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RESEARCH ARTICLE

P450 aromatase: a key enzyme in the spermatogenesis of the Italian wall lizard, *Podarcis sicula*

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ABSTRACT

P450 aromatase is a key enzyme in steroidogenesis involved in the conversion of testosterone into 17β-estradiol. We investigated the localization and the expression of P450 aromatase in *Podarcis sicula* testes during the different phases of the reproductive cycle: summer stasis (July–August), early autumnal resumption (September), middle autumnal resumption (October–November), winter stasis (December–February), spring resumption (March–April) and the reproductive period (May–June). Using immunohistochemistry, we demonstrated that the P450 aromatase is always present in somatic and germ cells of *P. sicula* testis, particularly in spermatids and spermatozoa, except in early autumnal resumption, when P450 aromatase is evident only within Leydig cells. Using real-time PCR and semi-quantitative blot investigations, we also demonstrated that both mRNA and protein were expressed in all phases, with two peaks of expression occurring in summer and in winter stasis. These highest levels of P450 aromatase are in line with the increase of 17β-estradiol, responsible for the spermatogenesis block typical of this species. Differently, in autumnal resumption, the level of P450 aromatase dramatically decreased, along with 17β-estradiol levels, and testosterone titres increased, responsible for the subsequent renewal of spermatogenesis not followed by spermiation. In spring resumption and in the reproductive period we found intermediate P450 aromatase amounts, low levels of 17β-estradiol and the highest testosterone levels determining the resumption of spermatogenesis needed for reproduction. Our results, the first collected in a non-mammalian vertebrate, indicate a role of P450 aromatase in the control of steroidogenesis and spermatogenesis, particularly in spermiogenesis.

KEY WORDS: P450 aromatase, Spermatogenesis, Steroidogenesis, Non-mammalian vertebrates

INTRODUCTION

Spermatogenesis is a complex process under the control of both gonadotropins and testicular factors (Li and Arimura et al., 2003), including estrogens, which for a long time have been regarded as typically female hormones. However, in the last 10 years it has been shown in mammals that estrogens are involved in different testis events including spermatogonia division, spermatid differentiation, acrosome biogenesis and sperm motility (O'Donnell et al., 2001; Carreau et al., 2007). In addition, estrogens are involved in negative

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feedback on the pituitary gland to control gonadotropin secretion, hence an absence of estrogens and inappropriate estrogen exposure leads to disturbance in the delicate balance of the hypothalamic–pituitary–testis axis (O'Donnell et al., 2001). So, estrogens are now regarded as male hormones too. Such hormones are produced by the irreversible aromatization of androgens by P450 aromatase, and, once produced, they work by their nuclear and cell surface receptors (Carreau et al., 2003; O'Donnell et al., 2001; Carreau et al., 2007; Carreau et al., 2011). P450 aromatase is an enzymatic complex, composed of two proteins, a ubiquitous NADPH-cytocrome P450 reductase and a cytochrome P450 aromatase, which contains the steroid-binding site (Carreau et al., 2011). P450 aromatase is localized in the cellular endoplasmic reticulum of different areas of the body, including the testis (Carreau et al., 2001). In humans, P450 aromatase is the product of a single gene, the CYP 19 located in the q21.1 region of chromosome 15, whose expression is under the control of tissue-specific promoters (Carreau et al., 2011). In particular, in the testes, aromatase expression is regulated by cAMP through interaction of the gonad promoter with the transcription factor CREB (cAMP response element binding protein) (Lynch et al., 1993; Carlone and Richards, 1997). In the rat testis, the distribution of aromatase changes during development such that the enzyme is located within Sertoli cells in immature animals and in Leydig and germ cells in mature animals (Bourguiba et al., 2003; Carreau, 2003; Carreau et al., 2006, 2011).

In non-mammalian vertebrate testes, investigations on P450 aromatase are limited to a few species: the trout *Oncorhynchus mykiss* (Delalande et al., 2015), the eel *Anguilla anguilla* (Peñaranda et al., 2014), the frogs *Xenopus laevis* (Urbatzka et al., 2007) and *Pelophylax esculenta* (Burrone et al., 2012), and the lizard *Podarcis sicula* (Cardone et al., 2002; Lance, 2009). In particular, in *P. sicula*, our experimental model, the partial coding sequence of *P450 aromatase* is known (gi 257359521), and it has been shown that the P450 aromatase inhibition by fadrozole modifies spermatogenesis, as well as the epididymis morphology (Cardone et al., 2002). More recently, we hypothesized also that in *P. sicula*, P450 aromatase activity could be under the control of Pituitary Adenylate Cyclase Activating Peptide (PACAP) (Rosati et al., 2016) and Vasoactive Intestinal Peptide (VIP) (Rosati et al., 2015), as such neuropeptides induce an increase of 17β-estradiol levels in testis culture. Finally, it is well known that in *P. sicula*, estrogens play a key role in the control of spermatogenesis; in particular, such hormones are responsible for the spermatogenesis block, a condition known as 'refractoriness' (Angelini and Botte, 1992).

