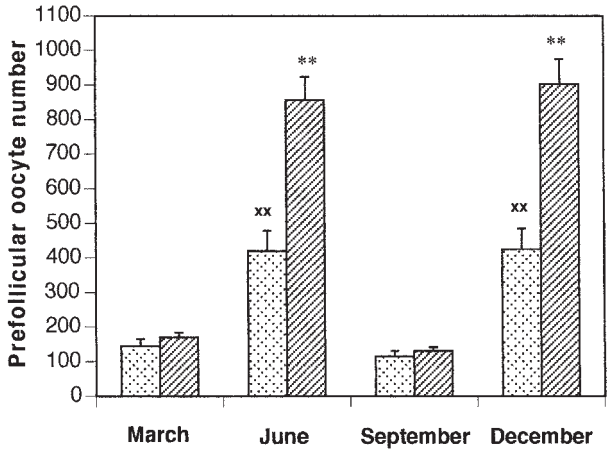

Figure 1 Changes in prefollicular oocyte number in control (cross-hatched bars) and FSH-treated (hatched bars) animals in the four most significant months of the ovarian cycle. Values shown are means ± S.D. of the oocyte number per animal. The value of F determined by ANOVA was 40·2 (F at $P=0{\cdot}01$ with 7 and 49 degrees of freedom was 3·41). The results of the Tukey test run at $P=0{\cdot}01$ showed that control groups in June and December were significantly different (xx) from the groups in March and September, and that the FSH-treated groups were significantly different (**) from the relative controls.

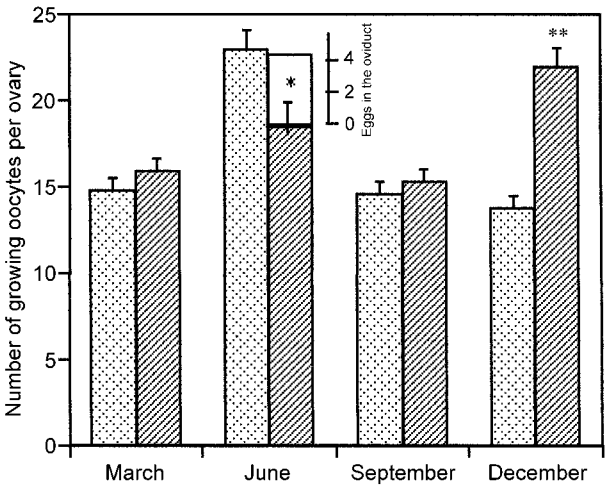

Figure 2 Number of growing oocytes in control (cross-hatched bars) and FSH-treated (hatched bars) animals in the four most significant months of the ovarian cycle. Values are shown as means ± S.D. The value of F determined with ANOVA was 17·5 (F at $P=0{\cdot}01$ with 7 and 37 degrees of freedom was 3·04). Significance at $P=0{\cdot}01$ (**) and P 0·05 (*) between FSH-treated groups and the corresponding controls was determined by Tukey test. The stacked bar (open bar) represents the number of eggs found in the oviduct of FSH-treated animals in June.

mean ± S.D. Protein content was analysed on membrane preparations according to Lowry *et al.* (1951).

Statistics

Statistical tests on experimental data sets were performed with programs contained in the Systat package, version 5.0 (SYSTAT Intelligent Software, Evanston, IL, USA).

Results

Effect of FSH on the ovary of P. sicula

In *P. sicula*, the number of prefollicular oocytes present in the ovary changes significantly throughout the year. The average number which is 144 in March, almost triples in June, in conjunction with the onset of the reproductive period, and decreases again to 110–120 in September. A new increase in the number of oocytes occurs in late fall: at this time, the ovary contains an average of 470 oocytes (Fig. 1).

As exogenous gonadotrophins have been shown to be fully bioactive in reptiles in which they are capable of inducing steroidogenesis (Callard *et al.* 1976, Lance & Callard 1978), we administered mammalian FSH to a group of female lizards during the four most significant periods of the reproductive cycle. The results reported in Fig.1 show that the oocyte number approximately doubles when the treatment is carried out in December and June, but remains unchanged in animals treated in September and March. The increase in prefollicular oocyte number observed in December both in control and FSH-treated females suggests the existence of an ovarian recrudescence before winter.

