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D-Asp: A new player in reproductive endocrinology of the amphibian *Rana esculenta*☆

Franca Raucci, Maria Maddalena Di Fiore*

Department of Life Sciences, Second University of Naples (SUN), Via Vivaldi 43, 81100 Caserta, Italy

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ABSTRACT

We investigated the involvement of D-Aspartic acid (D-Asp) on ovarian and testicular morphology of the green frog, *Rana esculenta,* and its effect on the testosterone production. The study has been performed throughout the reproductive cycle. In both ovary and testis a substantial amount of D-Asp is endogenously present and its concentration varies as function of reproduction. In the frog, D-Asp content is differently correlated with gonadal and plasmatic levels of testosterone, depending on the sex. In fact, the amount of the D-Asp is inversely linked with that of the testosterone in the ovary, while this correlation directly matched in the testis. *In vivo* short-term experiments, consisting of a single intra-peritoneal injection of D-Asp (2.0 μmol/g body weight), demonstrated that the enantiomer is significantly accumulated by both the ovary and testis, reaching after 3 h the highest uptake and thereafter decreasing to baseline values within 24 h. Furthermore, D-Asp influences the synthesis and/or the release of testosterone, causing a decrease of its level in the female, and an increase in the male, respectively. *In vivo* long-term experiments, D-Asp, chronically administered to the frogs of both sexes, enhances the maturation of both gonads, determining in the oocytes an higher accumulation of carbohydrate yolk plates in the ooplasm, and stimulating the spermatogenesis in the testis. Taken altogether, our results show that D-Asp operates differently in female and male frog gonads, indicating that it has different targets in the reproductive machinery depending on the sex.

1. Introduction

D-Aspartic acid (D-Asp) is an endogenous amino acid occurring as a free compound in several animal phyla [1]. In rat brain, this enantiomer has been localized in various neurons, including the hippocampus, but also in the hypothalamo-neurohypophyseal system suggesting its role in neurotransmission and neurosecretory activities [2–5].

However, the occurrence of D-Asp is not only restricted to the nervous tissues. Several evidences have established its involvement in endocrine system. In fact, the relationship between D-Asp and endocrine activity has been documented in the adenohypophysis, in the pineal gland and in the testis, where D-Asp is involved in hormone synthesis and release [4–9]. Specifically to rat testis, developmental changes in the levels of D-Asp and testosterone parallel each other closely: they both increase to maximum levels at sexual maturity. Moreover, D-Asp is contained in spermatids [10] and in both Leydig [6,11] and Sertoli cells [6]. Experiments performed either *in vitro* on isolated testis [12] or *in vivo*, by intraperitoneal administration of D-Asp to adult male rats [4], showed that this D-amino acid is accumulated by the gonad, and also induces a significant increase in luteinizing hormone, testosterone, progesterone [4] and prolactin [7] in the blood. These findings propose a novel role for D-Asp as a regulator of reproductive activity, suggesting a specific implication in the local regulation of androgen production. Our research group has focused some studies on the role of D-Asp in the gonads of the lizard, *Podarcis sicula*, a seasonal breeding vertebrate. In lizard female, D-Asp enhances follicular production of 17β-estradiol by up-regulating the local aromatase activity [13] and also promotes the maturation of oocyte follicular epithelium [14]. Furthermore, it has been demonstrated that D-Asp is rapidly taken up by Leydig cells of injected lizards and its rise is coupled with a significant increase in testosterone levels with a consequent augment of mitotic activity of the germinal epithelium of the testis [15,16]. On the other hand, D-Asp may play a role in hormonal regulation, as it stimulates testosterone synthesis in the testis, increasing the mRNA level of a steroidogenic acute regulatory protein (StAR) [17]. In our previous papers, the role of D-Asp in endocrine control of reproduction has been also investigated in the gonads of *Rana esculenta* [18,19]. However,

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* Corresponding author. Tel.: +39 0823 274527; fax: +39 0823 274571.
E-mail address: mariam.difiore@unina2.it (M.M. Di Fiore).

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although those pioneer studies clearly suggest a function of the D-enantiomer in local hormonal secretion, they miss information concerning the specific operating target, if any, of D-Asp in the physiology of reproduction in the different sexes. Thus, our intend here is to extend and complete the study of the role of D-Asp in the both ovary and testis of *R. esculenta*, in order to investigate if this D-amino acid has different targets in the reproductive machinery of female and male, respectively. Therefore, we first morphologically characterized the ovary and testis in different phases of reproductive cycle of *R. esculenta*. Then, we examined the occurrence of endogenous D-Asp in the gonads of both sexes as function of testosterone titers. To gain insight into the functional significance of D-Asp in the local production of testosterone, we also studied the uptake of D-Asp in the ovary and testis, respectively; after that we examined its role in the androgen output. Moreover, to extend the knowledge on the characterization either on ovarian or testicular activity, we described the histological and histochemical features of gonads as a consequence of D-Asp treatment. These observations have been carried out either throughout varied phases of the reproductive cycle and/or following *in vivo* experimental conditions.

2. Materials and methods

2.1. Reproductive aspect and sexual cycle of R. esculenta

The green frog *R. esculenta* is a seasonal breeder living in the Mediterranean area. Adult animals have a body length of about 6.5–7.0 cm and a body weight of about 8.5–10.5 g. The reproductive cycle of this amphibian begins at the end of 2nd year of age and includes the transition between high and low activities of the gonads. Briefly, the sexually quiescent frogs emerge in January–February (pre-reproductive period); soon, gonads and secondary sexual characteristics (SSC) start their development and in little time become reproduction functional (March–June, reproductive period). In this period, the frogs mate and eggs are deposited. Then from July to December (post-reproductive period), despite the still favorable temperature and photoperiod, gonadal activity gradually declines and SSC regress [20,21].

In the female, during the sexual cycle, besides specific modifications in plasma progesterone and 17β-estradiol concentrations, a sustained titer of circulating testosterone occurs and it is greater than that found in the male [22]. After few weeks of gonadal quiescence in summer (post-reproductive period), the vitellogenesis slowly resumes and, starting in late October, a set of new follicles progressively grows to become ripe at the end of the next winter (recovery period) [23,24]. In the female, increased concentrations of plasma testosterone are observed during the recovery period and in the early phase of the reproductive period [22,23].

In the male, plasma androgen titers are low in pre-reproductive period; then they rapidly increase in early spring becoming reaching higher concentration in reproductive period. After the reproductive period they start to fall, recording the lowest value in post-reproductive period. The rise of testosterone level during the reproductive phase is beneficial for spermatogenesis and for the development and maturation of the SSC [20,21]. Also in the testis, Raucci et al. [19] demonstrated that the augment of testosterone titers causes an increase of seminiferous ampulae area, in the amount of spermatid and sperm contents.