On the basis of this evidence, we have hypothesized that putative changes of the aromatase expression in different periods of the reproductive cycle could be responsible for the blocking and unblocking mechanisms described in *P. sicula* spermatogenesis. So, we decided to investigate the localization and expression of P450 aromatase in the testis of the Italian wall lizard, *P. sicula* (Rafinesque 1810), to verify the involvement of such an enzyme in the control of

P. sicula testis activity. The investigations were carried out in the different phases of the reproductive cycle: summer stasis (July–August), early (September) and mid-autumnal resumption (October–November), winter stasis (December–February), spring resumption (March–April) and the reproductive period (May–June). Stases are periods of refractory or blocked testicular activity, whereas resumption is the period of sperm production. Furthermore, these periods are significant in the spermatogenesis of *Podarcis* for both seminiferous tubule organization and plasma sex hormone profiles. Indeed, during the summer stasis, seminiferous tubules are composed of spermatogonia and Sertoli cells only, an organization similar to that of immature rats (Carreau et al., 2011). Then, in early- and mid-autumnal resumption, when spermatogenesis renewal occurs, tubules are composed of spermatogonia, spermatocytes I (early autumnal), spermatocytes II, spermatids and few non-useful spermatozoa (mid-autumnal). Testis morphological features of mid-autumn resumption do not change and the composition remains the same through the winter stasis and spring resumption periods. On the contrary, during the reproductive period, the tubules consist of germ cells in all the differentiating stages with numerous spermatozoa, ready to be ejaculated (Angelini and Botte, 1992).

Using immunohistochemistry, real-time PCR, semi-quantitative blots and hormonal immunoassay, we demonstrate that P450 aromatase is widely expressed in germ and somatic cells and that the levels of both mRNA and protein change during the different phases of the *P. sicula* reproductive cycle. These data strongly suggest an active role of P450 aromatase in the control of *P. sicula* steroidogenesis and spermatogenesis, as changes in aromatase levels are followed by changes in sex hormones. Moreover, the positivity recorded in late stages of spermatogenesis, particularly in spermatids, is indicative of a direct action of P450 aromatase on spermioistogenesis.

MATERIALS AND METHODS
Animals
Sexually mature males of *P. sicula* were collected in Campania (southern Italy) during different periods of the reproductive cycle (see Introduction). The animals were collected in the same year (2013); they were maintained in a soil-filled terrarium and fed *ad libitum* with *Tenebrio molitor* larvae, for approximately 15 days, the time required to ameliorate the capture stress (Manzo et al., 1994). The experiments were approved by institutional committees (Ministry of Health of the Italian Government) and organized to minimize the number of used animals. The animals were killed by decapitation after deep anaesthesia with ketamine hydrochloride (325 pg g^{-1} body mass; Parke-Davis, Berlin, Germany). Sexual maturity of each animal was determined by morphological parameters and by histological analysis (Agnese et al., 2014a,b,c; Rosati et al., 2014a,b). We collected five animals (samples) for each period of the reproductive cycle.

The testes were quickly removed. For each animal, one testis was fixed for 24 h in Bouin's solution, dehydrated and embedded in paraffin wax. Consecutive sections of 7 μm thickness were placed on polylysine glass slides (Menzel-Glaser, Braunschweig, Germany) and utilized for immunohistochemistry procedures. The other testis was stored at −80°C and used for RNA and protein extractions and for steroid determinations.