FSH also affects the number of growing oocytes in the gonad. Between September and March, the average number of growing oocytes is 14, and during the reproductive period (i.e. June) this number rises to 23. Following FSH treatment, significant changes in the oocyte number are observed in animals treated in December and June. In December, under the effect of FSH stimulation, the oocyte number rises to 22, which corresponds to the number usually found during the reproductive period. In June, the oocyte number apparently decreases to about 18, but at the same time an average of four mature eggs are found in the oviduct (Fig. 2). Hence, FSH administered during the reproductive period does not cause a further increase in the oocyte number, but it stimulates the differentiation of large oocytes into mature eggs.

Sensitivity of ovarian adenylate cyclase activity to gonadotrophin throughout the ovarian cycle

We have previously shown that FSH stimulates adenylate cyclase activity in follicular cell membranes and increases cAMP production in isolated ovaries in the lizard (Borrelli *et al.* 1997). For this reason, we decided to use FSH-

stimulated adenylate cyclase as a suitable biochemical marker of the ovarian activity in *Podarcis sicula*. We have not used gonadotrophin-releasing hormone (GnRH) to increase endogenous FSH artificially, because it has been reported that GnRH, besides its known secretagogue activity, may act directly on the ovary (Sirotkin *et al.* 1994, Zanagnolo *et al.* 1996, Yano *et al.* 1997).

The results in Table 1 show that, under environmental temperature conditions, the sensitivity of the lizard ovarian adenylate cyclase to FSH changes throughout the year. The results of one tailed paired *t*-tests show that adenylate cyclase activity is significantly enhanced by the presence of FSH in the reaction mixtures. The enzyme activity is moderately but still significantly sensitive to hormone in March ($t=5{\cdot}3$, $n=6$, $P=0003$) and in September ($t=8{\cdot}2$, $n=6$, $P<0001$), with 30–40% stimulation over the basal level. The sensitivity of adenylate cyclase to gonadotrophin increases in line with the reproductive season and during the winter stasis: FSH stimulation reaches a level of about 100% in June ($t=8{\cdot}4$, $n=7$, $P<0001$) and in December ($t=10{\cdot}7$, $n=6$, $P<0001$).

Effect of temperature on adenylate cyclase sensitivity to FSH stimulation

The effects of temperature treatments were tested by comparing the responsiveness of ovarian adenylate cyclase to FSH stimulation in groups of animals maintained at the experimental temperatures of 28 °C or 4 °C and at the seasonal temperatures. The results of these experiments, reported in Table 1, were subjected to an ANOVA statistical test with a covariance model, using temperature and period of the year as independent variables, the FSH-stimulated activity as dependent variable and the basal activity as covariate. Before analysing the data, homogeneity of slopes was tested with respect to categorial variables: the results proved the absence of significant interactions between covariate and temperature ($F=2{\cdot}2$, $P=0{\cdot}095$), and covariate and period ($F=2{\cdot}4$, $P=0{\cdot}096$). The analysis of the covariance model showed that the effect of the interaction of period and temperature on FSH-stimulated activity was significant ($F=3{\cdot}1$, $P=0{\cdot}01$). This prompted us to perform an ANOVA with Tukey post-hoc tests. In order to minimise the effects that might invalidate ANOVA, in this test we used the logarithmic transformation of the whole data set. The diagrams in Fig. 3 show the effect produced by FSH on ovarian adenylate cyclase of animals belonging to three different experimental groups kept at the environmental temperature, at 28 °C and at 4 °C. In March, the sensitivity of adenylate cyclase to FSH stimulation in animals kept at 28 °C is significantly higher ($F=5{\cdot}8$, $n=6$, $P=0{\cdot}013$) than the other two groups. In June, the sensitivity to hormone stimulation decreases in animals at 28 °C ($F=17$, $n=7$, $P<0{\cdot}01$), whereas the responsiveness to FSH is not significantly affected by a temperature of 28 °C applied in

