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Environmental influence on testicular MAP kinase (ERK1) activity in the frog *Rana esculenta*

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Summary

Recent studies suggest a role for ERK1 in the regulation of spermatogonial proliferation. In this report the frog *Rana esculenta*, a seasonal breeder, was used as a model to study the possible effect on ERK1 of photoperiod and temperature. Adult male *R. esculenta* were subjected to several combinations of light and temperature at different times of the year to elucidate the regulation of ERK1 testicular activity in the spermatogonial proliferation by these environmental factors.

Western blot analysis shows that under controlled experimental conditions an increase of temperature and photoperiod in November, characterized by a decrease in primary spermatogonial mitosis, induces ERK1 activity and spermatogonial proliferation, as confirmed using the proliferating cellular nuclear antigen (PCNA) as an early molecular marker. In contrast, a decrease in temperature and photoperiod in March, with an increase of primary spermatogonial mitosis, impairs ERK1 activity and spermatogonial proliferation.

In conclusion, our data clearly show for the first time in a non-mammalian vertebrate that the temperature and the photoperiod exert a role in the spermatogonial proliferation *via* ERK1 activity.

Key words: ERK1, spermatogenesis, photoperiod, proliferation, frog, *Rana esculenta*.

Introduction

The amphibians are the first tetrapods and therefore occupy a phylogenetic position of great interest. As cold-blooded vertebrates, amphibians are greatly susceptible to environmental fluctuations. In fact, most of the anurans and urodeles exhibit a markedly seasonal testicular cycle (circannual) (Lofts, 1974; Rastogi, 1976; Rastogi et al., 1978, 1983). Among environmental factors involved in the amphibian breeding cycle the most relevant are the photoperiod and the temperature. Studies on the influence of light demonstrated that photoperiod may potentially modify testicular activity in the frog *Rana esculenta* (Rastogi et al., 1976); in particular, it has been suggested that the light has only a permissive role in facilitating the temperature response that directly influences the annual testicular cycle in *R. esculenta* (Di Matteo et al., 1981). Temperature, on the other hand, has been shown to play an important role in synchronizing the different phases of the seasonal testicular cycle in several amphibian species (Rastogi et al., 1978).

Signal transduction mechanisms promoting mitosis of spermatogonia are poorly understood in vertebrate and invertebrate animals. Although little else is known of the early physiological consequences of this environmental cue on whole animals, downstream molecular pathways are demonstrably activated and are locally involved in initiating spermatogonial stem cell mitosis.

At least two signaling pathways are active; one is steroid based, the other is mitogen-activated protein kinase (MAPK) based. Recent evidence also suggests that these otherwise distinct pathways may interact in promoting mitosis of spermatogonial stem cells (Chieffi et al., 2000b, 2002).

MAPKs play a crucial role in signal transduction, mainly by activating gene transcription, including c-Myc, Ets1, Elk1 and c-Jun, *via* translocation into the nucleus (Karin, 1995; Waskieewicz and Cooper, 1995; Cobb, 1999). Among the members of this family the most extensively studied are p44 and p42 MAPK, also known as extracellular signal-regulated kinases (respectively ERK-1 and ERK-2), Jun amino-terminal kinase (JNK1/2) and p38. ERKs are expressed ubiquitously and closely related to the yeast protein kinases involved in pheromone induced mating (Nielsen et al., 1993). For example, in the frog *R. esculenta* and in the lizard *Podarcis s. sicula* testis different levels of ERK1/2 activity are present during the annual reproductive cycle (Chieffi et al., 2001, 2002); in addition, it has been shown that 17-β estradiol induces ERKs phoshorylation in frog (*R. esculenta*) (Chieffi et al., 2000b) and lizard (*P. s. sicula*) testis (Chieffi et al., 2002), and JNK1 has different activity in frog (*R. esculenta*) (Chieffi, 2003) and lizard *(P. s. sicula*) testis during the annual reproductive cycle (Chieffi et al., 1999).

The present work on *R. esculenta* (Amphibia, Anura) was undertaken to evaluate the influence of light and temperature, in different phases of the testicular cycle, on MAPK (ERK1) testicular activity. In this paper we present evidence that ERK1 testicular activity is also under temperature and photoperiodic control.