Immunohistochemistry
The immunohistochemistry procedure was performed as described elsewhere (Prisco et al., 2009; Agnese et al., 2012, 2013; Del Giudice et al., 2011, 2012). Briefly, slides were immersed in 0.1 mol l^{-1} citrate buffer, pH 6.0, in a microwave oven (2×10 min) for antigen retrieval. Sections were rinsed with 2.5% H$_2$O$_2$ to inactivate endogenous peroxidases and with normal goat serum (Pierce, Rockford, IL, USA) to reduce non-specific background. Rabbit anti-P450 aromatase (Santa Cruz Biotechnology, Dallas, TX, USA) antibody diluted 1:200 in normal goat serum was applied overnight at 4°C. The reaction was revealed with a biotin-conjugated goat anti-rabbit secondary antibody and an avidin–biotin–peroxidase complex (ABC immunoperoxidase kit, Pierce), using DAB as chromogen. Sections were counterstained with Mayer's hematoxylin. Negative controls were carried out by omitting primary antibody. Immunohistochemical signal was analyzed with Axioskop System (Zeiss, Oberkochen, Germany). Three investigators individually blindly ranked the intensity of staining (i.e. zero, weak, positive, intense) using a light microscope; afterwards, one researcher took digital photographs. The staining intensity shown in Table 1 was determined according to the majority of the opinions.

Real-time PCR
Total mRNA was extracted using TRI Reagent (Sigma). The concentration and integrity of the RNA samples (three different samples for each period considered) were determined by absorbance at 260 nm and by formaldehyde–agarose gel electrophoresis. RNA (1 μg) was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). The real-time PCR reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System using the Power SYBR Green Master Mix PCR (Applied Biosystems, Foster City, CA, USA). We performed experiments in a technical triplicate for each sample (animal). For internal standard control, the expression of β-actin was quantified simultaneously. The primers used in real-time PCR were: Aromatase ForPs 5′-TCCCGAGAGCATGAGATACCA-3′; Aromatase RevPs 5′-AAGTCCACGACTGGCTGGTATC-3′; β-ACTIN ForPs 5′-TATCC-ACGAGACCACCTTC-3′; and β-ACTIN RevPs 5′-TCCACCAATC-CAGACAGAG-3′. The fold change in expression was obtained using the 2$^{-\Delta\Delta CT}$ method. Results were graphed using Graphpad 5.0 Software (San Diego, CA, USA); statistical analysis was carried out by ANOVA

Table 1. Distribution of P450 aromatase after immunohistochemical investigation during the reproductive cycle in *Podarcis sicula*

	L	S	SPG	SPC I	SPC II	SPT	SPZ
Summer stasis	+	+++	+				
Early autumnal resumption	++	−	−	−			
Mid-autumnal resumption	+	+	−	++	++	++	++
Winter stasis	+	+	−	++	++	++	++
Spring resumption	+++	+	+	+	+	+++	+++
Reproductive period	++	+	+	−	−	++	++

L, Leydig cells; S, Sertoli cells; SPG, spermatogonia; SPC I, II, spermatocytes I, II; SPT, spermatids; SPZ, spermatozoa. +, faint positivity; ++, intense positivity; +++, more intense positivity; −, negative result.

with Bonferroni correction; a *P*-value of <0.05 was considered statistically significant.

