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Review article

Estradiol and reproduction in the South American toad *Rhinella arenarum* (Amphibian, Anura)

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ABSTRACT

Rhinella arenarum is a South American toad with wide geographic distribution. Testes of this toad produce high amount of androgens during the non reproductive season and shift steroid synthesis from androgens to 5α-pregnanedione during the breeding. In addition, plasma estradiol (E_2) in males of this species shows seasonal variations but, since testes of *R. arenarum* do not express aromatase, the source of plasma E_2 remained unknown for several years. However, the Bidder's organ (BO), a structure located at one pole of each testis, is proposed to be the main source of E_2 in male's toads since it expresses several steroidogenic enzymes and is able to produce E_2 from endogenous substrates throughout the year. In addition, there were significant correlations between plasma E_2 and total activity of BO aromatase, and between plasma E_2 and the amount of hormone produced by the BO *in vitro*. In the toad, apoptosis induced by *in vitro* treatment with E_2 was mostly detected in spermatocytes during the breeding and in spermatids during the post-reproductive season, suggesting that this steroid has an important role in controlling spermatogenesis. However, *in vitro* treatment with E_2 had no effect on proliferation. This evidence suggests that the mechanism of action of E_2 on amphibian spermatogenesis is complex and more studies are necessary to fully understand the role of estrogens regulating the balance between cellular proliferation and apoptosis. In addition, in *R. arenarum in vitro* studies suggested that E_2 has no effect on CypP450c17 protein levels or enzymatic activity, while it reduces 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I) activity during the post reproductive season. As well, E_2 regulates FSHβ mRNA expression all over the year suggesting a down regulation process carried out by this steroid. The effect on LHβ mRNA is dual, since during the reproductive season estradiol increases the expression of LHβ mRNA while in the non-reproductive season it has no effect. In conclusion, the effect of E_2 on gonadotropins and testicular function is complex, not clearly understood and probably varies depending on the species. The aim of the current article is to review evidence on reproductive endocrinology and on the role of estradiol regulating reproduction in amphibians, with emphasis on the South American species *Rhinella arenarum*.

1. Introduction

Rhinella arenarum is a South American toad characterized for having wide geographic distribution, from the Department of Cochabamba (Bolivia) to the Department of Chubut in Argentine, and from Rio Grande do Sul in Brasil to the Andes Mountains (Gallardo, 1965; Vaira et al., 2012). This toad inhabits regions with tropical climate and others with temperatures near 0 °C, and humid areas with more than 1000 mm annual of precipitation and others with less than 500 mm. In addition, some populations inhabit regions located at sea level and others at 2500 m over the sea (Incachaca, Bolivia). This species has an extensive reproductive

Abbreviations: BO, Bidder's organ; 3β-HSD/I, 3β-hydroxysteroid dehydrogenase/isomerase; 5α-DHT, 5α-dihydrotestosterone; E_2, Estradiol; 17, 20αDP, 17,20α-dihydroxy-4-pregnen-3-one; CypP450c17, Cytochrome P450 17-hydroxylase, C17,20-lyase; Erβ, E_2 receptor β; EDCs, Endocrine Disrupting Chemicals; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; GCs, Glucocorticoids; MR, Mineralocorticoid; GR, Glucocorticoid receptors; 11β-HSD, 11β-hydroxysteroid dehydrogenase; PCNA, Proliferating cell nuclear antigen; PGCs, Primordial germ cells.

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season with a high dependency on the local weather conditions. As a general pattern, the breeding season begins in the early spring (end of September or beginning of October) and can extend until February depending on precipitations, being classified as an opportunistic breeder whose reproductive behavior correlates with the heavy rains of spring and summer (Gallardo, 1974). This author has also established that the breeding season of populations surrounding Buenos Aires City extend from the end of September to the beginning of December.

The present review aims to summarize the new evidence on reproductive endocrinology of male toads, with emphasis on the South American species *Rhinella arenarum* and on the role of estradiol regulating testicular function and gonadotropins. We begin describing steroidogenesis and spermatogenesis in amphibians; then, we will focus on the Bidder's Organ as the main source of estradiol in male toads and we review evidence on hormonal control of amphibian reproduction. Finally, we will analyze the effect of estradiol on testicular steroidogenesis, on apoptosis and proliferation as key elements of spermatogenesis, and on gonadotropins as main regulators of amphibian reproduction.

