

## Immunolocalization of aromatase P450 in the epididymis of *Podarcis sicula* and *Rattus rattus*

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### ABSTRACT

The goal of this study was to evaluate P450 aromatase localization in the epididymis of two different vertebrates: the lizard *Podarcis sicula*, a seasonal breeder, and *Rattus rattus*, a continuous breeder. P450 aromatase is a key enzyme involved in the local control of spermatogenesis and steroidogenesis and we proved for the first time that this enzyme is represented in the epididymis of both *P. sicula* and *R. rattus*. In details, P450 aromatase was well represented in epithelial and myoid cells and in the connective tissue of *P. sicula* epididymis during the reproductive period; instead, during autumnal resumption this enzyme was absent in the connective tissue. During the non-reproductive period, P450 aromatase was localized only in myoid cells of *P. sicula* epididymis, whereas in *R. rattus* it was localized both in myoid cells and connective tissue. Our findings, the first on the epididymis aromatase localization in the vertebrates, suggest a possible role of P450 aromatase in the control of male genital tract function, particularly in sperm maturation.

**Key words:** P450 aromatase; 17 $\beta$ -estradiol; reproduction; epididymis; vertebrates.

*This paper is dedicated to Piero Andreuccetti, who greatly contributed to the work herein described. Magister vitae et scientiarum and wonderful colleague and mentor, he died suddenly on September 28, 2019.*

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## Introduction

It is well known that androgens and gonadotropins play crucial role in spermatogenesis, and in the development and function of the male reproductive system.<sup>1</sup> However, in the last 10 years it has been shown that also estrogens, for long time regarded as typically female hormones, are involved in the regulation of this process.<sup>2-7</sup> Estrogens are produced by the irreversible aromatization of androgens promoted by P450 aromatase. These hormones need to bind their own nuclear or cell surface receptors to perform their cellular activity.<sup>3,8-10</sup> P450 aromatase is located in the cellular endoplasmic reticulum of testis and various other districts.<sup>10</sup> In the testis, aromatase expression is regulated by cAMP through interaction of the gonad promoter with the transcription factor CREB (cAMP response element binding protein).<sup>11,12</sup> In non-mammalian vertebrate testis, investigations on P450 aromatase have been conducted in different species as: the trout *Oncorhynchus mykiss*,<sup>13</sup> the fish *Anguilla anguilla*,<sup>14</sup> the frog *Xenopus laevis*<sup>15</sup> and *Pelophylax esculenta*,<sup>16</sup> and the reptile *Podarcis sicula*.<sup>5,17,18</sup> In *P. sicula* testis, it has been shown that the P450 aromatase, as well as some testis local factors [pituitary adenylate-cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP)],<sup>19-21</sup> alters the balance between testosterone and 17 $\beta$ -estradiol levels, which acts as an on/off switch for spermatogenesis.<sup>5,19-24</sup> Investigations on P450 aromatase in mammalian testis are few. In particular, in rat testis the distribution pattern of aromatase changes during development: the enzyme is located within Sertoli cells in immature animals; instead, it is localized in Leydig and germ cells level in mature ones.<sup>9,25-27</sup>

Furthermore, also the investigations on the presence of P450 aromatase in epididymis are limited. In non-mammalians, as *P. sicula*, some investigations showed that inhibition of P450 aromatase by fadrozole changes the epididymal cell morphology.<sup>17</sup> Moreover, the rat epididymal epithelial cells, as well as cell cultures of epididymis, showed the presence of P450 aromatase *mRNA*.<sup>28,29</sup>

The aim of this work was to localize for the first time the aromatase in the vertebrate epididymis, as well as to compare how the distribution of this enzyme changes in the epididymis of two experimental models with different reproductive strategies. In particular, using immunohistochemical approach, our intention was to evaluate the presence of P450 aromatase in the epididymis of the seasonal breeder *P. sicula* and of the continuous breeder *R. rattus* which share the tubular organization of the testis. In *Podarcis*, the variation of the spermatogenic cycle during the different period of reproduction, not only change testis structure but also is associated with different epididymis organization. Specifically, epididymis epithelium during the reproductive period (May-June), is constituted of basal stem cells and columnar cells. The columnar cells present two nuclei and are quite active in the synthesis of large dense vacuoles that, once released their content in the epididymal lumen, take part in epididymal fluid organization.<sup>30-32</sup> On the contrary, during the non-reproductive period (July-August), basal cells are still present, instead the columnar cells present a different morphology: they have a single nucleus and do not show the presence of large dense vacuoles indicating that they are not in active synthesis. Moreover, differently from what occurs during the reproductive period, spermatozoa are absent in the epididymis lumen during the non-reproductive period.<sup>31</sup> Finally, during autumnal resumption (November-December), the epididymis epithelium presents a structure similar to the reproductive period, indeed are present the columnar cells in which are evident the secretion vacuoles; however these vacuoles are few compared to reproductive periods, as well as are few the spermatozoa evident in the lumen, because not useful for reproduction.<sup>30-32</sup>