Preparation of testis homogenates

Testis samples of each reproductive phase were homogenized with a Potter homogenizer at 4°C with 5 ml of RIPA buffer containing 50 mmol l^{-1} Tris-HCl (pH 7.4), 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 10% NP-40 and protease inhibitor cocktail (0.3 μmol l^{-1} aprotinin, 1 μmol l^{-1} leupeptin, 1 μmol l^{-1} E-64, 130 μmol l^{-1} bestain, 1 mmol l^{-1} EDTA and 2 mmol l^{-1} AEBSF). Homogenates were centrifuged at 7000 *g* for 10 min at 4°C. Total protein amounts of samples were determined using the BCA protein assay reagent kit (Pierce).
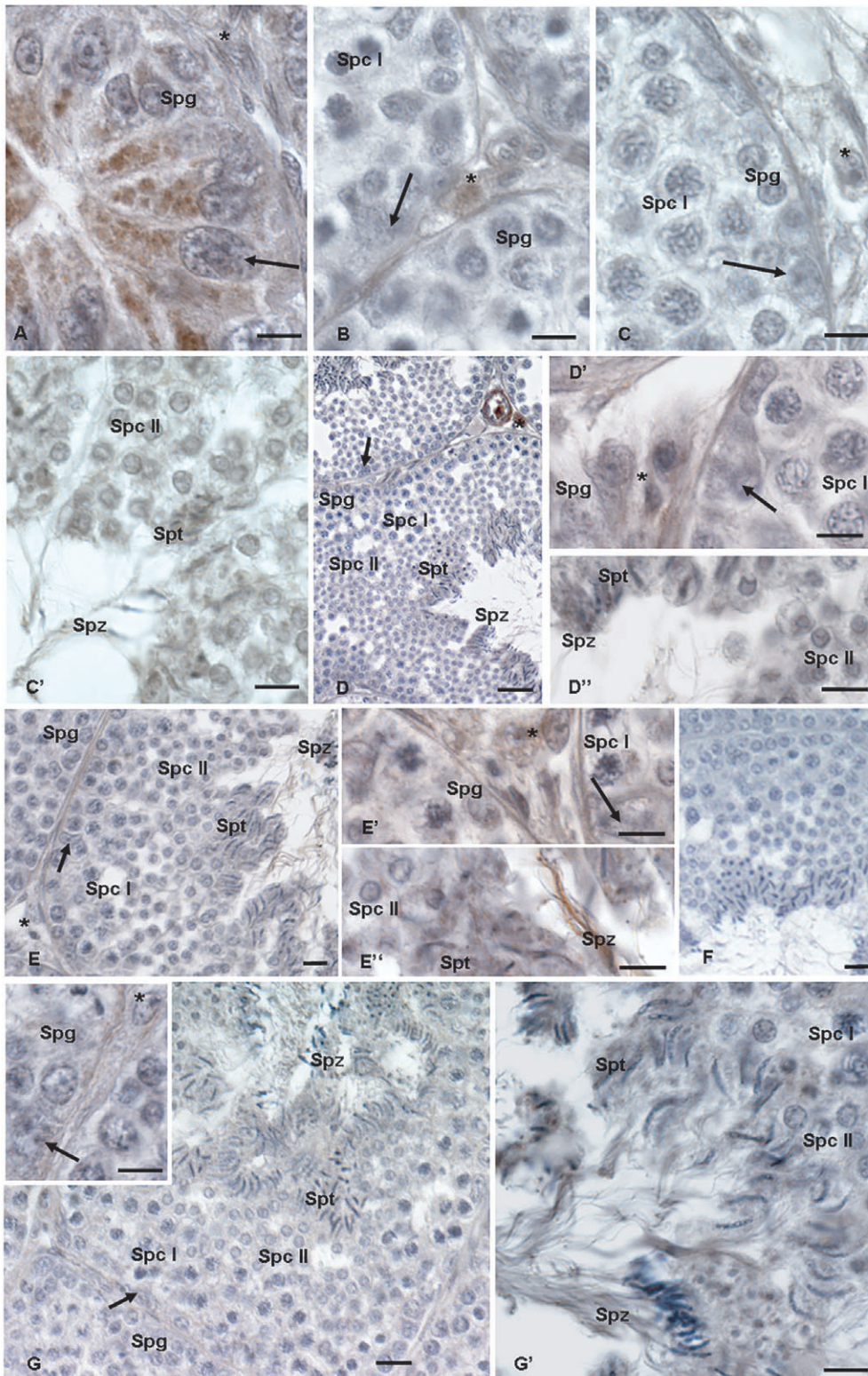
Fig. 1. Immunohistochemical localization (brown areas) of P450 aromatase in *Podarcis sicula* testis during the different phases of the reproductive cycle. (A) Summer stasis. Immunohistochemical signal is evident in Sertoli (arrow) and Leydig (asterisk) cells, and spermatogonia (Spg). (B) Early autumnal resumption. Signal is evident only in Leydig cells (asterisk). (C,C′) Mid-autumnal resumption. Signal is evident in spermatocytes I (Spc I), spermatocytes II (Spc II), spermatids (Spt) and spermatozoa (Spz), as well as in Sertoli (arrow) and Leydig (asterisk) cells. (D–D″) Winter stasis. Signal occurs in spermatocytes I (Spc I), spermatocytes II (Spc II), spermatids (Spt) and spermatozoa (Spz), and in Sertoli (arrow) and Leydig (asterisks) cells. (E–E″) Spring resumption. A quite strong signal occurs in Leydig (asterisk) cells, spermatids (Spt) and spermatozoa (Spz); a faint signal is evident in Sertoli (arrow) cells, spermatogonia (Spg), and spermatocytes I (Spc I) and II (SpcII). (F) No signal is present in control sections. (G,G′) Reproductive period. Signal is evident in Leydig (asterisk) cells, spermatids (Spt) and spermatozoa (Spz); a faint signal occurs in Sertoli (arrow) cells and in spermatogonia (Spg). Inset shows signal in Leydig (asterisk) and spermatogonia (Spg) cells. Scale bars: 20 μm (D,F), 10 μm (E,G) and 5 μm (A,B,C,C′,D′,D″,E′,E″,G inset,G′).