2. Testicular functions: Steroidogenesis and spermatogenesis

2.1. Steroidogenesis

As in other amphibians, *R. arenarum* testes are composed of two well-separated compartments, the interstitial tissue, containing steroidogenic cells or Leydig cells, and the germinal tissue, organized in seminiferous lobules. In amphibians, like other vertebrates, Leydig cells synthesize testosterone but also 5α-dihydrotestosterone (5α-DHT; Canosa et al., 1998; Kime, 1980).

Over the past years, several studies regarding testicular steroidogenesis in amphibians also showed that this group produces not only several androgens but also C_{21}-steroids such as progesterone, 5α-pregnan-3,20-dione, 3α-hydroxy-5α-pregnan-20-one, 5α-pregnan-3α,20-diol and 17-hydroxy-4-pregnen-3,20-dione (Canosa et al., 2003; Kim et al., 1998), suggesting that dehydrogenation in positions C_3 and C_5 is a frequent feature in amphibian testes. In addition, toad testes shift the steroid production from androgens to C_{21}-steroids during the breeding season (Canosa and Ceballos, 2002a). Testes from non-reproductive toads synthesize androgens through a complete 5-ene pathway (Fernández Solari et al., 2002) while during the breeding season the recovery of 5-ene steroids and androgens is significantly reduced while progesterone and its 3α/5α-reduced derivatives increase. Moreover, animals in reproductive condition exhibit a significant decrease in plasma androgens while 5α-pregnanedione increases, suggesting that the *in vitro* experiments highly correlate with the *in vivo* results (Canosa and Ceballos, 2002a, Denari and Ceballos, 2006). Even if low levels of plasma androgens during the reproductive season have been also found in males of other amphibian species (D'Istria et al., 1974; Itoh et al., 1990; Specker and Moore, 1980), only in *R. arenarum* this decrease has been associated to an increase in C_{21}-steroids.

In addition, plasma concentration of sexual steroids throughout the reproductive cycle was determined not only in *R. arenarum* but also in males of other amphibians (Canosa and Ceballos, 2002a; Canosa et al., 2003; Denari and Ceballos, 2006; Houck et al., 1996) and a relationship between androgens and the development of secondary sexual characters and/or the expression of sexual behavior has been clearly established (Itoh et al., 1990; Madelaire et al., 2017; Woodley, 2007). In *Pelophylax esculentus*, testosterone levels increase during autumn, they reach highest concentrations during winter and they decrease during spring (D'Istria et al., 1974; Fasano et al., 1989, 1993; Pierantoni et al., 2002; Rastogi et al., 1976; Rastogi and Iela, 1980). Moreover, in some species such as *R. arenarum* (Canosa et al., 2003; Denari and Ceballos, 2006), *Bufo japonicus* (Itoh et al., 1990) and *Taricha granulosa* (Specker and Moore, 1980), the lowest level of androgens occurs during the breeding and, although androgens reach the highest concentration at the beginning of the breeding season (end of winter – beginning of spring), sexual activity occurs when androgens are declining (Canosa et al., 2003; D'Istria et al., 1974; Rastogi et al., 1976; Specker and Moore, 1980).

The presence of high plasma and testicular levels of estradiol (E_2) were described in *P. esculentus* (Fasano et al., 1989; Polzonetti-Magni et al., 1984) but in *R. arenarum* no estrogen biosynthesis could be detected in the testis. This difference could be due to the fact that male toads possess Bidder's organs (BO) attached to the testes, which have been suggested to be the main source of E_2 (see below).

2.2. Spermatogenesis

As mentioned before, amphibian testes are organized in two compartments with different functions, the interstitial and the germinal tissue. Spermatogenesis proceeds in the lobules and after mitosis, spermatogonia I differentiate into spermatogonia II, which in turn differentiate into spermatocytes, resulting in spermatids and finally in mature spermatozoa. In anamniotes, lobular organization differs from the tubular one found in mammals (Pudney, 1998). In the first group, the unit of spermatogenesis is the spermatocyst, which is formed when a Sertoli cell encloses a primary spermatogonium with its cytoplasmic processes. In these groups, as spermatogenesis proceeds the spermatocysts migrate down the seminiferous lobule until they reach the efferent ducts where spermatozoa are released. In anurans, the cyst wall breaks down before spermiation occurs, and these open cysts form the wall of the seminiferous lobule. Spermiation takes place during the amplexus when spermatozoa are detached from Sertoli cells (Pudney, 1998).