2.2. Animals

Adult females and males of the green frog *R. esculenta* were caught in marshes (Castelvolturno, Italy) during the three main phases of their reproductive cycle, namely, October (pre-reproductive period), February–March (reproductive period) and July (post-reproductive period). Some animals were killed soon after capture, whereas others were transferred to the laboratory terrarium and maintained in a photothermal regime identical to the corresponding period of the year. Captive frogs were fed of meal worms and *Drosophila* fruit flies *ad libitum*. Mortality rates were low (<8%). These frogs were used for *in vivo* experiments. To evaluate the D-Asp and testosterone content in the gonads and testosterone level in the blood, ten animals of both sexes and, for each phase of their sexual cycle, were utilized soon after capture. They were anesthetized through immersion in a 1:1000 tap water solution of MS-222 (tricaine methylsulfonate, Sigma). Thereafter, blood was collected through the insertion of a heparinized glass capillary in the heart conus arteriosus; the blood samples were then centrifuged to obtain plasma, which was frozen in liquid nitrogen. Ovaries and testes were removed and immediately frozen in liquid nitrogen and/or fixed by immersion in Bouin's fluid.

2.3. In vivo short-term experiments

To determine the D-Asp effect on gonadic testosterone production, both short-term and long-term experiments were carried out. In the first experiment (short-term), 60 female and 60 male frogs collected during pre-reproductive phase and post-reproductive periods, respectively, were distributed into two groups ($n = 10$ each). Animals from the first group ($n = 10$) were injected i.p. with 2.0 μmol D-Asp (Sigma)/g body weight dissolved in 0.25 ml amphibian saline. This dose was chosen on the basis of preliminary experimental tests. Ten injected frogs from each group were killed at different set times within a period of 24 h (0, 3, 6, 9, 12 and 24 h after D-Asp injection, respectively). The animals of the second group ($n = 10$) received 0.25 ml amphibian saline alone, and, therefore, were used as controls. The frogs were dissected, and gonads (oocytes and testis) and plasma were obtained as reported above and stored in liquid nitrogen until use.

2.4. In vivo long-term experiments

In another set of experiments, ten frogs, separated by sex, were sorted into groups, each formed by five animals. The first received i.p. 2.0 μmol/g body weight of D-Asp dissolved in 0.25 ml of amphibian saline, every 2 days for 2 weeks. The frogs of second group were injected with solvent alone (physiological saline), and they were used as controls. Two days after the last injection, the animals were killed and used as reported above. The testes and the oocytes were rapidly fixed and processed for histology and histochemistry as below.

The methods of capture and dissection and the captive rearing conditions were in accordance with Italian law (D. L.vo 116/92) and authorized by the appropriate Italian government administrative office (Servizio veterinario della A.S.L. 44, Prot. Vet. 22/95).

2.5. Tissue preparation for D-Asp determination

Samples of testis and ovary were homogenized with 0.2 M perchloric acid (PCA) in a 1:10 ratio and centrifuged at 30,000 × *g* for 20 min. The supernatant was loaded on a column of cation-exchange resin (AG 50W-X8 resin, hydrogen ionic form, 200–400 mesh, Bio-Rad), using a column of 1 cm diameter and an amount of resin corresponding to 1 ml of settled resin for each ml of PCA supernatant. The resin was previously activated by treatment with 6 M HCl and then washed with 0.001 M HCl. Next, the samples were absorbed on the resin, and the column was washed with 10 ml of 0.01 M HCl and then eluted with 8 ml of 4 M NH_4OH. The ammonia eluted was dried by evaporation in small Petri dishes on a hot plate at 40–60 °C under a hood. The dry residue was dissolved in 2 ml distilled water, which contained free amino acids without salts,

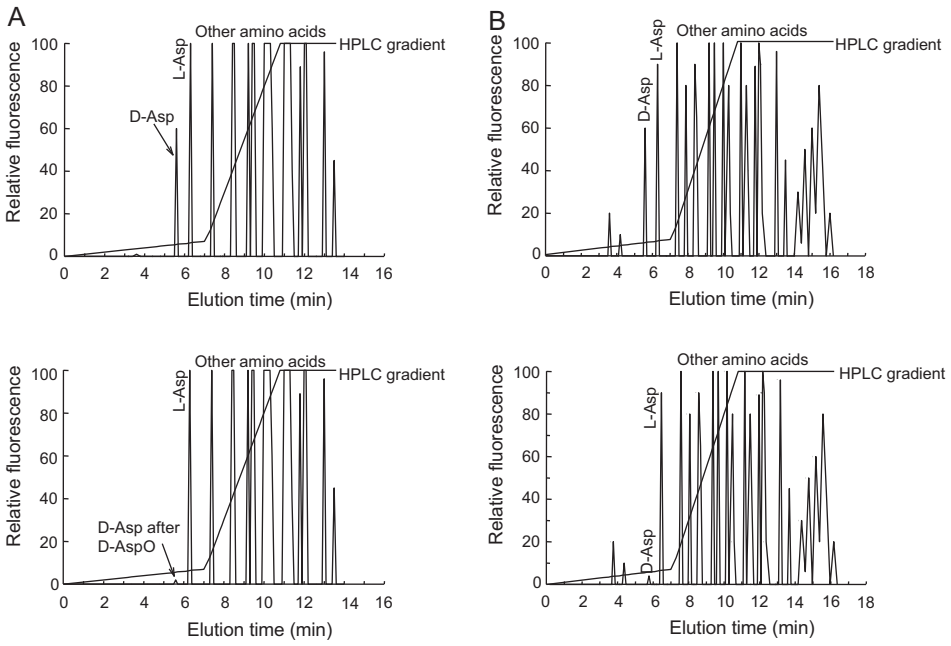

Fig. 1. HPLC determination of D-Asp. Panel A, upper part: Typical example of HPLC analysis of D-Asp and other amino acids of a standard amino acids mixture consisting of 50 pmol of D-Asp and 100 pmol of each of the following amino acids: L-Asp, L-Glu, L-Ser, L-Thr, Gly, L-Ala, L-Cys, L-Val, L-Met, L-Tyr, L-Leu, L-Phe, L-His and L-Lys. Panel A, lower part: The same mixture of amino acids standard as in upper part, but after incubation with D-aspartate oxidase (D-AspO). Panel B, upper part: HPLC analysis of free amino acids from a testis tissue. The amount of amino acids injected corresponds to the amino acids came from 0.1 mg of fresh tissue. Panel B, lower part: The same sample as in panel upper part, but after treatment with D-AspO.

lipids and other organic compounds. However, these samples still contained pigments and molecules that can interfere with the enzymatic assay of D-amino acids. Therefore, they were still purified on an Octadecyl C-18 resin as follows. The samples were slowly passed through a Sep-pak C-18 cartridge containing 400–600 mg resin (the cartridge had been previously activated with methanol or acetonitrile and washed with distilled water). After the samples had passed through the cartridge, the cartridge was washed with 2 ml distilled water, and all eluates were combined and concentrated to 200 μl with a Savant centrifuge or left to evaporate in small Petri dishes at 40–50 °C under the hood.

