Enhancement of aromatase activity by D-aspartic acid in the ovary of the lizard *Podarcis s. sicula*

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The present study investigated the role of D-aspartic acid (D-Asp) in ovarian steroidogenesis and its effect on aromatase activity in the lizard, *Podarcis s. sicula*. It was determined that D-Asp concentrations vary significantly during phases of the reproductive cycle: they vary inversely with testosterone concentrations and directly with oestradiol concentrations in the ovary and plasma. Experimental treatment showed that administration of D-Asp induces a decrease in testosterone and an increase in oestradiol, and that treatment with other amino acids (L-Asp, D-Glu and D-Ala) instead of D-Asp has no effects. Experiments *in vitro* confirmed these results. Furthermore, these experiments showed an increase in aromatase activity, as the addition of D-Asp either to fresh ovarian tissue homogenate or to acetonic powder of ovarian follicles induced a significant increase in the conversion of testosterone to oestradiol. Aromatase activity is four times greater in the presence of D-Asp than in its absence. However, almost equivalent values of the two K_m values (both approximately 25 nmol l^{-1}) indicate that aromatase has the same catalytic properties in both cases.

Introduction

The D-form of aspartic acid (D-Asp) has been found in the nervous and endocrine tissues of several invertebrate and vertebrate species (D'Aniello and Giuditta, 1977, 1978; Dunlop *et al.,* 1986; Neidle and Dunlop, 1990; D'Aniello *et al.,* 1992a,b, 1995; Hashimoto *et al.,* 1993a,b). It is generally accepted that, in nervous tissue, D-Asp can function either as a neurotransmitter or as a neuromodulator (for a review, see D'Aniello *et al.,* 1998). However, only a few studies have investigated the physiological role of D-Asp in endocrine tissue. These studies, mainly in rats, indicate that D-Asp is indirectly involved in steroidogenesis, in that it serves to activate the hormonal cascade of the hypothalamus–hypophysis–gonadal axis (D'Aniello *et al.,* 1996). In addition, studies *in vitro* on isolated rat testicular cells have shown that D-Asp plays a direct role in the synthesis of testosterone (Nagata *et al.,* 1999a,b). However, the physiological role of D-Asp has been studied only recently in a low vertebrate species, namely, the green frog, *Rana esculenta* (Di Fiore *et al.,* 1998a). It was found that in female frogs, D-Asp suppresses the production of testosterone in isolated ovarian follicles, and that this suppression occurs only when the ovarian follicles are engaged in active processes of vitellogenesis. This observation is significant as it has also been demonstrated that testosterone is involved in the induction of vitellogenesis in frog liver: specifically, testosterone is converted by a local aromatase to oestradiol, which is needed to activate vitellogenesis (Di Fiore *et al.,* 1998b; Assisi *et al.,* 2000). These findings indicate that D-Asp may enhance aromatase activity, and consequently decrease testosterone concentrations and increase oestradiol concentrations. Hence, it was hypothesized that D-Asp serves as a paracrine factor in the regulation of the vitellogenetic processes that characterize the reproductive cycle of low vertebrates. This hypothesis was tested in the oviparous lizard *Podarcis s. sicula,* because the hormonal activity controlling its vitellogenetic processes is well known (Licht, 1984; Paolucci, 1989; Paolucci and Di Fiore, 1994); therefore, this lizard provides a good model of vitellogenesis. Experiments were carried out *in vivo* and *in vitro* to test the hypothesis.

Materials and Methods

Animals

Adult female lizards were captured in the countryside (Caserta) during three main periods of their reproductive cycle: the pre-reproductive (October–March), reproductive (April–June) and post-reproductive (July–September) phases. In females at the pre-reproductive phase, both the ovary and the oviduct are quiescent. Only small pre-vitellogenetic follicles are present in the ovary. The ovary of females at the reproductive phase usually contains several growing

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follicles, and two or three mature follicles. The oviduct is well developed and secretory. In the late breeding phase, several post-ovulatory follicles can also be detected in the ovary. The ovary of females at the post-reproductive phase lacks growing or mature follicles, but contains several atretic follicles, in addition to many pre-vitellogenetic follicles. The oviduct is in a regressive phase.