Regarding rats, epididymis epithelium consists of four types of cells: basal, tight, main and apical cells.<sup>33-39</sup> Basal cells are located on the basement membrane and do not interact with the epididymal

lumen. This type of cells is responsible of the production of factors involved in immune responsivity, electrolytic secretion as well as regulation of main and tight cells activity.<sup>39</sup> Tight cells present, around the nucleus, a thin cytoplasm with typical extensions projecting towards the lumen of the epididymis.<sup>38</sup> The main cells have a large basal nucleus centrally located, and a well-developed secretion apparatus, which synthesizes proteins in the epididymal fluid. This synthesis apparatus is recognizable also in the apical cells, which, differently from the main cells, are not in contact with the basement membrane.<sup>38</sup>

More recently, in the epididymis of *P. sicula*, as well as *R. rattus*, we have demonstrated that VIP/VPACR system is widely represented, suggesting that such a system could play an active role in the reproduction of vertebrates, in particular sperm maturation and fertilization.<sup>40,41</sup>

Now, the present immunohistochemical investigation demonstrates that P450 aromatase is represented in the epididymis of both experimental models, suggesting that this protein complex could play an active role in vertebrate reproduction, mainly in sperm maturation and fertilization.

## Materials and Methods

Male specimens of *P. sicula* lizards, sexually mature, were collected in Campania (southern Italy; Latitude: 41° 19'54 "N; Longitude: 13° 59'29 "E) during reproductive period (May 2013), non-reproductive period (July 2013) and autumnal resumption (November 2013). After capture, the lizards were maintained in a soil-filled terrarium and fed *ad libitum* with *Tenebrio molitor* larvae, for approximately 15 days, the time required to reverse capture-related stress.

*R. rattus* epididymis of sexually mature animals, were kindly gifted by prof. M.P. Mollica, Department of Biology, Federico II University of Naples.

The experiments were permitted by institutional committee (Ministry of Health of the Italian Government) and organized to minimize the number of animals utilized for the experiments (6 animals for each species have been used).

After deep anesthesia with ketaminehydrochloride (325 pg/g of body mass; Parke-Davis, Berlin, Germany), animals were killed by decapitation and sexual maturity of each animal was determined using morphological parameters and histological analysis.

## Immunohistochemistry

Paraffin-embedded Bouin's fixed testis with epididymis were cut at 5  $\mu$ m sections and used for immunohistochemistry analysis, as previously reported.<sup>42-49</sup> Briefly, slides were dewaxed and heat treated in microwave (2 x 10 min), using 0.1 M citrate buffer (pH 6.0) for antigen retrieval. After washed in PBS, sections were first rinsed with 2.5% H<sub>2</sub>O<sub>2</sub> for 40 min to inactivate endogenous peroxidases and then blocked for 1h with normal goat serum (Pierce, Rockford, IL, USA) to reduce non-specific background. Sections were incubated overnight at 4°C with the primary antibody Rabbit anti-P450 aromatase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:200 in normal goat serum and this antibody have been previously validated both in *P. sicula*<sup>5</sup> and in *R. rattus* testis.<sup>46</sup> The day after, the reaction was revealed with a biotin-conjugated goat anti-rabbit secondary antibody (Kit Pierce, diluted 1:2000 in normal goat serum) and an avidin-biotin-peroxidase complex (ABC immunoperoxidase Kit, Pierce), using diaminobenzidine (DAB) as chromogen. Sections were counterstained with Mayer's hematoxylin. Negative controls were performed by omitting incubation with primary antibody. Immunohistochemical signal was analyzed with Axioskop System (Zeiss, Oberkochen, Germany).