2.6. HPLC method for the determination of D-aspartic acid

The content of D-Asp in the purified sample was determined specifically by the HPLC method associated with the use of the enzyme D-aspartate oxidase (D-AspO) (EC 1.4.3.1.). This procedure is based on the chromatographic separation of D-Asp from other amino acids and through the oxidation of D-Asp with D-AspO, as previously described [4]. The purified D-AspO enzyme was obtained by overexpression and purified according to the described procedure [25].

Briefly, 5–20 μl of the sample obtained as previously described (Section 2.5) was mixed with 2–10 μl of 0.1 M NaOH (to bring the pH to 9.0) and 0.01 M sodium borate buffer (pH 8.0) to obtain a final volume of 100 μl. Finally, 5 μl of OPA-NAC reagent (prepared by mixing 20 mg of OPA and 10 mg of NAC in 4 ml 50% methanol) was added; after 2 min, 20 μl was injected onto a C-18 Supelcosil HPLC column (0.45 cm × 25 cm, Supelco Inc., Belafonte, PA) using the Beckman-Gold HPLC system. The column was eluted with a gradient consisting of solvent A (5% acetonitrile in 30 mM sodium citrate buffer, pH 6.0) and solvent B (90% acetonitrile in distillate water), as follows: 0–7% B over 7 min, then to 100% B over 4 min, staying at 100% B for 4 min, and returning to 0% of B in 2 min at a flow rate of 1.2 ml/min. Amino acid derivatives were detected fluorometrically using a wavelength at 330 nm excitation and 450 nm emission. A standard curve was obtained under the same conditions using 5 μl of a standard solution of an amino acid mixture consisting of 50 pmol of D-Asp and 100 pmol of each of the following amino acids: L-Asp, L-Glu, L-Ser, L-Thr, Gly, L-Ala, L-Cys, L-Val, L-Met, L-Tyr, L-Leu, L-Phe, L-His and L-Lys. It was observed that D-Asp eluted at 5.5 min, L-Asp at 6.5 min, followed by the other amino acids (Fig. 1). To determine the amount of peak area due to D-Asp, a parallel sample was incubated with 2 ml of purified D-AspO for 15 min at 37 °C and chromatographed as above. The total disappearance or the reduction of peak area corresponding to D-Asp elution peak confirmed the presence of D-Asp and gave the exact amount of the content of D-Asp.

Recovery studies were performed by adding D-Asp at different concentrations to a sample homogenate, and D-Asp was determined before and after addition of D-AspO. The percent (%) recovery obtained was 94–108%. The inter-assay coefficient of variation (CV) was evaluated at several points along the standard curve in three different assays. The CV was 4.5–8.5% in the range 10–100 pmol of D-Asp. The data were statistically analyzed by the STATVIEW program, version 4 (Abacus Concepts, Berkeley, CA, USA). This method allowed us to determine a minimum amount of D-Asp corresponding to a 10 pmol/assay mixture.

2.7. Testosterone assay in gonads and in plasma

Sex steroid in the gonad and plasma was determined with enzyme immunoassay (EIA) kits (Biochem Immuno Systems, Bologna, Italy). The sensitivity of testosterone detection was 6 pg (intra-assay variability 5.6%, interassay variability 9.3%). The addition of D-Asp to the standard curve did not modify the assay sensitivity. Samples (ovaries and testes) were homogenized 1:5 (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. Plasma samples (100–200 μl) were vortexed with ethyl ether (1:10, v/v) for 5 min and centrifuged