Soon after capture, the lizards were divided into two groups. Lizards in the first group were anaesthetized by short cold exposure, and blood samples were collected by inserting a heparinized glass capillary into the heart. The blood samples were centrifuged at 800 g for 15 min and the plasma was stored at –20°C for analysis by sex steroid assay. The lizards were examined visually to determine the phase of development of their reproductive organs. The ovaries were excised and washed with cold isotonic saline solution (0.7% (w/v) NaCl) to eliminate any blood residue, weighed, and either used immediately for aromatase assay or stored at –80°C for the determination of D-Asp and hormonal content.

The second group of lizards was reared in a laboratory terrarium maintained at a photothermal regimen consistent with the period of the year, and fed mealworms and fresh vegetables *ad libitum*. Mortality rates were low (< 10%). These lizards were used for experiments *in vivo*.

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

Experiments *in vivo*

Experiments were conducted *in vivo* to determine the specificity and uptake time of D-Asp. Lizards were injected i.p. with an appropriate volume of a 0.5 mol l^{-1} amino acid solution in 0.7% saline to administer 2.0 µmol amino acid g^{-1} body weight, a dose chosen on the basis of preliminary experimental results. D- and L-amino acids (Sigma, Milan) were used for specificity testing. For determination of the uptake of amino acids, lizards were killed at set times within an 18 h period, and used in the first group described above. Control lizards were injected with saline. All tests were performed on groups of at least five animals.

Sex steroid assays in plasma and ovarian tissues

Plasma concentrations of sex steroids were determined using enzymeimmunoassay kits (Biochem Immuno Systems, Bologna). The following limits of detection were observed: for testosterone, assay sensitivity was 6 pg (intra- and interassay coefficients of variation were 5.6 and 9.3%, respectively); and for oestradiol, assay sensitivity was 6 pg (intra- and interassay coefficients of variation were 5.3 and 7.5%, respectively). The addition of D-Asp to the standard curve (range 0.01–1.0 µmol) did not modify the assay sensitivity (crossreactivity = 2%). Plasma samples (100–200 µl) were vortexed with ethyl ether (1:10; v/v) for 5 min and centrifuged at 3000 g for 10 min. The upper phase (ethyl ether) was transferred into a glass tube. Three 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 0.5 ml of 0.05 mol sodium phosphate buffer l^{-1}, pH 7.5, containing 10 mg BSA ml^{-1}, and then used for the assay.

Tissues were homogenized (1:5; w/v) with distilled water. The homogenate was 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. Recovery of sex steroids from the plasma and tissues was 85 and 80%, respectively. Pooled ether extracts were dried and used for the enzymeimmunoassay.

Preparation of samples for assay of aromatase activity

The ovaries were dissected and all vitellogenetic follicles were separated carefully under a light microscope using small scissors. All procedures were conducted at 4°C to minimize enzyme inactivation. Intact follicles were homogenized with Krebs–Ringer nutrient medium (1:1; w/v), supplemented with antibiotics (50 iu penicillin l^{-1}, 50 iu streptomycin l^{-1} and 100 iu nistatin l^{-1}; Life Technologies, Milan) and used for aromatase assay.

A second aromatase assay was performed using acetone powder rather than fresh tissue. Acetone powder was prepared by homogenizing follicles with cold acetone (1:3; w/v) and the suspension was centrifuged at 3000 g for 10 min. The extraction was repeated three times. Traces of acetone were removed from the pellets under a nitrogen flow and the pellets were stored at –20°C until use.

Assay of aromatase activity

Aromatase activity in vitellogenetic follicles was measured by evaluating the conversion rate of testosterone to oestradiol *in vitro* as described by Di Fiore *et al.* (1998b).