Marerials and methods

Animals

Adult male frogs *Rana esculenta* L. were captured in November and March (*N*=30/month) in the vicinity of Naples. Frogs under various treatments were maintained in plastic tanks and fed meal worms twice a week *ad libitum*. For each experiment animals were injected with 50 mg 100 ml^{-1} of colchicine 24 h before evaluating mitotic index. Animals were killed by decapitation under anaesthesia with MS222 (0.05% in aqueous solution, Sigma Chemical Co., St Louis, MO, USA), and the testes were removed immediately and stored at –80°C (*N*=30 testes/month) until processed for western blot analysis, or quickly prepared for histological examinations (*N*=30 testes/month). For light microscopy, testes were fixed in Bouin's fluid and embedded in paraffin by standard procedures. 5 μm sections were stained with Hematoxylin and Eosin.

Photo-thermal treatments

Frogs contained in plastic tanks were housed in photo-thermostatic chambers where varying combinations of photoperiods and temperatures were controlled with high precision (±5 min light; ±1°C) (Rastogi et al., 1978).

Experiment A: in November 30 frogs were housed for 2 weeks under a 12 h:12 h (light:dark) photoperiod and a temperature of 22°C. Experiment B: in March 30 frogs were housed for 2 weeks under a 8 h:16 h (light:dark) photoperiod and a temperature of 4°C.

Protein extract preparations

Frozen frog testes (10 testes/month/experiment) were homogenised directly into lysis buffer containing 50 mmol l^{-1} Hepes, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 10% glycerol, 1% Triton-X-100 (1:2 w/v), 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) 1 mg aprotinin, 0.5 mmol l^{-1} sodium orthovanadate, 20 mmol l^{-1} sodium pyrophosphate, (Sigma), and clarified by centrifugation at 14 000 ***g*** 10 min. Protein concentrations were estimated using a modified Bradford assay (Bio-Rad, Melville, NY, USA).

Antibody

The antibodies were purchased from the following sources: (1) polyclonal anti-phospho-p44 MAP kinase (Thr202/Tyr204) antibody (#9101S, New England Biolab, MA, USA), raised in rabbit, (2) polyclonal anti-ERK1 (#sc-94-G, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) raised in rabbit against epitope corresponding to an amino acid sequence conserved in frog, chicken, murine and human, (3) mouse monoclonal antibody against recombinant Proliferating Cellular Nuclear Antigen (PCNA, Dako Corporation, Denmark).

Western blot analysis

25 or 40 mg of total protein extracts were boiled in Laemmli buffer for 5 min before electrophoresis. The samples were subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Immobilon Millipore Corporation, Bedford, MA, USA); complete transfer was assessed using prestained protein standards (Bio-Rad). The membranes were treated for 2 h with blocking solution (5% no fat powdered milk in 25 mmol l^{-1} Tris, pH 7.4; 200 mmol l^{-1} NaCl; 0.5% Triton X-100, TBS/T), and then the membranes were incubated for 1 h at room temperature with the primary antibody, (1) against phospho-ERK1 (diluted 1:2000), (2) against ERK1 (diluted 1:2000), (3) against PCNA (diluted 1:1000). After washing with TBS/T and TBS, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:5000) for 45 min (at room temperature) and the reactions were detected using the enhanced chemiluminescence (ECL) system (Amersham Life Science, UK).

Statistical analysis

The primary spermatogonial mitotic index was expressed as the number of metaphases per total primary spermatogonia counted ×100 in three randomly chosen sections/animal. Values are expressed as means ± s.d. Significance of differences was evaluated using one-way analysis of variance (ANOVA) followed by Duncan's test for multigroup comparisons.

Results

Experiment A

In November (8°C; 8 h:16 h light:dark), the testis showed all stages of spermatogenesis, but the spermatogonial mitotic index was rather low and there were several cysts of degenerating primary and secondary spermatocytes (I and II SPC) and spermatids (SPT).