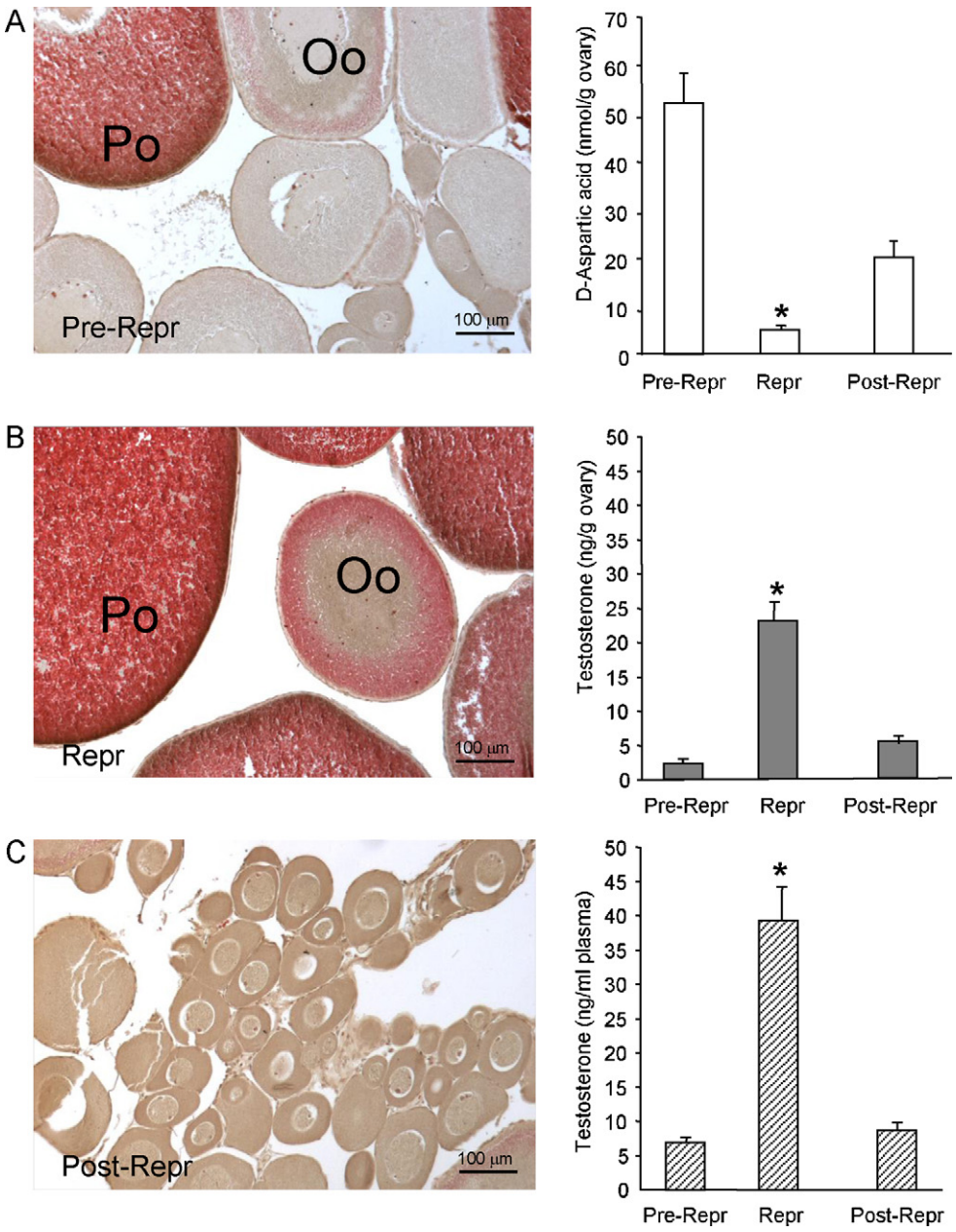

Fig. 2. Oocyte morphology. Sections of follicles of *Rana esculenta* during pre-reproductive, reproductive and post-reproductive periods. Haematoxylin/eosin. Concentrations of D-aspartic acid in the ovary (A), and ovarian (B) and plasma (C) titers of testosterone in female green frog, *Rana esculenta*, during the sexual cycle. Each point represents the mean value ± S.D. from ten individual female frogs. Pre, pre-reproductive period; Repr, reproductive period; Post, post-reproductive period. *$P < 0.01$. Oo = oocyte; Po = peripherical ooplasm.

at 3000 × *g* for 10 min. The upper phase (ethyl ether) was transferred to a glass tube. Two extractions were performed. The pooled ether phases were left to evaporate on a hot plate at 40–50 °C under a hood. The residue was dissolved in a 0.5 ml sodium phosphate buffer 0.05 M, pH 7.5, containing BSA at a concentration of 10 mg/ml, and then utilized for the assay.

Testosterone recovery was 80% from tissues and 85% from plasma. Pooled ether extracts were dried and then utilized for the enzyme immunoassay as previously reported.

2.8. Histology and histochemistry

After dissection, ovaries and testes were rapidly immersed in Bouin's fluid. Gonads of both sexes were serially sectioned at 5 μm and stained with haematoxylin/eosin (H/E) and trichrome-Mallory. Oocyte polysaccharides were detected by PAS reagent [26] (Humason 1979) and alcian blue/PAS (AB/PAS) [27] (Bancroft and Stevens 1990). Follicular stages were classified as described by Rastogi et al. [23].

2.9. Statistical analysis

The data were expressed as mean ± S.D. for ten animals per group. Differences between groups were then evaluated by one one-way ANOVA followed by Duncan's multiple-range test. The criterion for significance was $P < 0.05$ and $P < 0.01$.

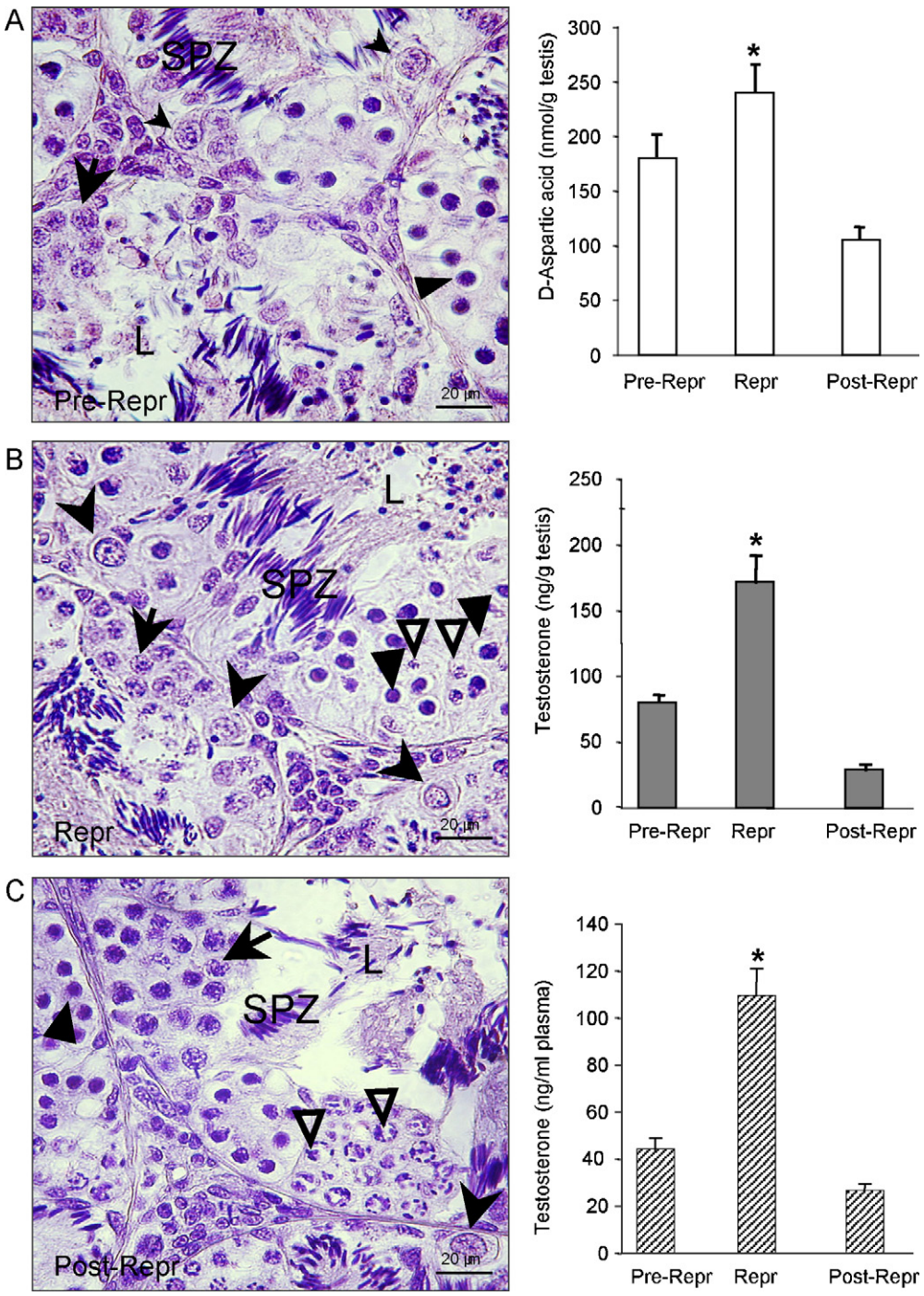

Fig. 3. Testis morphology. Histological sections of testis of *Rana esculenta* during pre-reproductive, reproductive and post-reproductive periods. Arrowhead, I SPG; arrow, II SPG; ▲, I SPC; △, II SPC; L, lumen. Haematoxylin/eosin. Seasonal changes in D-Aspartic acid (A) and testosterone content detected in both testis (B) and plasma (C) of green frog *Rana esculenta*, during the reproductive cycle. Results are expressed as mean ± S.D. obtained from ten individual male frogs. Pre, pre-reproductive period; Repr, reproductive period; Post, post-reproductive period. *$P < 0.01$.