Preparation of samples for amino acid analysis

Tissues were homogenized with 0.5 mol perchloric acid (PCA) l^{-1} in a 1:10 ratio and centrifuged at 30 000 g for 20 min. The supernatant was adjusted to pH 7.5–8.5 by the addition of 5 mol KOH l^{-1}, cooled for 30 min at 0°C and the potassium perchlorate precipitate was removed by centrifugation as described above. The supernatant was adjusted to a pH of approximately 2.5 with 1 mol HCl l^{-1}, and the amino acids were purified on a cation exchange column (AG 50W-X8 resin, hydrogen ionic form, 200–400 mesh, Bio-Rad). The sample was loaded on to a column (1 cm $\times$ 3 cm) equilibrated with 0.01 mol HCl l^{-1}, and after washing with 10 ml of 0.01 mol HCl l^{-1}, the sample was eluted with 8 ml of 4 mol NH$_4$OH l^{-1}. The eluates were dried by evaporation in small Petri dishes on a hot plate at 40–60°C under a hood. The dry eluates were dissolved in 1 ml of 0.01 mol HCl l^{-1} and purified by slowly passing them, by means of a syringe, through a Sep-pak C-18 cartridge (300 mg; Waters, Milan) that had been activated with methanol or acetonitrile, and subsequently washed with

Table 1. Testosterone and oestradiol concentrations in the plasma and ovary, and D-aspartate (D-Asp) content in the ovary of the lizard, *Podarcis s. sicula,* during the main phases of the reproductive cycle

Phases of the reproductive cycle	Plasma (ng ml^{-1})		Ovary (ng g^{-1} tissue)		D-Asp (nmol g^{-1} tissue)	
	Testosterone	Oestradiol	Testosterone	Oestradiol	Enzymatic	HPLC
Pre-reproductive	5.54 ± 0.29	1.50 ± 0.15	23.0 ± 2.2	6.7 ± 1.1	5.54 ± 0.42	5.32 ± 0.44
Reproductive	1.54 ± 0.13	2.37 ± 0.13	7.0 ± 1.5	8.9 ± 1.3	7.97 ± 0.58	7.81 ± 0.78
Post-reproductive	7.16 ± 0.53	0.75 ± 0.06	31.5 ± 3.4	4.2 ± 0.7	1.56 ± 0.21	1.63 ± 0.26

Each value represents the mean of five determinations ± SD.
Reproductive versus post-reproductive $P < 0.01$; post-reproductive versus pre-reproductive $P < 0.01$.

distilled water. The cartridge was eluted twice with 2 ml 0.01 mol HCl l^{-1} to recover all the amino acids from the eluates. The resulting eluates were combined, and either dried using a Savant centrifuge or left to evaporate in small Petri dishes at 40–50°C under a hood. The dry residues were dissolved in 200 µl of 0.01 mol HCl l^{-1} and analysed for D-Asp content.

Determination of D-Asp: enzymatic UV and HPLC methods

These two assay procedures are identical to those described by Di Fiore *et al.* (1998a).

A standard curve was obtained by applying the enzymatic method to D-Asp solutions of known concentrations (D'Aniello *et al.*, 1993; Tedeschi *et al.*, 1994).

Determination of proteins

Proteins were determined by the method of Lowry *et al.* (1951) using BSA as the standard.

Statistical analysis

Data were analysed by ANOVA/MANOVA methods followed by Student–Newman–Keuls' or Duncan's tests. Kinetics were analysed using linear regression analysis.