Western blot

A total of 40 µg of proteins of each sample was boiled for 5 min in SDS buffer [50 mmol l^{-1} Tris-HCl (pH 6.8), 2 g 100 ml^{-1} SDS, 10% (v/v) glycerol, 0.1 g 100 ml^{-1} Bromophenol Blue], loaded on a 12.5% SDS/polyacrylamide gel, and transferred to nitrocellulose using a Mini Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 1 h at room temperature with TBS-T buffer [150 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris HCl (pH 7.4) 0.1% Tween 20] containing 5% non-fat milk powder. The blots were incubated overnight with rabbit anti-human P450 aromatase antibody (Santa Cruz Biotechnology) diluted 1:200 in TBS-T containing 2.5% non-fat milk powder. Then, the membranes were washed three times with TBS-T and incubated for 1 h with anti-rabbit biotinylated IgG diluted 1:200 in TBS-T containing 2.5% milk. Finally, the membranes were incubated with an avidin–biotin–peroxidase complex (ABC immunoperoxidase kit, Pierce), using DAB as chromogen. In contrast, for semi-quantitative western blots, after washing the primary antibody with TBS-T, the blots were incubated for 1 h with anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:3000 in TBS-T containing 2.5% per 200 ml milk. The proteins were visualized by enhanced chemiluminescence using 2.5 mol l^{-1} Luminol. Western blots were analyzed using the C-Digit Chemiluminescent Western Blot Scanner (Li-Cor); Image Studio Software was utilized to determine optical density of the bands. To monitor equal loading of gel lanes, the blots were stripped and re-probed using an anti human-β-actin (endogenous control) polyclonal antibody diluted 1:200. Results were graphed using Graphpad 5.0; statistical analysis was carried out using ANOVA with Bonferroni correction; a P-value of <0.05 was considered statistically significant.

Sex steroid assays in the testis

Sex steroid determinations in the testis were conducted using enzyme immunoassay (EIA) kits (DiaMetra, Spello, Italy). The following limits of detection were observed: for testosterone, sensitivity was 50 pg ml^{-1} (intra-assay variability 4.0%, inter-assay variability 9.0%); for 17β-estradiol, sensitivity was 3 pg ml^{-1} (intra-assay variability 6.0%, inter-assay variability 7.5%). Testes were homogenized 1:10 (w/v) with distilled water. The homogenate was then mixed vigorously with ethyl ether (1:10 v/v) and the ether phase was withdrawn after centrifugation at 3000 *g* for 10 min. Three extractions were performed. Pooled ether extracts were dried and then utilized for the enzyme immunoassays as previously reported (Raucci et al., 2005). Sex steroid recovery was 80% from tissues. Statistical analysis was carried out using ANOVA with Bonferroni correction; a P-value of <0.05 was considered statistically significant. Five individual males for each period were used to test hormone levels.

RESULTS

Cellular localization of P450 aromatase

Immunohistochemistry revealed that P450 aromatase was widely distributed in both germ and somatic cells during the six phases of the reproductive cycle (Fig. 1, Table 1). In summer stasis, a quite strong signal was recorded in the cytoplasm of Sertoli cells (Fig. 1A), while it was faint within spermatogonia and Leydig cells (Fig. 1A). In early autumnal resumption, the signal occurred only in the cytoplasm of Leydig cells (Fig. 1B), while in mid-autumnal resumption, it was evident in spermatocytes I and II, spermatids and spermatozoa (Fig. 1C,C'), as well as in Sertoli and Leydig cells (Fig. 1C); no immunoreactive signal was found in spermatogonia (Fig. 1C,C'). In winter stasis, when the tubules present the same organization