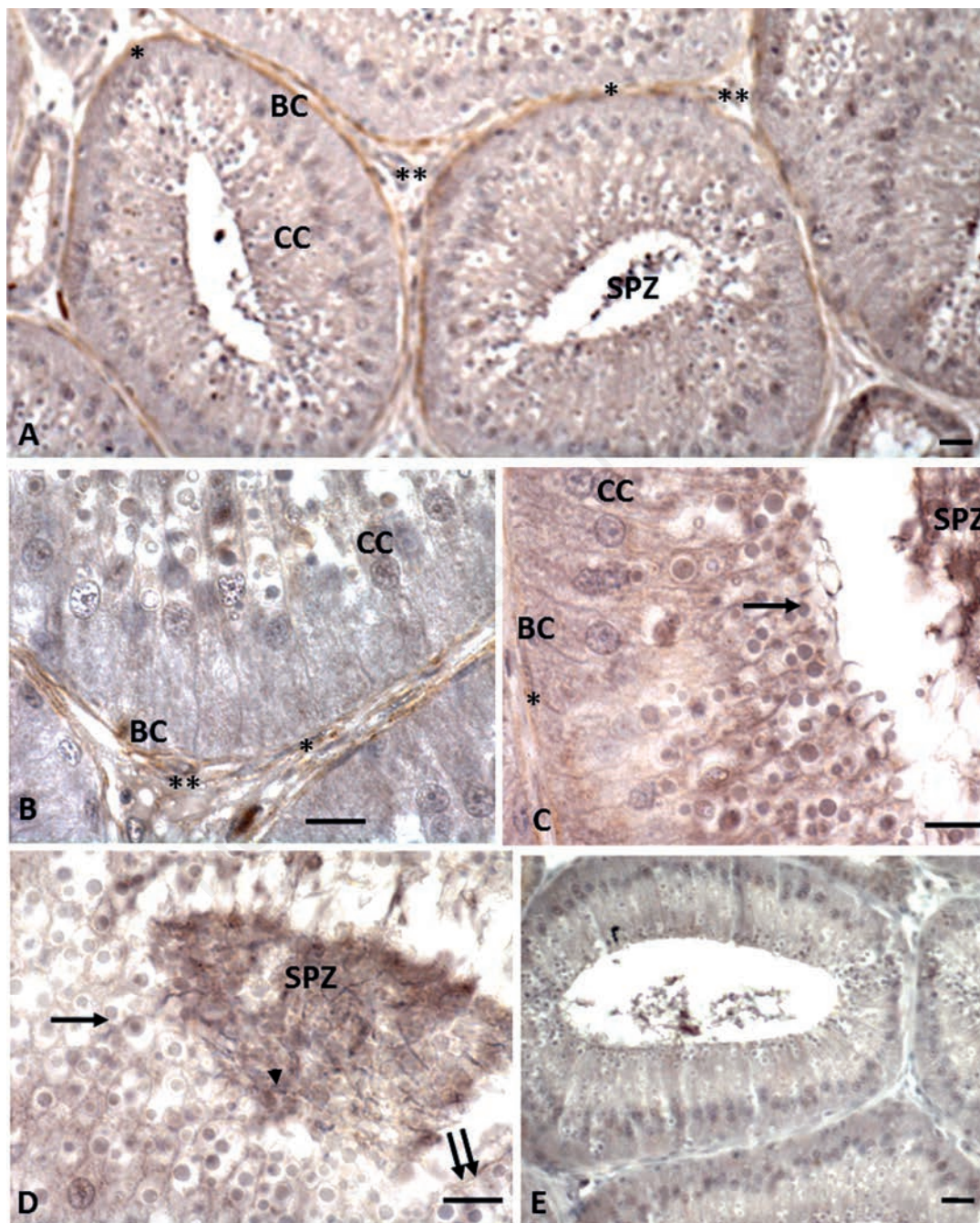
## Results

### *Podarcis sicula*

#### *P450 aromatase localization in epididymis during reproductive period*

Immunohistochemistry analysis showed the presence of the enzyme P450 aromatase in the epididymis of the lizard *P. sicula* during the reproductive period. Specifically, P450 aromatase has

been detected in both basal and columnar cells of the epididymis epithelium, in myoid cells, connective cells and in the spermatozoa present in the lumen (Figure 1 A-D). In particular, in columnar cells, the enzyme is localized in the cytoplasm and also in the large dense vacuoles present in the cytoplasm. Positive vacuoles for P450 aromatase were identified also in the epididymal lumen, where they were blended with labeled spermatozoa at level of acrosome and tail (Figure 1 B-D). In Figure 1E it is possible to note the absence of signal for P450 aromatase in the negative control.