Table 1 Basal and FSH-stimulated adenylate cyclase activity (expressed as pmoles cyclic AMP/mg protein) in the gonads of female lizards kept at environmental and experimental temperatures. The values are the means of adenylate cyclase activity ± S.D. measured in individual gonads from *n* animals

	March			**June**			**September**			**December**		
	Basal	+FSH	*n*	Basal	+FSH	*n*	Basal	+FSH	*n*	Basal	+FSH	*n*
Temperature												
Environmental	187·8 ± 89·1	247·5 ± 123·4	6	205·9 ± 50·4	431·7 ± 94·9	7	202·8 ± 50·4	291·0 ± 71·2	6	177·2 ± 58·1	351·3 ± 93·3	6
28 °C	134·2 ± 49·5	247·8 ± 78·2	6	191·1 ± 18·4	301·4 ± 25·0	7	179·8 ± 48·9	272·0 ± 55·5	6	133·3 ± 45·2	283·2 ± 43·5	6
4 °C	154·0 ± 45·3	216·6 ± 51·2	7	164·7 ± 70·9	396·4 ± 107·2	7	201·0 ± 29·5	397·7 ± 43·5	6	165·7 ± 29·3	248·3 ± 51·7	6

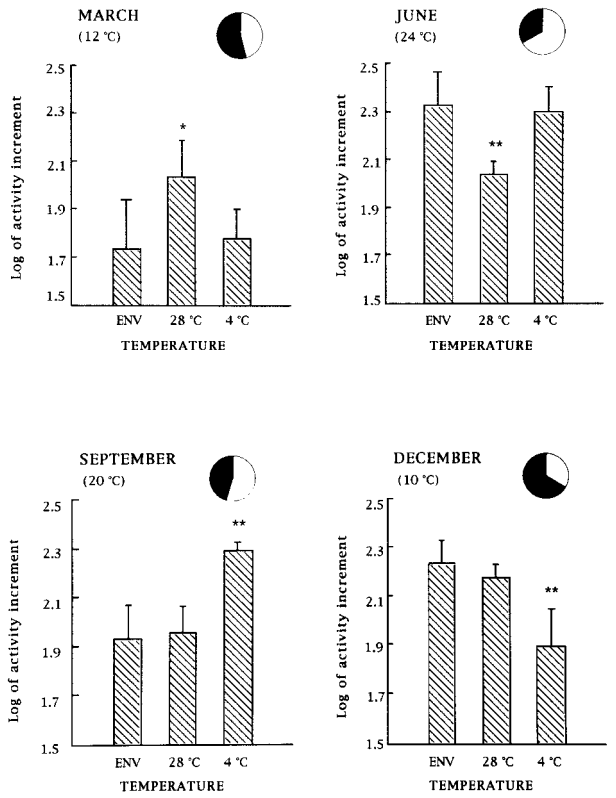

Figure 3 FSH-induced increments in ovarian adenylate cyclase activity in animals kept at experimental (28 °C and 4 °C) and environmental (Env) temperatures during the most significant months of the reproductive cycle. The activity increments produced by FSH, logged to the base 10, were calculated from the data reported in Table 1. Significance was assessed by ANOVA using Tukey one tailed test (** significant at 1% level; * significant at 5% level). The average seasonal temperatures and the natural photoperiods (pies) are shown at the top of each diagram.

September and December. The response to the low temperature (4 °C) shows an entirely different pattern: the temperature treatment results in no significant effect on the enzyme sensitivity to hormone stimulation in March and June, whereas in September the enzyme stimulation is significantly higher ($F=22{\cdot}7$, $n=6$, $P<0{\cdot}01$) in animals kept at 4 °C. Finally, in December, the enzyme sensitivity to FSH decreases in the group at 4 °C ($F=16{\cdot}4$, $n=6$, $P<0{\cdot}01$). It is noteworthy that a temperature of 28 °C in March and in June shifts the sensitivity of adenylate cyclase to FSH towards values typical of June and September respectively, while exposure to a temperature of 4 °C in September and December induces a condition typical of December and March respectively.