previously reported, the immunohistochemical signal showed the same localization recorded in samples of autumnal resumption (Fig. 1D,D',D''). In spring resumption, the signal was differently localized: strong in Leydig cells, spermatids and spermatozoa, and faint in Sertoli cells, in spermatogonia and spermatocytes I and II (Fig. 1E,E',E''). Finally, in the reproductive period, a strong immunohistochemical signal was evident within Leydig cells, spermatids and spermatozoa, while it was faint in Sertoli cells and in the spermatogonia; no signal was found in spermatocytes I and II
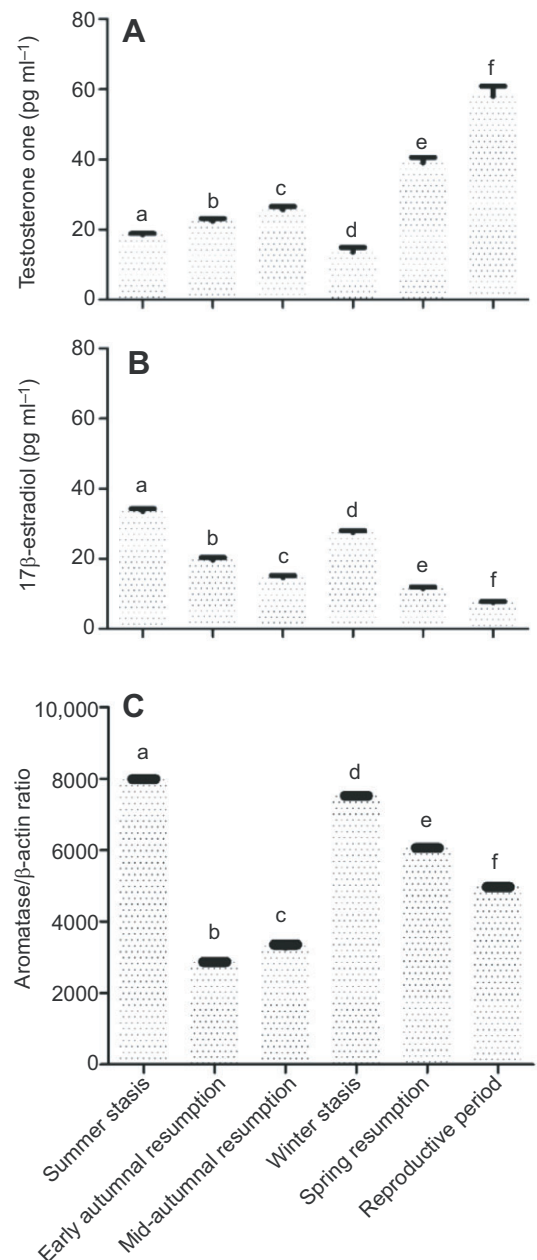

Fig. 2. Hormone levels and *P450 aromatase* mRNA profile in *Podarcis sicula* testis during the different phases of the reproductive cycle. (A) Endogenous testosterone and (B) 17β-estradiol levels. Each value represents the mean±s.d. of five determinations. (C) Real-time PCR for *P450 aromatase* mRNA. *P450 aromatase* mRNA showed a cyclic trend. Note that the highest level was recorded in summer stasis, while the lowest one was recorded in autumnal resumption. Different letters in all panels correspond to statistically significant differences ($P<0.05$).
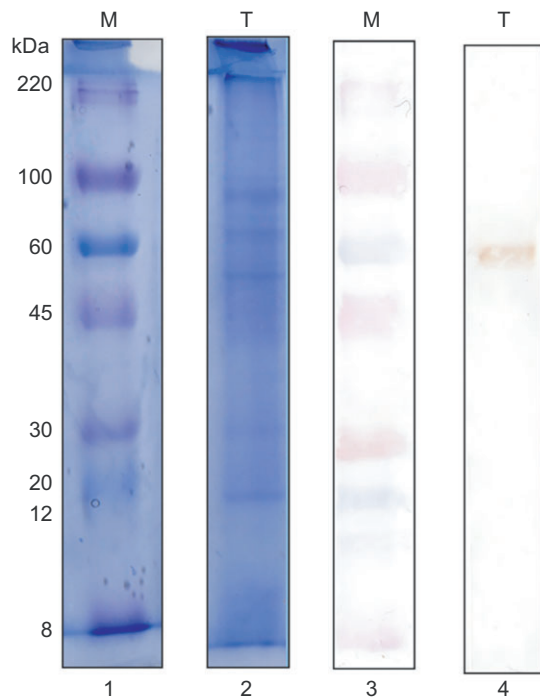
Fig. 3. Western blot for P450 aromatase in *Podarcis sicula* testis. Lane 1, protein ladder after running on a 12.5% SDS/polyacrylamide gel; Lane 2, proteins extracted from whole testis after running on a 12.5% SDS/polyacrylamide gel; Lane 3, protein ladder on nitrocellulose membrane; Lane 4, the band of 60 kDa corresponds to P450 aromatase. M, molecular weight markers; T, testis.