Frogs were housed for 2 weeks under a 12 h (12 h:12 h light:dark) photoperiod at 22°C. In order to assess spermatogonial proliferation we analyzed activation of the ERK1 isoform; in fact, a recent paper reports that ERK1 protein is present in the SPG of the frog *R. esculenta* and its activation is necessary for spermatogonial proliferation (Chieffi et al., 2000b). These conditions induced an increase of ERK1 phosphorylation status (Thr202/Tyr204) after 1 and 2 weeks with respect to the control (Fig. 1). The spermatogonial mitotic index (SPG-MI) was consistent with the increased ERK1 activity. An increase of SPG-MI after 1 and 2 weeks with respect to the control was observed (Fig. 2A). In addition, the increase of primary SPG proliferation was

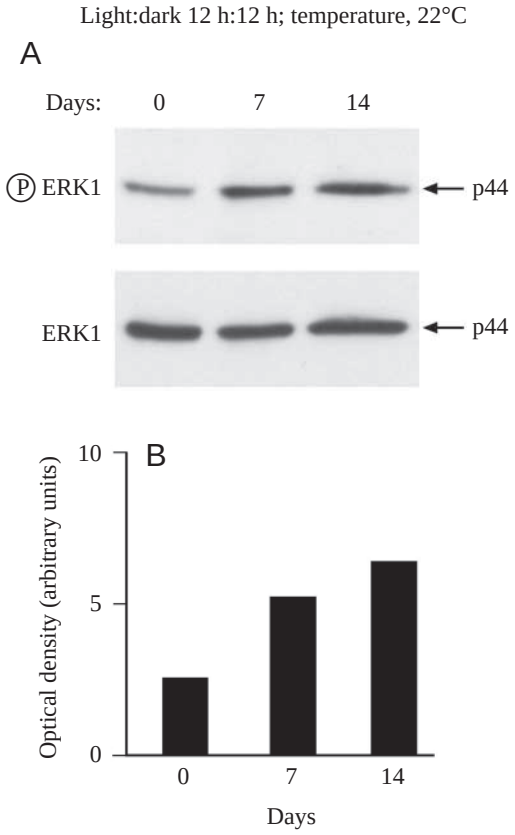

Fig.·1. Western blot detection of ERK1 protein in the testicular extracts of *R. esculenta* at various times, 22°C and 12h:12h photoperiod. (A) Proteins (25·mg/lane) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against phospho-ERK1 and ERK1 protein. A specific band was observed of 44·kDa (determined by comparison with comigrating size markers). The blot is representative of three separate assays. (B) The amount of activated ERK1 was quantitated by using ImageQuant 5.2 Program and normalized by total ERK1. The values shown are representive of three separate experiments.

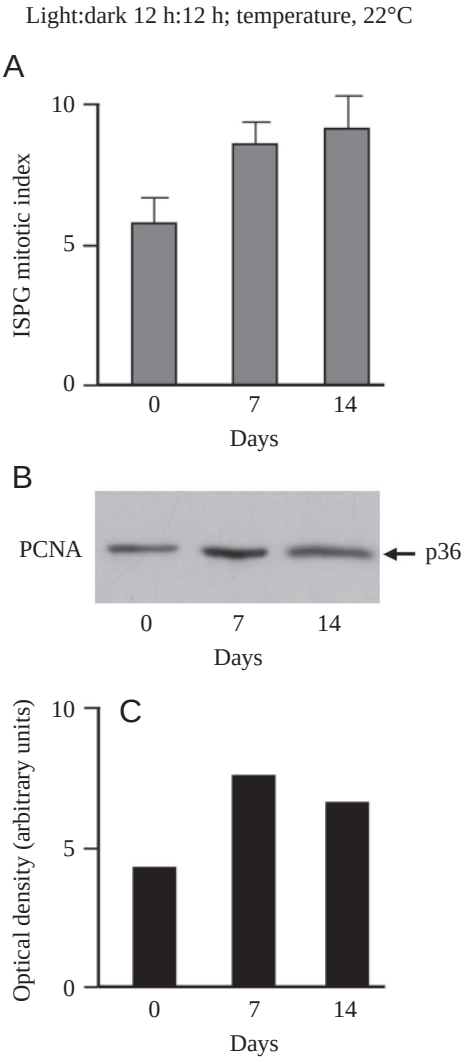

Fig.·2. (A) Mitotic index of primary spermatogonia (I SPG) in the frog *R. esculenta* testis at various times, 22°C and 12h:12h photoperiod. The I SPG was expressed as the number of metaphases per total primary spermatogonia counted ×100 in three randomly chosen sections/animal. Values are means ± S.D. Differences were considered significant at $P<0.01$. (B) Western blot detection of PCNA protein in the cytosolic testicular extracts of *R. esculenta* at various times, 22°C and 12h:12h photoperiod. Proteins (40·µg/lane) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against PCNA protein; a specific band was observed of 36·kDa (determined by comparison with comigrating size markers). (C) The amount of PCNA was quantitated using ImageQuant 5.2 Program. The values shown are representative of three separate experiments.

confirmed using PCNA as an early molecular marker (Chieffi et al., 2000a); in fact, this nuclear protein is expressed in G_1–S phases of SPG. Western blot analysis showed an increase in the amount of PCNA after 1 and 2 weeks with respect to the control (Fig. 2B,C).