Discussion

In the present report, we describe the effect of FSH on the ovarian activity in *Podarcis sicula* and the influence of temperature on the responsiveness of adenylate cyclase to FSH stimulation. Our results demonstrate that the effect of the *in vivo* FSH treatment on the ovary is particularly evident in two periods of the ovarian cycle, i.e. in early summer, at the onset of the breeding period, and in early winter, when the ovary undergoes a resumption of oogonial recruitment in spite of the unfavourable environmental conditions. When the hormone is administered in June it brings about an increase in the number of prefollicular oocytes, and stimulates oocyte growth as well as egg deposition. In December, FSH treatment causes effects similar to those observed in June, but fails to induce egg deposition. The reproductive cycle is also characterised by changes in the responsiveness of adenylate cyclase activity to FSH: the periods of maximal sensitivity to hormone stimulation, one in June and another in December, coincide with the two peaks of ovarian activity. All these observations together suggest that, in the females of this species, a partial recrudescence of ovarian activity occurs in early winter. This phenomenon, described also for the male gonad (Angelini *et al.* 1986), can be explained by assuming that *P. sicula* originally had two seasonal ovulatory waves, one in the summer and another in the fall, similar to the lizards normally living in warm habitats (Angelini & Ghiara 1984). According to Arnold (1989), *P. sicula* was originally a warm-adapted species living in North Africa or the Middle East, and only later did it migrate to southern Europe. Our hypothesis is that when this species moved to a thermal regime with cold winters, it adapted to the new environmental conditions by suppressing the ovulatory wave in the fall, in order to leave a single reproductive episode in spring, a time suitable for maximal offspring survival.

The contrast between the bimodal pattern of ovarian activity and the pattern of the seasonal temperatures, which in southern Europe show a single peak in spring-summer, indicates that a complex interaction exists between endogenous control mechanisms and environmental temperature. For such a reason, we have studied the effect of temperature on the sensitivity of adenylate cyclase to FSH stimulation because the change in the activity of this enzyme seems to be correlated with the morphological modifications observed in the ovary during the reproductive cycle. In addition, the receptor–transducer–effector system constituting adenylate cyclase is a simpler model compared with the complexity of the whole ovary. Our data show that the sensitivity of ovarian adenylate cyclase to FSH may be cued by temperature according to a well-defined programme. If the applied temperature follows the trend of the natural thermal regime, the sensitivity of adenylate cyclase to FSH approaches the level typical of the coming season. Indeed, exposure to 28 °C in March and in June results in a sensitivity level to FSH that is shifted towards the values observed in June and in September respectively; whereas exposure to 4 °C in September and December induces a

condition typical, respectively, of December and March. The enzyme sensitivity does not vary if the temperatures applied do not agree with the trend of the natural thermal cycle, like 4 °C in March or June and 28 °C in September or December.

Our results suggest that temperature plays a very relevant role in the reproduction of *P. sicula*. However, this does not rule out the possibility that in *P. sicula*, as in other ectotherms, the annual reproductive cycle is regulated by the interplay between environmental temperature and photoperiod (Vivien-Roels 1985). It has been shown that the interaction of thermal and photoperiodic factors modulates the daily rhythm of melatonin production in reptilians (Firth & Kennaway 1989, Firth *et al.* 1989). In such a context, an interesting observation is that in frog retina, serotonin N-acetyl transferase activity, the key enzyme involved in the regulation of melatonin synthesis, is sensitive to differences in the environmental temperature (Valenciano *et al.* 1994). In this species, a nerve fibre connection has been demonstrated between the frontal organ, i.e. the intracranial component of the pineal complex, and skin (Guglielmotti *et al.* 1995).

In conclusion, the evidence presented here shows that gonadal processes are synchronised with the temperature variations through a mechanism that regulates ovarian activities, such as adenylate cyclase sensitivity to gonadotrophin stimulation. The anticipation of the natural thermal regime elicits a physiological response in the ovary that is typical of the coming season. The present data also support the hypothesis that remains of a second reproductive wave are still present in *P. sicula*. The suppression of this winter reproductive wave may be regarded as an adaptive strategy for offspring survival.

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