(Fig. 1G and inset, G′). No immunohistochemical signal was found in the control sections (Fig. 1F).

Real-time PCR

Real-time PCR showed that the *P450 aromatase* mRNA profile significantly changes over the course of the annual reproductive cycle (Fig. 2). The highest level was recorded during summer stasis; such a level dramatically decreased in early and mid-autumnal resumption; then, in winter stasis, it suddenly increased, reaching the second peak of reproductive cycle; finally, in spring resumption and in the reproductive period, *P450 aromatase* mRNA decreased once again. The differences among the six time periods were statistically significant ($P<0.05$).

Western and semi-quantitative blot

Western blot investigation showed only one band at 60 kDa positive to rabbit anti-P450 aromatase antibody (Fig. 3); the band corresponded to the molecular weight of P450 aromatase, demonstrating the validity of rabbit anti-human P450 aromatase antibody (Santa Cruz Biotechnology) in *P. sicula* testis as well. Semi-quantitative blot analysis showed that the band corresponding to P450 aromatase (60 kDa) was detectable in all periods although with different intensity; in contrast, the band corresponding to β-actin (45 kDa) was present in all periods with equal intensity (Fig. 4A). In particular, the protein showed the same distribution of mRNA (Fig. 4B). The highest level occurred in summer stasis; then, it dramatically decreased in early autumnal resumption, and again increased in winter stasis. Finally, the P450 aromatase level again lowered in spring resumption and in the reproductive period. Differences among the six periods analyzed were statistically significant ($P<0.05$).
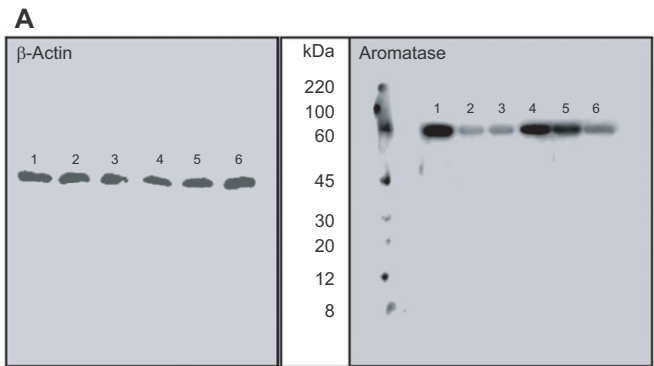

Fig. 4. Semi-quantitative blot for P450 aromatase in *Podarcis sicula* testis. (A) Semi-quantitative blot: Lane 1, summer stasis period; Lane 2, early autumnal resumption period; Lane 3, mid-autumnal resumption period; Lane 4, winter stasis period; Lane 5, spring resumption period; Lane 6, reproductive period. β-actin represents the endogenous control. (B) Note the cyclic trend of aromatase with the highest level in summer stasis, and the lowest in autumnal resumption. Different letters correspond to statistically significant differences ($P<0.05$).

Endogenous sex hormone concentrations in the testis during the annual reproductive cycle

Fig. 2 reports the profiles of endogenous levels of sex hormones (testosterone and 17β-estradiol) in the testis of adult male during the major phases of the annual reproductive cycle. Testosterone concentration was higher during spring resumption and peaked in the reproductive period, whereas it was low in summer and winter stasis. In contrast, the 17β-estradiol level was low in early and mid-autumnal resumption and during the reproductive phase, but significantly higher during the summer and winter stasis.

DISCUSSION

The aim of this paper was to verify the localization and expression of P450 aromatase together with the profile of sex hormones to highlight the role of this enzyme in the control of spermatogenesis during the annual reproductive cycle of *P. sicula*. Indeed, it is well known that P450 aromatase controls the synthesis of 17β-estradiol, one of the most relevant factors that locally controls spermatogenesis in non-mammalian (Cardone et al., 2002; Lance, 2009; Urbatzka et al., 2007; Burrone et al., 2012; Peñaranda et al., 2014; Delalande et al., 2015) and mammalian (O'Donnell et al., 2001; Carreau et al., 2007) vertebrates.