Results

Free D-Asp content in the ovary and sex hormone concentrations in the ovary and plasma during the main phases of the sexual cycle

Variations in the content of D-Asp in the ovary and of sex hormones in the ovary and plasma in *Podarcis s. sicula* are summarized (Table 1). The determination of D-Asp content in the ovary was performed using both enzymatic and HPLC methods. These methods produced similar results (Table 1). In the ovary, free D-Asp concentrations varied with the phase of the reproductive cycle (Table 1: D-Asp enzymatic $F_{(4,14)} = 436.85$; D-Asp HPLC $F_{(4,14)} = 595.38$). The concentration of D-Asp was high in females at the reproductive phase, considerably lower in females at the post-reproductive phase and intermediate in females at the

pre-reproductive phase. The differences in D-Asp concentrations at various phases of the reproductive cycle were significant: reproductive versus post-reproductive, $P < 0.01$; post-reproductive versus pre-reproductive, $P < 0.01$; and pre-reproductive versus reproductive $P < 0.01$.

The concentration of testosterone was high in females at the post-reproductive phase and low in females at the pre-reproductive phase, and reached its minimum value in females at the reproductive phase (Table 1: plasma $F_{(4,14)} = 847.04$, $P < 0.01$; ovary $F_{(4,14)} = 1013.60$, $P < 0.01$). In contrast, the plasma and ovarian concentrations of oestradiol were low in post- females at the reproductive phase, and high in females at the pre-reproductive phase. Oestradiol concentrations were highest in females at the reproductive phase (Table 1: plasma $F = 352.15$, $P < 0.01$; ovary $F = 98.69$, $P < 0.01$). In addition, the patterns of oestradiol concentrations were similar to the patterns of D-Asp concentrations.

Effect of D-Asp in vivo on sex hormone concentrations in ovary and plasma

The administration of D-Asp to female lizards affected the concentration of sex hormones (plasma testosterone $F_{(4,24)} = 272.95$, $P < 0.01$; ovarian testosterone $F_{(4,24)} = 2029.7$, $P < 0.01$; plasma oestradiol $F_{(4,24)} = 8.5$, $P < 0.01$; ovarian oestradiol $F_{(4,24)} = 3517.9$, $P < 0.01$). At 3 h after amino acid treatment, D-Asp was the only amino acid of those tested that induced a decrease in plasma testosterone ($P < 0.01$ versus control) and an increase in plasma oestradiol ($P < 0.01$ versus control) (Fig. 1a). The effects of D-Asp on the concentration of these sex hormones in the ovary were similar to those observed in plasma (Fig. 1b). However, the administration of D-Asp to animals at the pre-reproductive and reproductive phases had similar effects to those in post-reproductive animals, but the effects were less marked (not shown).

Pattern of D-Asp uptake in vivo in the ovary, and comparison with sex hormone concentration patterns in the ovary and plasma

D-Asp (2 µmol g^{-1} body weight) administered i.p. to female lizards was taken up significantly by the gonads
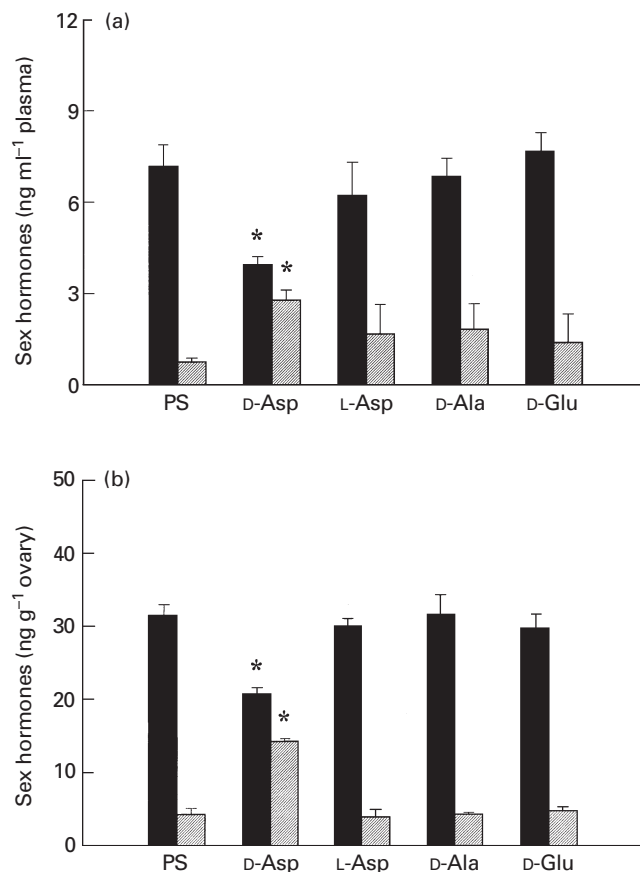
Fig. 1. Effects of administering different amino acids (2 μmol g⁻¹ body weight) on plasma (a) and ovarian (b) concentrations of testosterone (■) and oestradiol (▨) in post-reproductive lizards (*Podarcis s. sicula*) evaluated 3 h after administration. Each value represents the mean ± SD of five determinations. PS: physiological solution; D-Asp: D-aspartate; L-Asp: L-aspartate; D-Ala: D-alanine; D-Glu: D-glutamate. Data were analysed by MANOVA followed by Duncan's test. *$P < 0.01$ versus PS.