Experiment B

The initial controls captured in March at a temperature of ca. 18°C (12 h:12 h light:dark photoperiod) showed very active spermatogenesis. Treatment at 4°C (8 h:16 h light:dark) induced a decrease of ERK1 phosphorylation status (Thr202/Tyr204) after 1 and 2 weeks with respect to the control (Fig. 3). The SPG-MI was consistent with the decreased P-ERK1 activity, and decreased after 1 and 2 weeks with respect to the control (Fig. 4A). I SPG proliferation was monitored by western blot analysis, which showed a decrease in the amount of PCNA after 1 and 2 weeks with respect to the control (Fig. 4B,C).

Discussion

Male *R. esculenta* appear potentially to be continuous breeders, with annual testicular cycles depending upon environmental cues. It is well known that temperature is a primary factor in the regulation of the quiescent phase of the testicular cycle, whereas for stimulating and maintaining active spermatogenesis, both a relatively high temperature and long

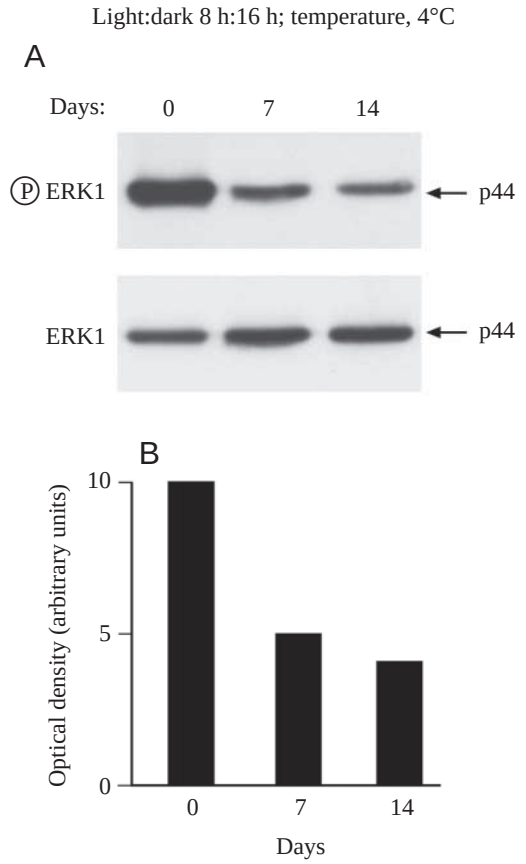

Fig.·3. (A) Western blot detection of ERK1 protein in the testicular extracts of *R. esculenta* at various times, 4°C and 8·h:16·h photoperiod. Proteins (25·mg/lane) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against phospho-ERK1 and ERK1 protein. A specific band was observed of 44·kDa (determined by comparison with comigrating size markers). (B) The amount of activated ERK1 was quantitated by using ImageQuant 5.2 Program and normalized by total ERK1. The values shown are representative of three separate experiments.