In *P. sicula*, it is well known that high titres of 17β-estradiol are responsible for the spermatogenesis block (Angelini and Botte, 1992) and that 17β-estradiol production is under the control of VIP (Rosati et al., 2015) and PACAP (Rosati et al., 2016), two neuropeptides involved in the control of lizard spermatogenesis (Agnese et al., 2010, 2012, 2014a,b,c, 2016; Rosati et al., 2014a,b, 2016).

Now, we show that the expression profile of *P450 aromatase* mRNA and protein changes throughout the reproductive cycle. Indeed, real-time PCR and semi-quantitative blot investigations demonstrated that both *P450 aromatase* mRNA and protein are expressed in all phases of the reproductive cycle, but with significant differences among the six different functional periods of spermatogenesis. In particular, the highest levels of mRNA and protein are observed in summer and winter stasis, contemporaneously with an increase of 17β-estradiol, responsible for the spermatogenesis block (Angelini and Botte, 1992). In contrast, in autumnal resumption, the level of *P450 aromatase* mRNA dramatically decreases, along with 17β-estradiol levels, and testicular testosterone titre increases, which is responsible for the subsequent renewal of spermatogenesis that is not followed by spermiation; the biological meaning of this spermatogenesis resumption that is not useful to reproduction could be linked to an evolutionary memory of a previous condition, in which reptiles exhibited an annual bimodal reproduction (Angelini and Botte, 1992). It is only with an intermediate titre of P450 aromatase, recorded in spring resumption and the reproductive period, that the resumption of spermatogenesis occurs accompanied by a production of spermatozoa useful for reproduction. The balance between expression of P450 aromatase and sex hormone titre is quite interesting. In particular, the highest levels of 17β-estradiol have been recorded in the stasis periods, when it is known to be involved in spermatogenesis block; in contrast, the highest levels of testosterone have been recorded in resumption periods, in particular in the reproductive period, when testosterone is responsible for the massive spermatozoa production and for development and maintenance of secondary sexual characteristics (Angelini and Botte, 1992). Therefore, in this system, the variations in P450 aromatase levels during the six phases of the annual reproductive cycle modify the balance between testosterone and 17β-estradiol levels, which acts as an on/off switch for *P. sicula* spermatogenesis.

In *P. sicula*, the production of P450 aromatase is due to the activity of germ and somatic cells during the different phases of the reproductive cycle; this is the first evidence so far reported in a non-mammalian vertebrate testis. In detail, Leydig cells are positive for P450 aromatase in all periods throughout the year, so that they represent an excellent source for androgens and estrogens as well. The same positivity recorded in Leydig cells occurs within Sertoli cells, except during autumnal resumption, when only Leydig cells are positive; this corresponds to the time when the testes are primarily involved in the synthesis of testosterone for resumption of spermatogenesis. The greatest presence of P450 aromatase within Sertoli cells is recorded during summer stasis, when these cells are likely involved in the massive production of estrogens, which are also recorded at the highest levels during this period; this condition is similar to that of immature rats (Carreau et al., 2011). In contrast, the massive estrogen production occurring during the winter stasis may be due to germ cells, as the positivity recorded in Sertoli cells is faint compared with summer stasis. Finally, the positivity to P450 aromatase in germ cells during the different phases of the reproductive cycle strongly suggests that P450 aromatase controls the proliferative and meiotic phases of gametogenesis through

estrogen production; particularly, the high and constant positivity to P450 aromatase recorded in spermatids and spermatozoa, as reported in mammalian testes (Carreau et al., 2007), is indicative that spermiogenesis is under the control of estrogens.

Once synthesized, estrogens may control spermatogenesis in an intracrine or paracrine fashion through interaction with estrogen receptor beta. Indeed, the presence of such a receptor was recorded within the Sertoli and germ cells of lizard testes (Chieffi and Varriale, 2004), as well as in mammalian testes (Carreau et al., 2006). Then, estrogen receptor beta activates kinases Akt-1 (Russo et al., 2005) and ERK 1/2 (Chieffi et al., 2002), which, through the phosphorylation of transcription factors, activate the proliferation and differentiation of germ cells present in the lizard testis.

In conclusion, the present results demonstrate that germ and somatic cells are differently involved in the production of P450 aromatase and that the enzyme level modifies in accordance with the levels of sex hormones, thus controlling the steroidogenesis and gametogenesis in the lizard testis. Based on these data and results shown elsewhere (Agnese et al., 2014a,b,c; Rosati et al., 2014a,b, 2015), we can conclude that *P. sicula* is an excellent model with which to study the mechanisms that control the activity of the vertebrate testis.

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