(Fig. 2a; $F(3,19) = 346.42$, $P < 0.01$). In females at the post-reproductive phase, the uptake of D-Asp in the ovaries reached a peak at 3 h after administration ($P < 0.01$), and then progressively decreased to its initial value within 18 h. The ovarian testosterone concentration decreased 3 h after D-Asp administration ($P < 0.01$ versus basal values, $F(3,19) = 23.98$), and then increased to its initial value, whereas, the ovarian oestradiol concentration increased 3 h after D-Asp administration ($P < 0.01$ versus basal values, $F(3,19) = 520.93$) and then progressively decreased to its initial value 18 h after administration. Plasma testosterone and oestradiol concentrations showed the same patterns as those in the ovaries (Fig. 2b; testosterone $F(3,19) = 87.81$, $P < 0.01$; oestradiol $F(3,19) = 59.09$, $P < 0.01$).

Aromatase activity assay in vitro

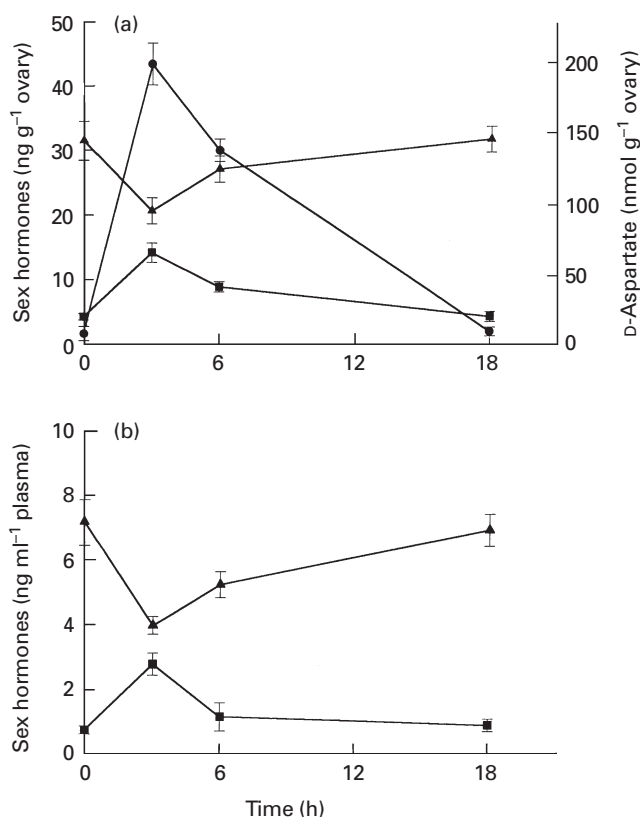Ovarian follicles converted testosterone to oestradiol (Fig. 3). The conversion rate was significantly increased by