**Figure 1.** Reproductive period: immunohistochemistry for P450 aromatase in *P. sicula* epididymis. Immunolocalization signal appears as brown areas. A-B-C-D: a signal for P450 aromatase is evident in basal (BC) and columnar (CC) cells, as well as in myoid cells (asterisk) and connective cells (double asterisk). Spermatozoa (SPZ) present in the lumen are also immunolabelled: signal occurs in acrosome (arrow-head) and tail (double arrow). Signal is also evident in the large dense vacuoles present both in columnar cells and in epididymal lumen intermingled with spermatozoa (arrows). No signal is evident in the negative control sections (E). Scale bars: A,E) 20  $\mu$ m; B,C,D) 5  $\mu$ m.

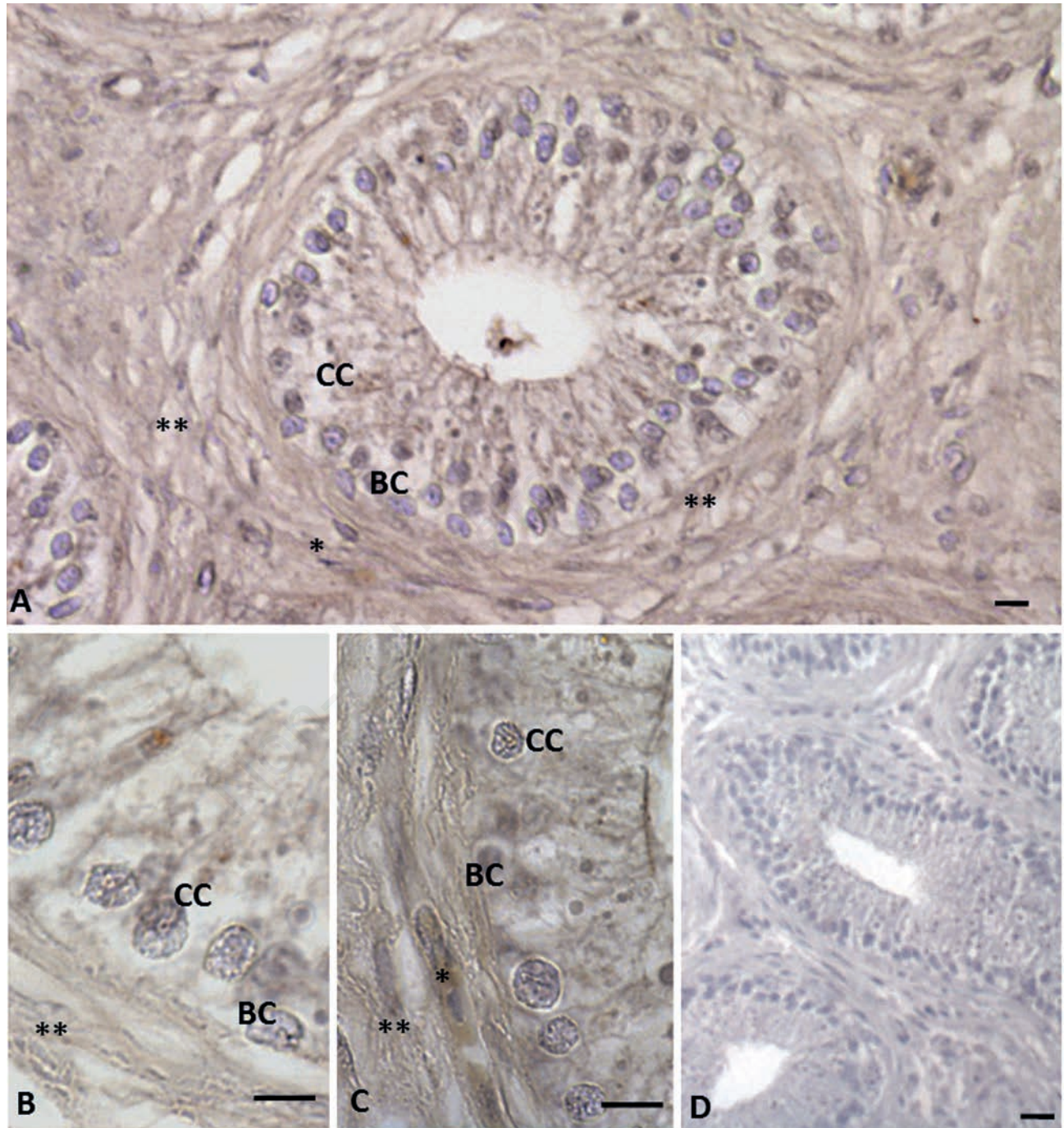


### P450 aromatase localization in epididymis during non-reproductive period

During the non-reproductive period, P450 aromatase showed a more limited pattern of distribution in the epididymis of *P. sicula*. In fact, the signal was found only in myoid cells (Figure 2 A-C) while basal, columnar cells, as well as connective cells are not positive for P450 aromatase (Figure 2 A-C). Controls obtained by omitting the primary antibody showed no positive reaction (Figure 2D).

### P450 aromatase localization in epididymis during autumnal resumption

During the autumnal resumption, immunohistochemistry performed with anti-P450 aromatase antibody, showed a similar distribution to reproductive period in lizard epididymis. In details, the immunohistochemical signal was evident within cytoplasm of columnar cells, as well as in few large dense vacuoles present in the cytoplasm and in the epididymal lumen, where they were intermingled with the spermatozoa, which result stained for P450



**Figure 2.** Non-reproductive period: immunohistochemistry for P450 aromatase in *P. sicula* epididymis. Immunolocalization signal appears as brown areas. A,B,C) Positivity for antibody is evident only in myoid cells (asterisk); no signal is evident in basal (BC) and columnar (CC) cells, as well as connective cells (double asterisk). D) Negative control section shows no signal. Scale bars: A,D) 20  $\mu\text{m}$ ; B,C) 5  $\mu\text{m}$ .