3. Results

3.1. HPLC profile in a standard amino acids mixture and in a sample testis tissue before and after the incubation with D-aspartate oxidase

Fig. 1 shows a typical example of HPLC analysis of D-Asp. The panel A reports the separation peaks of D-Asp and of L-amino acids (L-Asp, L-Glu, L-Ser, L-Thr, Gly, L-Ala, L-Cys, L-Val, L-Met, L-Tyr, L-Leu, L-Phe, L-His, L-Lys) in a standard mixture before (upper part) and after (lower part) incubation with D-aspartate oxidase (D-Asp). In this last case the peak corresponding to D-Asp (retention time, 5.5 min) is disappeared, indicating thus that it was actually D-Asp. Fig. 1 panel B, shows HPLC analysis of free amino acids from a sample of testis tissue before (upper part) and after (lower part) treatment with D-AspO. The reduction of the peak

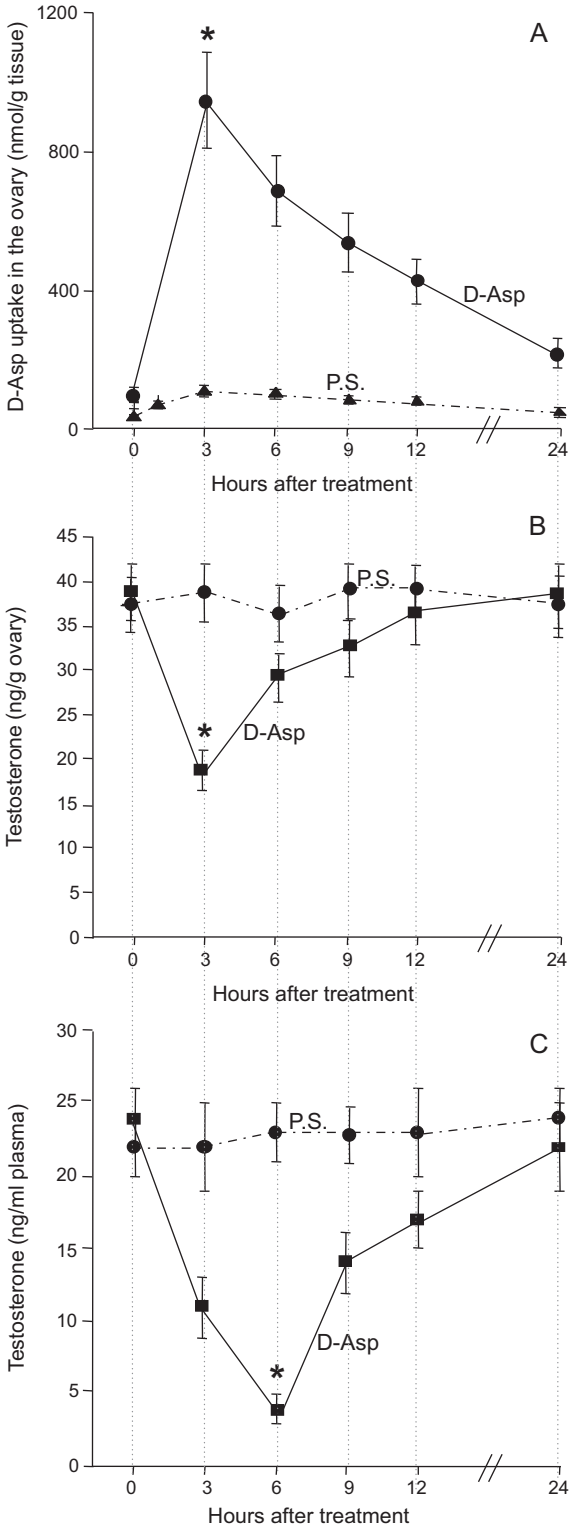

Fig. 4. Trend of ovarian D-Asp (A), ovarian testosterone (B) and plasma testosterone (C) in *Rana esculenta* after the injection of the D-Asp (2.0 μmol/g body weight, black line) and vehicle alone (control, dashed line) at different set time (from 0 to 24 h). Data are the mean ± S.D. obtained from ten individual animals. Black line, treated animals; dashed line, control. $^{*}P < 0.01$.

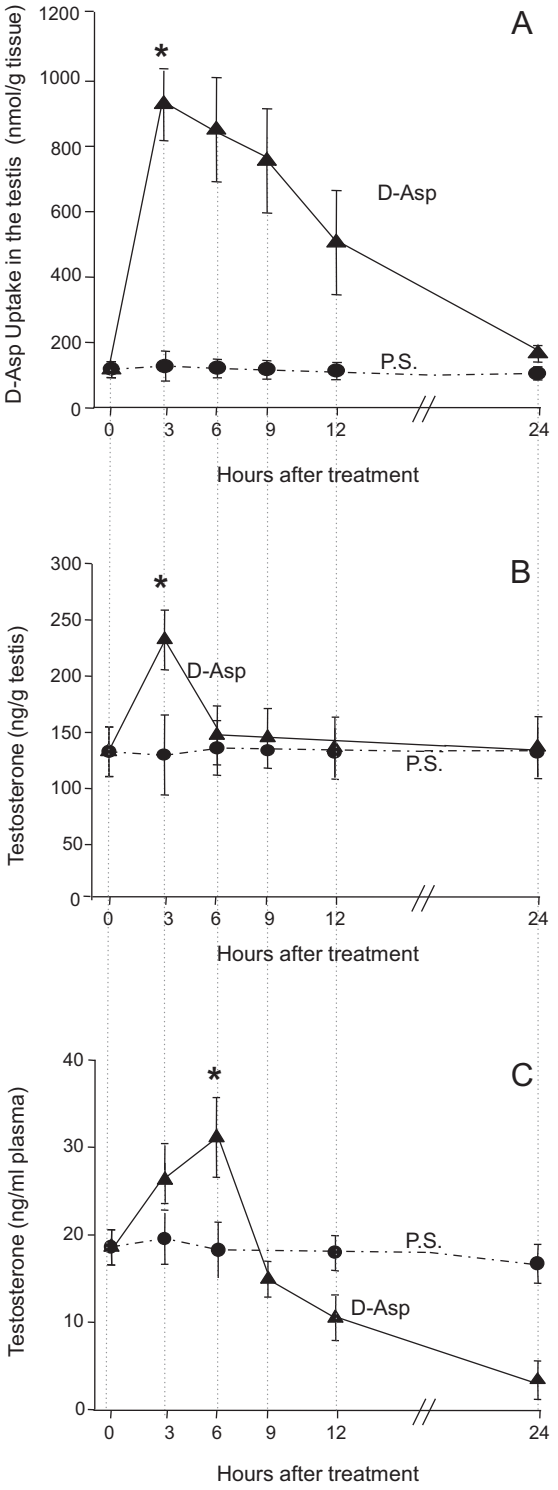

Fig. 5. Pattern of uptake of testicular D-Asp (A) and concentrations of testosterone in the testis (B) and plasma (C) of *Rana esculenta* after initial administration of the D-Asp (black line) and in the control (dashed line). Each value represents mean ± S.D. of ten determinations; $^{*}P < 0.01$.