Fig. 2. Concentrations of D-aspartate (D-Asp) (●) in the ovary at different times after initial administration of the amino acid (2 μmol g⁻¹ body weight), and effects of D-Asp on ovarian (a) and plasma (b) concentrations of testosterone (▲) and oestradiol (■) in the post-reproductive lizards (*Podarcis s. sicula*). Each value represents the mean ± SD of five determinations.
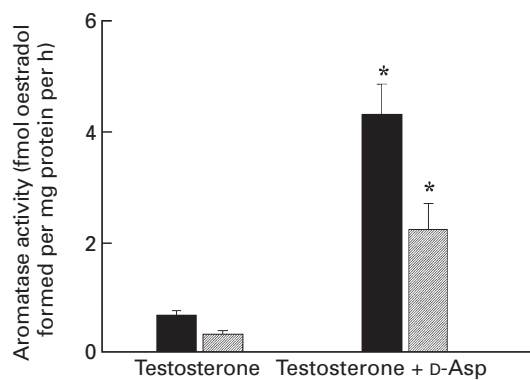

Fig. 3. Effects of D-aspartate (D-Asp) on aromatase activity in the fresh tissue (■) and acetonic powder extracts of ovarian follicles (▨) of ovaries of post-reproductive lizards (*Podarcis s. sicula*). Each value represents the mean ± SD of five determinations. *$P < 0.01$ versus testosterone alone.

the addition of D-Asp into the incubation wells containing fresh ovarian tissue ($P < 0.01$ versus control, $F(1,9) = 244.07$). An increase in the rate of conversion was also observed when D-Asp was added to incubation wells containing
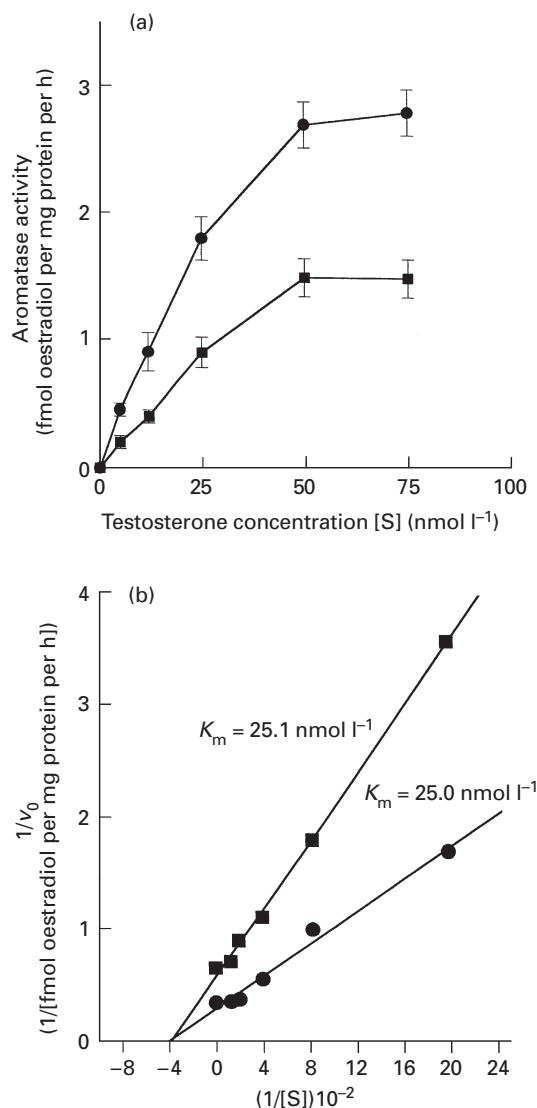
Fig. 4. Aromatase activity in the ovary of post-reproductive lizards (*Podarcis s. sicula*). Each value represents the mean ± SD of five determinations. (a) Velocity plot: ●, D-aspartate; ■, control. (b) Hanes plot using linear regression analysis: ●, D-aspartate; ■, control. [S]: substrate concentration (testosterone).

acetone powder extracts from the ovarian follicles ($P < 0.01$ versus control, F (1,9) = 74.52). This conversion rate, evaluated on the basis of substrate concentration (Fig. 4a), was four times greater in the presence of D-Asp than in the controls. However, the Michaelis–Menten plots that were analysed using linear regression analysis (Fig. 4b) showed nearly equivalent K_m values for the controls and for D-Asp (25.1 nmol l^{-1} for control, 25.0 nmol l^{-1} for D-Asp).