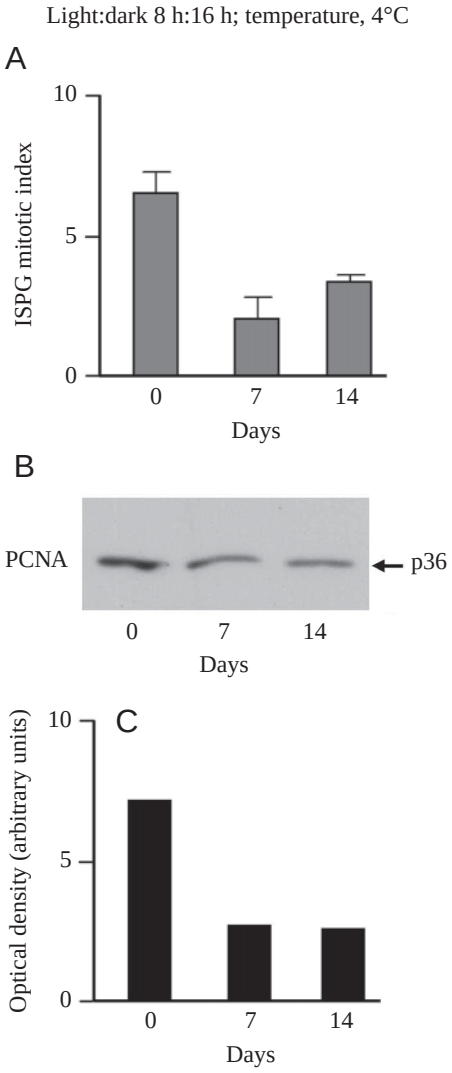

Fig.·4. (A) Mitotic index of primary spermatogonia (I SPG) in the frog *R. esculenta* testis at various times, 4°C and 8·h:16·h photoperiod. The I SPG was expressed as the number of metaphases per total primary spermatogonia counted ×100 in three randomly chosen sections/animal. Values are expressed as means ± S.D. Differences were considered significant at $P<0.01$. (B) Western blot detection of PCNA protein in cytosolic testicular extracts of *R. esculenta* at various times, 4°C and 8·h:16·h photoperiod. Proteins (40·μg/lane) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and then incubated with antibody raised against PCNA protein; a specific band at 36 kDa (by comparison with comigrating size markers). The blot is representative of three separate assays. (C) The amount of PCNA was quantitated by using ImageQuant 5.2 Program. The values shown are representative of three separate experiments.

photoperiod are important (Rastogi et al., 1978); in particular, it has been demonstrated that the light only has a permissive role, facilitating the temperature response in the regulation of spermatogenesis of *R. esculenta* (Di Matteo et al., 1981).

Using the male green frog *R. esculenta* spermatogenic system, we experimentally manipulated spermatogenesis using environmental cues. The objective of this study was to analyse MAPK (ERK1) spermatogonial activity in the regulation of proliferation induced by temperature and photoperiod. In experiment A, our data clearly show that an increase of photoperiod and temperature induces a ISPG proliferation through ERK1 activation. In addition, experiment B shows that a decrease of temperature and photoperiod in spring caused an intense spermatogonial proliferation, with a downturn in ERK1 activation and, consequently, mitotic spermatogonial division.

In testicular germ cells ERKs are predominantly confined to the nuclei, where they might also regulate gene transcription and substrate phosphorylation which, in turn, regulate cell proliferation (Chieffi et al., 2000, 2002). Since changes in the state of activation of ERKs also correlate with spermatogenetic activity, it is likely that these enzymes play a key role in the regulation of the testicular epithelium proliferation (Chieffi et al., 2000b). Recently, it has also been reported that the mitogenic c-Src/p21ras/MAP kinase signal transducting pathway, which is known to be stimulated by different growth

factors in different systems, is activated by 17-β estradiol (Migliaccio et al., 1996; Watters et al., 1997); for example, it has been demonstrated that estrogens induce spermatogonial proliferation by activation of the MAP kinase cascade which, in turn, promotes transcription of the immediate early genes (Chieffi et al., 2000, 2002).

In *R. esculenta*, not only the pineal complex but also the eyes intervene in the normal testicular activity (Rastogi, 1976). In fact, the light influences the hypotalamo–hypophyseal system, with the mediation of pineal gland/eyes (Rastogi, 1976; d'Istria et al., 2003), in the secretion of gonadotropins that regulate the synthesis of mitogen factors (i.e. growth factors, estrogens) by Sertoli and Leydig cells that induce and regulate spermatogonial proliferation (Rastogi, 1976). In this scenario MAPK (ERK1) seems to be a primary factor in the phosphorylation of different nuclear transcriptional factors necessary for the spermatogonial proliferation (Chieffi et al., 2000, 2002). It is important to note that our data clearly demonstrate that ERK1 activation of spermatogonial proliferation in the frog *R. esculenta* is also under temperature and photoperiodic control. It is well documented that temperature plays an important role in the control of spermatogenesis (Rastogi, 1976); in fact, under natural conditions temperatures of up to 25°C and photoperiod up to ca. 16 h of light daily are favorable for active winter spermatogenesis (Di Matteo et al., 1981). Experiments performed in autumn and winter revealed the importance of high temperature and normal photoperiod in the stimulation and maintenance of normal spermatogenesis (Rastogi et al., 1978).