at retention time of 5.5 min, demonstrated the occurrence of D-Asp in the sample and allowed to calculate the concentration of this D-amino acid in the sample by comparison with the standard curve.

3.2. Seasonal morphological changes of oocytes and trends of both D-Asp and testosterone during the reproductive cycle

The present morphological observations confirmed those already reported in the literature for this species. The ovaries of *R. esculenta* undergo an annual cycle of growth and developmental maturation depending to the reproductive season [23]. At pre-

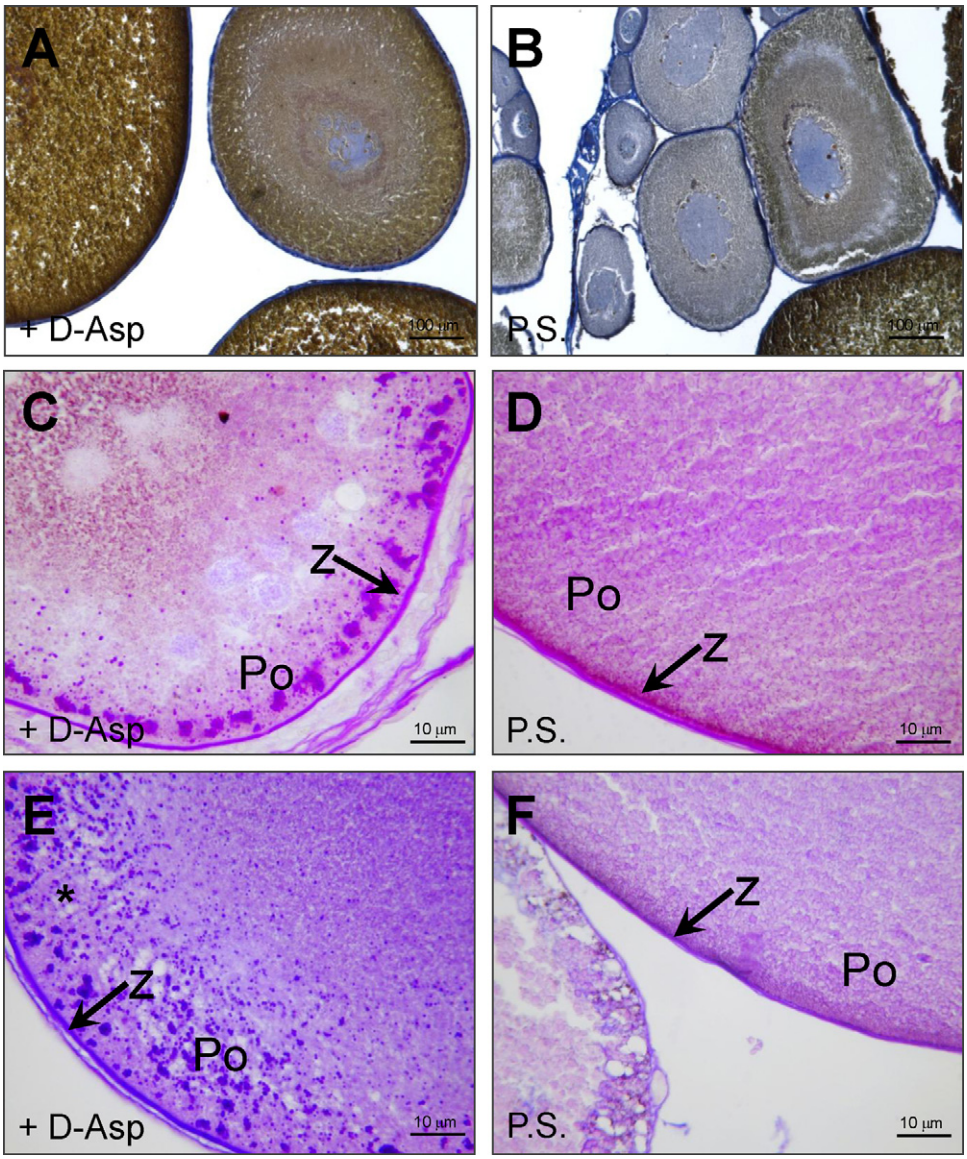

Fig. 6. Histological sections of D-Asp-treated (A) and control (B) oocytes of pre-reproductive frog. Mallory. D-Asp histochemistry in pre-reproductive oocytes in response to *in vivo* treatment. In the treated frogs (+D-Asp), an PAS-positive (C) and alcianophilia (E) are localized in proximity (asterisk) and at level to the zona pellucida (z) as compared with the control (D and F, respectively). C, D; E, F = alcian blue/PAS; Po = peripheric ooplasm; z = zona pellucida.

reproduction (Fig. 2, pre-repr), the majority of follicles appear to be pre-vitellogenic [23], although very few of them show some typical feature of vitellogenic follicles and other are in quiescent state. They present a germinal vesicle eccentrically located in the ooplasm and no estimable accumulation of yolk plates is observed in the oocyte (Oo) cytoplasm. Externally, they were covered by a single layer of flattened granulosa cells and by a thin theca layer. At reproduction (Fig. 2, repr), the follicles appeared fully engaged in vitellogenic. The ooplasm contained an abundance of fibrillar basophilic granules eosin-positive which appeared located in the oocyte cytoplasm with particular accumulation at the periphery (Po). Furthermore, in some oocytes the central ooplasm exhibited finely granular cytoplasm moderately eosin-affine. At this phase the zona pellucida is fully distinguishable. In post-reproductive frogs (Fig. 2, post-repr) the follicles were smaller and no indication of vitellogenesis was observed.