Discussion

The indirect physiological role that D-Asp plays in the hypothalamus–hypophysis–gonadal axis has been described

by D'Aniello *et al.* (1996). Nagata *et al.* (1999a, 1999b) proposed a direct local role for D-Asp in steroidogenesis in *in vitro* systems. The results from the present study support the hypothesis that D-Asp plays a direct local role in the enhancement of ovarian aromatase activity in the oviparous lizard *Podarcis s. sicula.* The hypothesis is supported by the relationships among the concentrations of D-Asp, testosterone and oestradiol that were observed (i) during the reproductive cycle of the lizard, (ii) under experimental treatments, and (iii) in experiments performed *in vitro*. Observations made throughout the reproductive cycle showed that D-Asp is present in the ovary and undergoes regular changes in concentration. The concentration of D-Asp in the ovary was high in females engaged in active processes of vitellogenesis, and low in females at the post-reproductive phase whose gonads were in a quiescent state. The highest concentration of D-Asp in the ovary coincides with the lowest concentration of testosterone in the ovary and plasma, and vice versa. These data are consistent with the findings in the green frog, *Rana esculenta* (Di Fiore *et al.*, 1998a). More importantly, in the lizard, high D-Asp concentrations observed in the ovary coincide with high oestradiol concentrations in the ovary and plasma, and vice versa. The administration of D-Asp confirmed these observations: there was a decrease in testosterone and an increase in oestradiol. Furthermore, this effect was specific to D-Asp, as administration of other amino acids had no effects.

The physiological mechanism underlying the inverse and direct relationships between D-Asp and, testosterone and oestradiol, respectively, were evident from the results of the experiments *in vitro*. The results showed that when D-Asp is added to an incubation medium containing testosterone and either homogenate or acetone powder extracts of ovarian follicles, a significant increase in aromatase activity occurs, which converts testosterone to oestradiol. Aromatase was characterized using linear Michaelis–Menten plots. Furthermore, the aromatase activity was four times greater in the presence of D-Asp than in its absence; however, the nearly equivalent values of the two K_m values (both approximately 25 nmol l^{-1}) indicate that aromatase has the same catalytic properties in both cases.

The physiological mechanism involved in the enhancement of aromatase activity by D-Asp has not been established. However, as the synthesis of new aromatase was not possible in the *in vitro* system used in the current study, it is possible that a pool of inactive aromatase is present, which is transformed into active forms by D-Asp. This hypothesis is consistent with findings that D-Asp enhances the activity of steroidogenic acute regulatory protein (StAR) through either gene expression or activation of inactive forms (Nagata *et al.*, 1999b).

Results from the present study indicate that D-Asp plays a role in ovarian steroidogenesis during periods of intense vitellogenesis. In the lizard *Podarcis s. sicula*, which is a good model for the vitellogenesis process, large amounts of vitellogenin are required during the pre-reproductive and

reproductive phases to produce the yolk required for follicular growth. It is well known that vitellogenin synthesis in the liver is an oestrogen-dependent process (Licht, 1984; Ho, 1987; Paolucci, 1989; Paolucci and Di Fiore, 1994). Therefore, as relatively high oestrogen synthesis is necessary for yolk production, it is possible that any factor capable of increasing oestradiol production in the ovary could have been positively selected. D-Asp may have been one of these factors as it (i) favours an increase in ovarian aromatase activity, (ii) increases oestrogen availability in vitellogenetic females, and (iii) indirectly helps to sustain high vitellogenin production in the liver.

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