In conclusion, our data demonstrate for the first time in a non-mammmalian vertebrate that exogenous factors such as temperature and photoperiod exert a role in the induction of spermatogonial proliferation through increased ERK1 activity.

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References

Chieffi, P., Picascia, A., Stanzione, R. and Tramontano, D. (1999). c-Jun phosphorylation (Ser-63) in the lizard, *Podarcis s. sicula*, testis. *J. Endocrinol.* **163**, 337-344.

Chieffi, P., Franco, R., Fulgione, D. and Staibano, S. (2000a). PCNA in the testis of the frog, *Rana esculenta*: a molecular marker of the mitotic testicular epithelium proliferation. *Gen. Comp. Endocrinol.* **119**, 11-16.

Chieffi, P., Colucci D'Amato, G. L., Staibano, S., Franco, R. and Tramontano, D. (2000b). Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *J. Endocrinol.* **167**, 77-84.

Chieffi, P., Colucci D'Amato, L. and Tramontano, D. (2001). Annual profile of mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) in the frog (*Rana esculenta*) testis. *Rend. Accad. Naz. Lincei* **12**, 19-28.

Chieffi, P., Colucci-D'Amato, G. L., Guarino, F., Salvatore, G. and Angelini, F. (2002). 17-β estradiol induces spermatogonial proliferation through mitogen-activated protein kinase (extracellular signal-regulated kinase 1) activity in the lizard (*Podarcis s. sicula*). *Mol. Reprod. Dev.* **61**, 218-225.

Chieffi, P. (2003). Changes in JNK1 activity in the frog (*Rana esculenta*) testis. *Mol. Reprod. Dev.* **66**, 398-402.

Cobb, M. H. (1999). MAP kinase pathways. *Progr. Bioph. Mol. Biol.* **71**, 479-500.

d'Istria, M., Palmiero, C., Serino, I., Izzo, G. and Minucci, S. (2003). Inhibition of the basal and oestradiol-stimulated mitotic activity of primary spermatogonia by melatonin in the testis of the frog, *Rana esculenta*, *in vivo* and *in vitro*. *Reproduction* **126**, 83-90.

Di Matteo, L., Minucci, S. and Rastogi, R. K. (1981). Influence of photoperiodism on high temperature-induced testicular recrudescence in the green frog, *Rana esculenta*. *Experientia* **37**, 149-150.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270**, 16483-16486.

Lofts, B. (1974). Seasonal changes in the functional activity of the interstitial and spermatogenetic tissues of the green frog, *Rana esculenta*. *Gen. Comp. Endocrinol.* **4**, 550-562.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Contempo, P., Nola, E. and Auricchio, F. (1996). Tyrosine kinase/p21^{ras}/Map-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J.* **17**, 2008-2018.

Nielsen, O. (1993). Signal transduction during mating and meiosis in *S. pombe*. *Trends Cell Biol.* **3**, 60-65.

Rastogi, R. K. (1976). Seasonal cycle in anuran (Amphibia) testis: the endocrine and environmental controls. *Boll. Zool.* **43**, 151-172.

Rastogi, R. K., Iela, L., Del Rio, G., Di Meglio, M., Russo, A. and Chieffi, G. (1978). Environmental influence on testicular activity in the green frog, *Rana esculenta*. *J. Exp. Zool.* **206**, 49-63.

Rastogi, R. K., Iela, L., Di Meglio, M., Di Matteo, L., Minucci, S. and Izzo-Vitiello, I. (1983). Initiation and kinetic profiles of spermatogenesis in the frog, *Rana esculenta* (Amphibia). *J. Zool. (Lond)* **201**, 515-525.

Waskieewicz, A. J. and Cooper, J. A. (1995). Mitogen and stress response pathways: MAP kinase cascade and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.* **7**, 798-805.

Watters, J. J., Campbell, J. S., Cunningham, M. J., Krebs, E. G. and Dorsa, D. M. (1997). Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* **138**, 4030-4033.