In the male toad *R. arenarum*, spermiation occurs as a consequence of mating, during the breeding season. Moreover, spermiation is also induced by *in vitro* treatment of testes with gonadotropins (Pozzi and Ceballos, 2000; Pozzi et al., 2006). In addition, in male toads of *B. japonicus* amplexus induces a surge of LH (Ishii and Itoh, 1992), suggesting that LH elicits spermiation under physiological conditions.

Regarding the mechanism underlying gonadotropin-induced spermiation, many years ago it was suggested that 17,20α-dihydroxy-4-pregnen-3-one (17,20αDP) was the spermiation-inducing hormone in the testis of the frog *Pelophylax nigromaculatus* since *in vitro* induction of spermiation by gonadotropin was accompanied by a marked elevation of 17,20αDP concentrations in incubation media (Kobayashi et al., 1993). However, in *R. arenarum* (Pozzi and Ceballos, 2000; Pozzi et al., 2006; Volonteri and Ceballos, 2010) and *L. catesbeianus* and *Leptodactylus ocellatus* (Rosemblit et al., 2006) spermiation is induced by a mechanism independent on steroid production.

2.3. Reproductive cycles in anurans

Three classes of male reproductive cycles can be defined in anurans according to their spermatogenesis: discontinuous, continuous and potentially continuous types, with continuous and discontinuous types being the two extremes. Some anurans display a discontinuous type of spermatogenesis in which primary spermatogonia lose their mitotic capacity when spermatocytes start their differentiation into spermatozoa. As a consequence, in these species spermatogenesis is completely interrupted during part of the year, usually during the winter and sometimes even in summer

(Cei et al., 1996; Guarino et al., 1993; Rastogi et al., 1986). On the other hand, the continuous type of spermatogenetic cycle is characterized by the presence of different stages of spermatogenesis throughout the year (Emerson and Hess, 1996; Kanamadi and Jirankali, 1992; Kao et al., 1993; Yoneyama and Iwasawa, 1985). A third type of spermatogenetic cycle was defined as potentially continuous (Rastogi et al., 1976; Villagra et al., 2014). In this case males are strictly seasonal, with moderate to strong suppression of spermatogenesis during the winter when there is a massive degeneration of cysts with spermatocytes and even spermatids. However, there are differences between the discontinuous and the potentially continuous types: in the discontinuous type spermatogenesis could not be stimulated by any cues while in the potentially continuous types spermatogenesis can be stimulated in the winter by appropriate thermal and hormonal signals (Rastogi et al., 1976).

Generally, tropical and subtropical species have continuous cycles, while temperate species have discontinuous or potentially continuous cycles (Lofts, 1987; Rastogi et al., 1976). It has been recently proposed that in the toad spermatogonial proliferation is higher during the reproductive season and that cysts in late stages of spermatogenesis (spermatocytes and spermatids) are removed from the testes by apoptosis immediately after the breeding, suggesting that according to different environmental or physiological cues spermatogenesis could continue and *R. arenarum* could be classified as having a potentially continuous cycle (Scaia et al., 2016).

3. The Bidder's organ: Steroidogenic characteristics

The BO is a structure characteristic of the Bufonidae family, and it has been historically compared to a rudimentary ovary because of the presence of previtellogenic follicles. Although the presence of BO in bufonid males is evident because they can be clearly distinguished from the testis, its presence in females is more difficult to determine. In tadpoles of *R. arenarum* the BO is evident since stage 26 of Gosner before sexual differentiation (Gosner, 1960; Sassone et al., 2014, Fig. 1). The presence of BO was described in several species bufonids such as *Anaxyrus woodhousii* (Pancak-Roessler and Norris, 1991), *B. japonicus* (Tanimura and Iwasawa, 1986), *Duttaphrynus melanostictus* (Deb and Chatterjee, 1963) and *Rhinella schneideri* (de Gregorio et al., 2016), among others.

It has been suggested that BO's development in different species is arrested by the differentiation of a functional male and female gonad. In *B. vulgaris*, gonadectomy in male and female toads induces the BO to reach a development similar to functional ovary in both sexes and vitellogenic follicles (Ponse, 1927). Due to this capacity, it has been suggested that the presence of BO in males could be a possible reproductive strategy (Tanimura and Iwasawa, 1986). Moreover, Pancak-Roessler and Norris (1991) also suggested that oogenesis inhibition in *Anaxyrus woodhousii* is due to functional testis, since bilateral orchiectomy caused an increase in BO's weight and a shift towards more advanced stages of oogenesis. The idea that testis physiology maintains BO differentiation repressed is also supported by the fact that in *Duttaphrynus melanostictus* the BO regresses as a consequence of testosterone administration (Deb and Chatterjee, 1963). Moreover treatment with Flutamide, an antiandrogenic medicine, decreases the number of atresic bidderian oocytes, supporting the idea that androgens encourage oocyte degradation in the BO of *Rhinella schneideri* (de Gregorio et al., 2016). These evidences suggest that oogenesis inhibition in the BOs could be due to testicular functions and probably because of a testicular product such as testosterone.