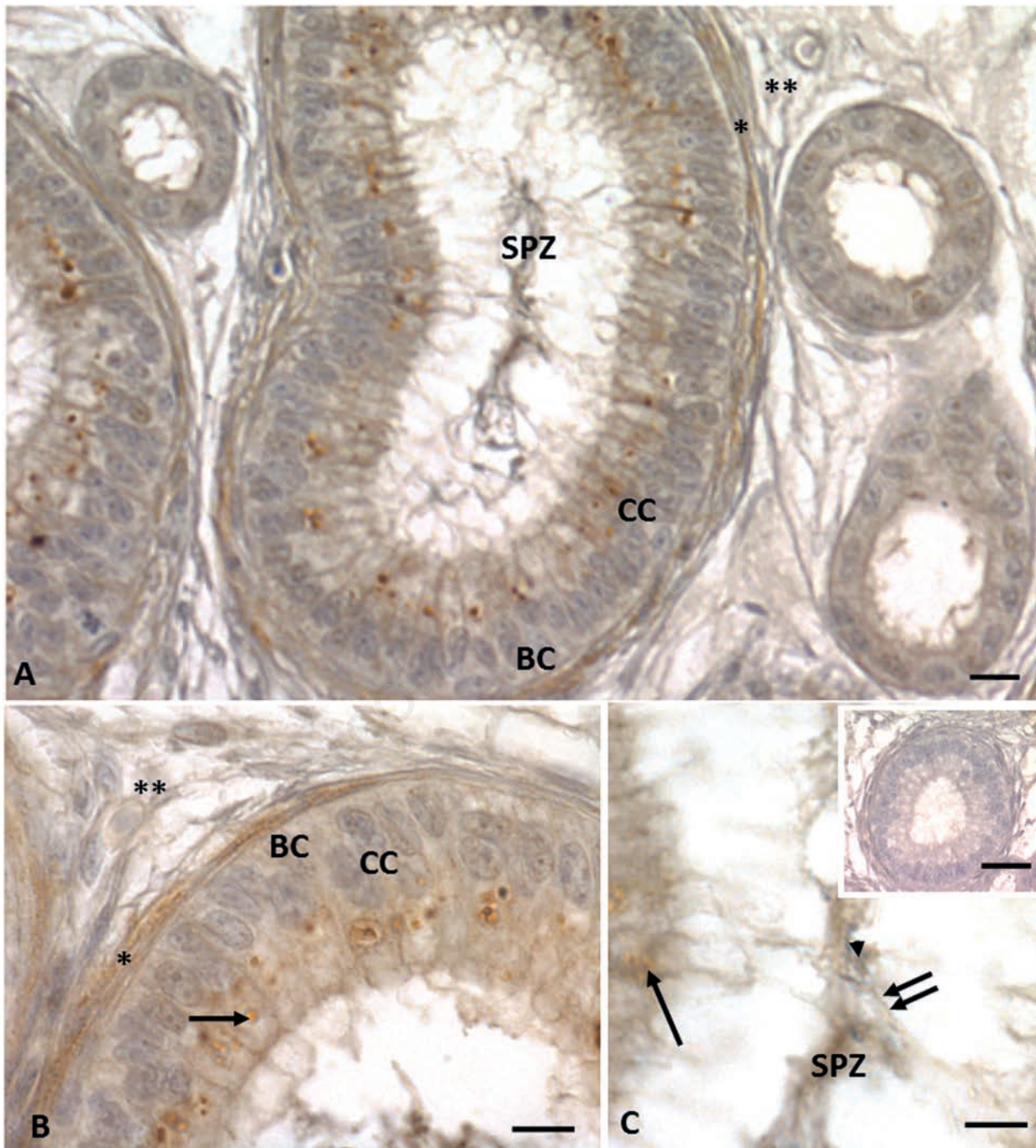


aromatase at both acrosome and tail (Figure 3 A-C). No signal occurred in epididymis cubic cells (Figure 3 A-B). In the spermatozoa, the signal was recognizable in both the acrosome and tail (Figure 3C). Immunolabelling for P450 was also observed in myoid cells; whereas no signal was evident in the connective tissue (Figure 3 A-B). Controls obtained by omitting the primary antibody were not immunolabelled (Figure 3C, insert).

### *Rattus rattus*

#### *P450 aromatase localization in epididymis*

Immunohistochemistry performed for P450 aromatase in *R. rattus* epididymis showed absence of signal in basal, tight, main and apical epithelium cells (Figure 4 A-C); instead it was possible to highlight the signal in myoid and connective cells (Figure 4 A-C).



**Figure 3.** Autumnal resumption: immunohistochemistry for P450 aromatase in *P. sicula* epididymis. Immunolocalization signal appears as brown areas. A signal for P450 aromatase is evident in columnar (CC) cells, as well as in myoid cells (asterisk). No signal is evident in basal cells (BC) and connective cells (double asterisk). Spermatozoa (SPZ) present in the lumen are also immunolabelled: signal occurs in acrosome (arrowhead) and tail (double arrow). Signal is also evident in the large dense vacuoles present both in columnar cells and in epididymal lumen intermingled with spermatozoa (arrows). No signal is evident in the negative control sections. Scale bars: A,C insert) 20  $\mu$ m; B,C) 5  $\mu$ m.