Fig. 2 also shows the endogenous level of D-Asp in the ovary and the concentration of testosterone in both gonad and plasma of female frogs during the sexual cycle. As indicated in Fig. 2A, in all phases of the cycle, substantial amount of free D-Asp were found in the ovary extract. This amino acid level showed significant variations depending on the phase of reproductive cycle. The highest value of D-Asp in the ovary was detected during pre-reproductive period (Fig. 2A, 52 ± 6 nmol/g ovary). At this time, testosterone content was the lowest recorded though the annual cycle (Fig. 2B, 2.3 ± 0.2 ng/g ovary; Fig. 2C, 7.1 ± 0.6 ng/ml plasma). At reproduction, D-Asp content dramatically decreased (Fig. 2A, 5 ± 1 nmol/g ovary), while that of the testosterone appeared the highest in both ovary (Fig. 2B, 23.1 ± 2.8) and plasma (Fig. 2C, 39.6 ± 4.8). Finally, during post-reproductive period, D-Asp level moderately increased (Fig. 2A; 20 ± 3 nmol/g ovary). At this stage, ovarian and circulating testosterone contents (Fig. 2B, 5.4 ± 0.5, ovary; Fig. 2C, 8.9 ± 0.9 plasma) were moderately low as compared with those observes during the reproductive period (Fig. 2B vs C).

3.3. Seasonal morphological changes in the testis and D-Asp and testosterone profiles during the reproductive cycle

Fig. 3 reports morphological modification of germinal epithelium of the testis through the sexual cycle. The testis of green

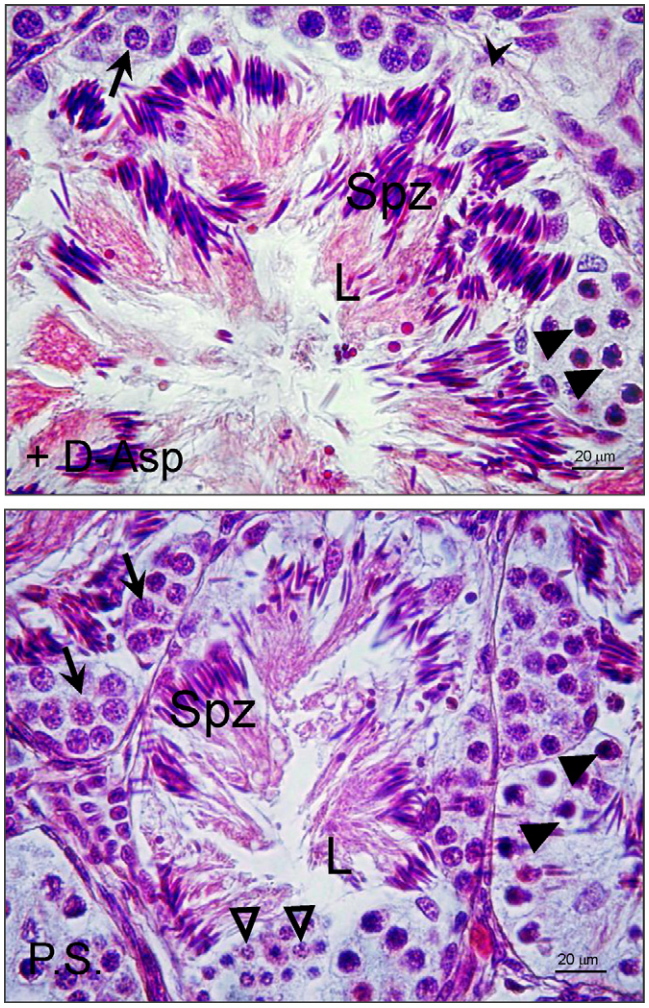

Fig. 7. Histological sections of D-Asp-treated (+D-Asp) and control (P.S.) testis of post-reproductive frogs. Haematoxylin/eosin. Seminiferous ampoules of treated animals (+D-Asp) showed a higher presence of the spermatozoa (spz) in the lumen (L) than the control (spz, P.S.). Arrowhead, I SPG; arrow, II SPG; ▲, I SPC; △, II SPC; L, lumen.

frog *R. esculenta* shows a cystic pattern, i.e. the germ cells proliferate in coordinated clusters enclosed in membranous cyst walls (ampoules). In the pre-reproductive period (Fig. 3, pre-repr), seminiferous ampoules of testis contained predominantly primary (I) and secondary spermatogonia (II SPG) (Fig. 3, pre-repr, arrowhead and arrow, respectively). Although primary (I, ▲) and secondary spermatocytes (II SPC, △) and spermatids (SPT) were present, their number was low because they underwent degeneration (Fig. 3, pre-repr, I SPC, ▲; II SPC, △). Some spermatozoa (SPZ) were present in the lumen (L). In the reproductive period (Fig. 3, repr), testicular epithelium contained the maximal number of I SPG (arrowhead) and appeared highly differentiated. Several II SPG (Fig. 3, repr, arrow) were present in the cysts and numerous I SPC were also detected (Fig. 3, repr, I SPC). Furthermore, II SPC were evident (Fig. 3, repr, II SPC, △) and the lumen (L) was full of SPZ. In the post-reproductive period (Fig. 3, post-repr), high numbers of cysts still contained I and II SPC although their decrease was closely associated with the formation of SPT. Not many SPZ were present in the lumen (L).

Fig. 3 also reports the fluctuations of both D-Asp and testosterone contents during the seasonal reproductive cycle. Testicular free D-Asp content varies as function of the reproductive cycle (Fig. 3A, pre-reproductive period, 105 ± 11 nmol/g testis; reproductive period, 240 ± 25 nmol/g testis; 105 ± 11 nmol/g testis). However, its variations in the testis positively matched with that of the testosterone. In fact, this hormone titer was higher during the reproductive phase in the testis (Fig. 3B, 17.2 ± 2.1 ng/g tissue) and in the blood (Fig. 3C, 109.5 ± 11.3 ng/g tissue). Otherwise, in the pre- and, particularly in the post-reproductive periods, the androgen titer was significantly lower than the reproductive period ($P < 0.01$), respectively (pre-reproductive: Fig. 3B, 8.0 ± 0.6 ng/g testis; Fig. 3C, 44.3 ± 5.2 ng/ml plasma; post-reproductive: Fig. 3B, 2.9 ± 0.4 ng/g testis; Fig. 3C, 26.7 ± 3.1 ng/ml plasma).

3.4. *In vivo short-term experiments on frog female: D-Asp uptake and testosterone level*

In the *in vivo* experiments, in which pre-reproductive female frogs were injected with D-Asp (2.0 μmol/g body weight), the administration of this amino acid was followed by a significant and fast accumulation of it in the ovary. In fact, D-Asp was rapidly taken up by the ovary, so that 3 h after injection, its concentration in the ovary changed from 52 ± 6 nmol/g tissue to 967 ± 117 nmol/g tissue (Fig. 4A, $P < 0.01$). At 6 h D-Asp content was still high (Fig. 4A, 672 ± 105 nmol/g tissue, $P < 0.01$). After that, D-Asp content progressively decreased reaching baseline values within 24 h (Fig. 4A, 210 ± 52 nmol/g tissue).