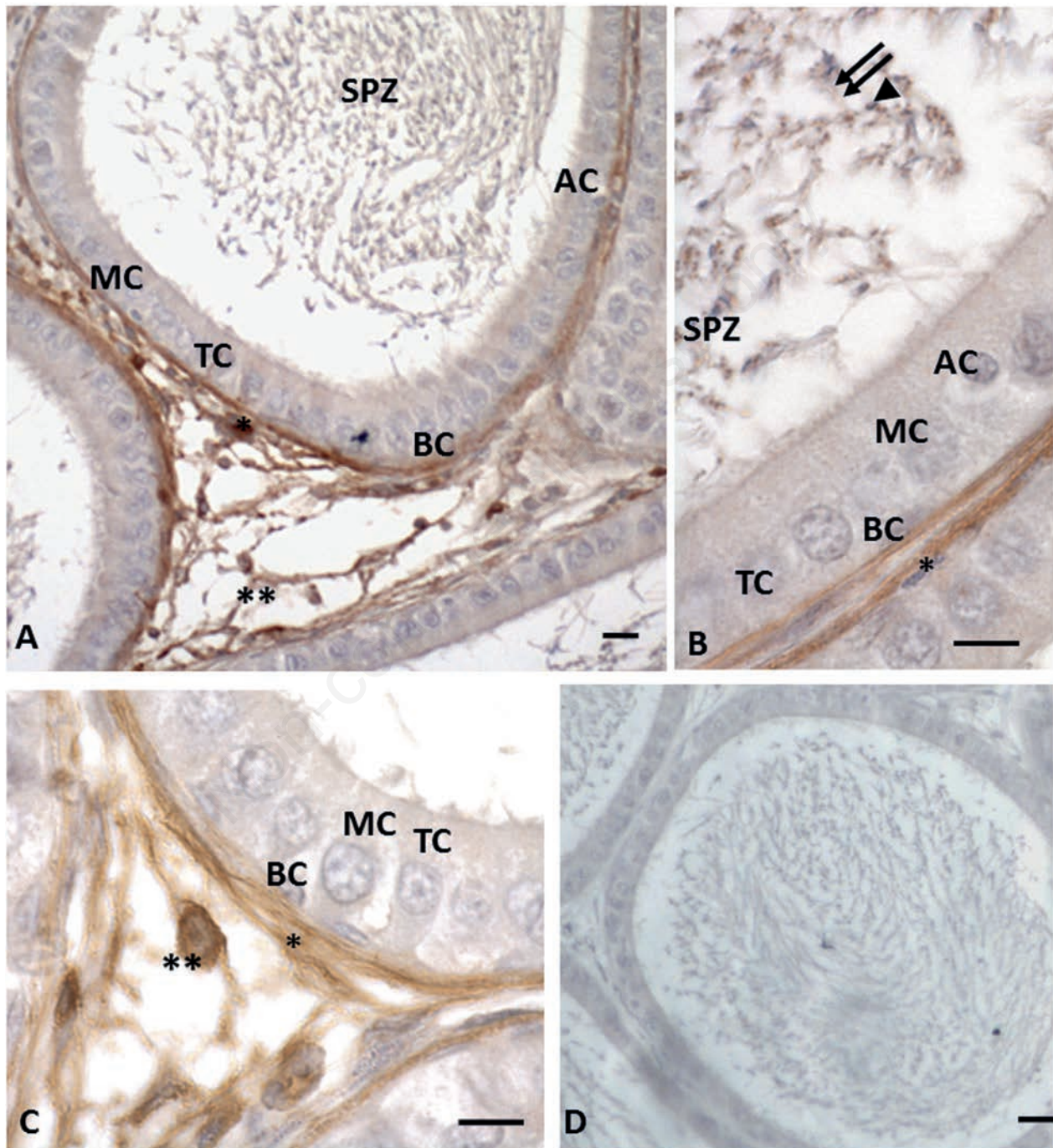


Moreover, P450 aromatase was detectable at the level of both acrosome and tail of the spermatozoa present within the epididymal lumen (Figure 4 A-B). Figure 4D shows negative controls.

## Discussion

P450 aromatase is an enzyme involved in the synthesis of  $17\beta$ -estradiol, one the most relevant factors that locally control

spermatogenesis.<sup>5,6,9,13,16,17</sup> The aim of this paper was to assess the localization of P450 aromatase in the epididymis of two vertebrates with a different reproductive strategy and with tubular testis organization: *P. sicula* lizard and *R. rattus*, to highlight the possible role of this enzymatic complex in the control of reproduction. In *P. sicula*, it is well known the high titers of  $17\beta$ -estradiol are responsible for spermatogenesis block,<sup>5,31</sup> and that P 450 aromatase acts as an on/off switch for spermatogenesis.<sup>5</sup> In addition, in *P. sicula*, has been shown also that ERs mRNA are distributed in the



**Figure 4.** *R. rattus* epididymis: P450 aromatase immunolabelling appears as brown areas. A,B,C) Anti-P450 aromatase immunolabelling; no signal is evident in basal (BC), tight (TC), main (MC) and apical (AC) cells; the positivity occurs in myoid cells (asterisk) and connective cells (double asterisk); the spermatozoa (SPZ) present in the lumen were immunolabelled: signal occurs in acrosome (arrowhead) and tail (double arrow). D) No signal is present in negative control sections. Scale bars: A,D) 20  $\mu$ m; B,C) 5  $\mu$ m.

efferent duct and epididymis in all reproductive cycle.<sup>50</sup> In *R. rattus*, it is well known that aromatase is present in somatic and germ cells of the testis and that estrogens are molecules involved in the spermiogenesis process.<sup>9</sup> If on the one hand previous studies conducted in reptiles and rats have shown the presence of aromatase mRNA and estrogen receptors in the epididymis, no data are available so far about the localization of the P450 aromatase protein in this reproductive district.

In this study, we proved for the first time that P450 aromatase was well localized in the epididymis of two vertebrates: *P. sicula*, and *R. rattus*. We compared two experimental models that have different reproductive strategies; in particular, the use of *P. sicula* has allowed us to assess the epididymis activity in reproductive, non-reproductive and in autumnal resumption stages. Based on the function of the aromatase positive cells, our results suggest that this enzyme could be involved in different functions as sperm maturation, sperm progression and fertilization, as recently reported for PACAP and VIP.<sup>40,51,52</sup> In details, an immunostaining for enzyme was found always in myoid cells and connective tissue as well as in acrosome and tail of spermatozoa present in the epididymal lumen.

The possible involvement of P450 aromatase in the sperm maturation stands out from the observations performed in *P. sicula* epididymis. In fact, a strong positivity for enzyme was reported also in the epithelial cells of epididymis during the reproductive period and autumnal resumption; during the non-reproductive period, the same positivity occurred only inside myoid cells. In particular, during the reproductive period, P450 aromatase labeling in the epithelial cells occurs within many large dense vacuoles, whose content is discharged into the lumen of epididymis where numerous spermatozoa are present. Thus, considering that P450 aromatase occurs both in cytoplasm and in large dense vacuoles present both in the columnar cells and in the lumen intermingled with spermatozoa, it could be hypothesized that the enzyme may contribute to sperm maturation. Moreover, the presence of P450 aromatase in spermatozoa, as previously reported in seminiferous tubules<sup>5,9</sup> strongly suggests that this enzyme could be involved in the acrosome biosynthesis and fertilization as reported for estrogens in rat and for PACAP and VIP in both experimental models.<sup>5,9,40,51</sup> On the other hand, the P450 aromatase positivity evident in myoid cells, considering the function of these cells, could suggest that estrogens promote the release of cell content in the lumen of epididymis (sperm progression). Differently, in autumnal resumption, the presence of P450 aromatase in the cytoplasm of epididymis epithelial cells as well as and in large dense vacuoles, few compared to reproductive period, suggest that this enzyme could be involved in the resumption of epididymal activity, although in this period spermatozoa are few and not useful for reproduction. This spermatogenesis resumption is not useful for reproduction and its biological meaning could be linked to an evolutionary memory of a previous condition in which reptiles exhibited an annual bimodal reproduction.<sup>31</sup>

Furthermore, it is known that at level of efferent duct the expressions of Aquaporin 1-9 is under the control of estrogens, therefore the aromatase present in the spermatozoa could be involved in the control of water resorption from the seminal fluid as well as the secretion of Cl<sup>-</sup> ions, as demonstrated in other systems.<sup>53</sup> In fact, mice deficient in ER $\alpha$  are infertile because present at the level of epididymis a seminal fluid most diluted and less qualitative.<sup>54</sup>

Moreover, the different localization showed in *P. sicula* during the various moment of reproductive cycle, with more wide presence in the reproductive period, is in agreement with distribution of P450 aromatase in the male reproductive system showed in other seasonal breeders.<sup>55-58</sup>

Finally, the wider distribution of the enzyme in the epididymis

of *P. sicula* compared to *R. rattus*, demonstrated that aromatase could play a key role in the control of structural and functional variations in the epididymis of a seasonal reproducer compared to a continuous reproducer. In fact, it is also possible to hypothesize that during the evolution, epididymis aromatase role has been reduced to advantage of other molecules, since in continuous reproducer the enzyme presents a more limited distribution.

In conclusion, P450 aromatase presence in *P. sicula* and *R. rattus* epididymis, strongly suggests that 17 $\beta$ -estradiol produced by enzyme could be involved in the control of reproduction, mainly in sperm maturation and fertilization, as well as in sperm mobility during the transfer across the epididymis.

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