Fig. 4 also shows the variation of testosterone level following D-Asp injection in reproductive frogs. After a single injection of D-Asp (2.0 μmol/g body weight), the androgen content significantly decreases during the first 3 h in ovary (Fig. 4B, from 37.6 ± 3.2 to 18.4 ± 2.5 ng/g tissue, $P < 0.01$) and 6 h in plasma (Fig. 4C, from 23.1 ± 2.8 to 3.4 ± 0.4 ng/ml, $P < 0.01$), respectively. However, this effect appeared to be reversible, being androgen level restored within 24 h after the treatments (Fig. 4B, ovary and Fig. 4C, plasma, respectively).

3.5. *In vivo short-term experiment on frog male: D-Asp uptake and testosterone level*

Fig. 5 reports the profile of D-Asp uptake by the gonad (Fig. 5A) together with those of the testosterone levels in the gonad (Fig. 5B) and in the plasma (Fig. 5C) of male frogs caught in the post-reproductive period and injected with a single dose of exogenous D-Asp (2.0 μmol/g body weight) or saline solution alone (P.S.). As shown in Fig. 5A exogenous D-Asp was rapidly taken up by the testis, reaching the highest accumulation after 3 h from the injection. At 6 h D-Asp content is still high; thereafter, it slowly decreased at 9 and 12 h, until to progressively reach the basal values within 24 h. During the first 3 h from D-Asp treatment, also testicular content of testosterone increased, while in the plasma the highest content of the androgen was observed at 6 h. Afterward, testosterone levels in both testis and plasma returned to baseline within 24 h.

3.6. *In vivo long-term experiment of frog female: ovary histochemistry*

D-Asp treatment affects the histochemical proprieties of oocyte (Fig. 6). D-Asp-treated pre-reproductive follicles (Fig. 6A) were more mature than the control (Fig. 6B) and acquired morphological features typically of reproductive follicles (compare Fig. 6A with Fig. 2, repr-period). Histochemically, a stronger PAS and AB/PAS staining were observed in the ooplasm of treated oocytes with an evident accumulation in proximity of peripheric ooplasm (Po) and zona pellucida (z) (Fig. 6B vs D, treated and control, respectively). In addition, an alcianofilia and PAS positivity were noticed at level of zona pellucida (z) of treated follicles (Fig. 6C and E, z, respectively)

while the relative controls appeared moderately stained (Fig. 6D and F, z, respectively).

3.7. *In vivo long-term experiments on frog male: testis morphology*

In another set of experiments, the testes of post-reproductive frogs were utilized for morphologic studies. As shown in Fig. 7A, in D-Asp-treated animals seminiferous ampoule activity rescued and the testis contained a numerous spermatozoa (SPZ) in the lumen as compared with the control (Fig. 7B). In the seminiferous ampoules of treated animals, several I and II spermatocytes were also found (Fig. 7).

4. Discussion

Seasonal breeders are good models for studying the involvement of D-Asp on the gonads because the effects of this molecule can be compared in response to the different phases of reproductive cycle. In our previous investigations we have already ascertained that in the amphibian *R. esculenta* the gonadal levels of this D-amino acid undergo regular changes in its concentration with regard to the reactivation of both ovarian and testicular functions [18,19]. Here, we report new results concerning not only the local action of D-Asp on the gonadal production of testosterone, but also showing the morphological changes caused by D-Asp on both ovary and testis. In this regard, the data presented in current paper mainly enlarge the information on the physiological role of the D-Asp in the gonad and give a deeper knowledge of the action of this D-amino acid in the reproduction of *R. esculenta* which appear to be different as function of the sex. In fact, both ovary and testis of *R. esculenta* show a very high and relatively rapid ability to take up and accumulate exogenously administered D-Asp. The comparison of D-Asp trend with testosterone content, in both gonads and plasma concentrations, show a differential relationship between the D-amino acid and the androgen hormone. More interestingly, this observation led us to propose that D-Asp is endowed with the control of the synthesis and/or the release of testosterone hormone by the gonad as function of the sex. In the ovary, in fact, a reverse relation is founded between D-Asp and testosterone levels during the cycle; conversely, in the testis this link results to be direct. The physiological mean of D-Asp in the gonadal functions has been ascertained by *in vivo* experiments. In the ovary, the exogenous D-Asp induces a significant decrease in both plasma and ovarian testosterone concentrations; vice versa, in the testis, D-Asp accumulation parallels with an increase of testosterone.

The results of present study agree with those obtained in lizard *P. sicula* [13–15] in which the administration of D-Asp causes in the female a decrease in testosterone content, while favoring in the male an increase of the androgen level. In the female lizard, the physiological mechanism underlying the inverse and direct relationship between D-Asp and sex steroid hormones was elucidated in our previous *in vitro* studies [13]. These data showed that when D-Asp is added to an incubation medium containing testosterone and either homogenate or acetone powder of follicles, the aromatase enzyme was 4-fold greater in the presence of D-Asp than in its absence. However, we cannot exclude the possibility that D-Asp can enhance steroidogenesis by affecting cholesterol production, and future studies will be necessary to explore this hypothesis.

As a direct consequence of D-Asp *in vivo* injection and/or an indirect effect of this amino acid on local testosterone production, the morphological profile of male and female frog gonads results modified. In D-Asp-treated pre-reproductive female the D-amino acid enhanced the maturation of the follicles promoting either a growing or the accumulation of nutritive substances in the oocyte cytoplasm. In fact, D-Asp-treated pre-reproductive follicles show a consistent accumulation of PAS positive granules in the cortical region of the ooplasm, a typical histochemical of feature of follicles engaged in the vitellogenesis [23].

In D-Asp-treated pre-reproductive male, the testis shows a cytological pattern of a reproductive gonad, with developed seminiferous ampoule and many spermatozoa located in the lumen. In the lizard, it has been recently shown that exogenous D-Asp raises the mitotic activity of the germinal epithelium by a sequential enhancement of the levels of expression of c-kit receptor protein, an augment of tyrosine kinases activity and an increase of proliferating cellular nuclear antibody (PCNA) expression level [16]. However, further studies are necessary to clarify the mechanism of action of D-Asp in the mitogen activity of the testis. Since androgens affect proliferation activity of the testis, an indirect effect of D-Asp on spermatogenesis cannot be excluded.

While considerable work still remains, evidences reported in this paper and in previous work to date, suggest that D-Asp is a critical regulatory molecule of gonads, having a different physiological role in female and male in term of testosterone production. An unresolved issue still remains to be elucidated: what is the mechanism of D-Asp action in both sexes? This is an intriguing question that deserves further study, which we are preparing to undertake.

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