The development of BO in adult males seems to be influenced by the environment and/or the reproductive status. For instance, the BO volume in reproductively active males of *A. woodhousii* was smaller than in non reproductive animals, suggesting that cues associated with reproduction regulates the development of this structure (Calisi, 2005). In addition, in *R. arenarum* BOs are generally smaller than testes but in some cases BOs are much bigger than the adjacent gonad, suggesting that there is a great variability in shape and size, particularly in the degree of follicle development (Scaia et al., 2011, Fig. 2). Regarding follicular cells, flat and round types have been described in the BO of *R. schneideri* (Freitas et al., 2015) and *R. arenarum* (Scaia et al., 2016). Moreover, in *R. arenarum* proliferation was measured in both cell types during the reproductive and the post-reproductive seasons (Scaia et al., 2016), suggesting that the BO is an active structure, experiencing not only active vitellogenesis but also proliferation of follicular cells.

As mentioned before, plasma E_2 was measured in males of several amphibian species and in most cases this hormone is synthesized by the testis (Fasano et al., 1989; Polzonetti-Magni et al., 1984). However, in *R. arenarum* testes do not express aromatase and, as a consequence, the source of plasma E_2 has remained unknown for several years (Canosa et al., 1998, Canosa and Ceballos, 2001). Since the BO was considered a rudimentary ovary containing follicular cells, it became one of the most conspicuous candidates for E_2 production. The presence of different steroidogenic enzymes has been described in several species such as *B. bufo, A. woodhousii* and *D. melanostictus* (Colombo and Colombo Belvedere, 1980; Ghosh et al., 1984; Pancak-Roessler and Norris, 1991). Furthermore, in the BO of *Rhinella marina* key genes for transcriptional factors (Sf1, Dax1) and the key enzyme for E_2 production, aromatase, are active during sexual development and maturation (Abramyan et al., 2010). Furthermore, in *R. arenarum* the BO expresses different steroidogenic enzymes, such as cytochrome P450 side-chain cleavage, 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I), cytochrome P450 17-hydroxylase, C17,20-lyase (CypP450c17) and aromatase (Scaia et al., 2011) and it is able to produce E_2 from endogenous substrates throughout the year (Scaia et al., 2013).

Plasma E_2 levels also show seasonal variations in several anuran species. In *P. esculentus*, plasma E_2 levels are highest during the breeding season (Varriale et al., 1986; Fasano et al., 1989), while in *R. arenarum* E_2 levels are lower during the pre-reproductive season than during the reproductive and post-reproductive ones (Scaia et al., 2013). In this species, seasonal variations in plasma E_2 correlate with seasonality of total activity of aromatase in the BO, since this enzymatic activity is also lower during the pre-reproductive season (Scaia et al., 2013). These results, together with the fact that there were significant correlations between plasma E_2 and total activity of BO aromatase, and between plasma E_2 and the amount of hormone produced by the BO *in vitro*, suggested that this organ could be the main source of estrogens in males toads. Moreover, proliferation in follicular cells was registered during the reproductive and the post-reproductive seasons when plasma E_2 is elevated, suggesting that the contribution of BOs to plasma E_2 could be due not only to amount of aromatase but to the number of follicular cells (Scaia et al., 2016). Furthermore, estrogen receptor β (ERβ) was localized in follicular cells of early vitellogenic follicles as well as in oocytes of previtellogenic and early vitellogenic ones, suggesting that E_2 could have an autocrine/paracrine action on bidderian steroidogenesis and oogenesis (Scaia et al., 2016).

Nowadays, some of the chemical compounds used as herbicides or pesticides act as Endocrine Disrupting Chemicals (EDCs), altering hormonal regulation and hence reproduction and development of organisms (Orton and Tyler, 2015). Amphibians are one of the most threatened vertebrate groups, with 31.62% of species under some endangered category (IUCN, 2017), and factors contributing to this worldwide trend also include human-induced pollution (Blaustein et al., 2003; Relyea and Mills, 2001). Estrogenic agents

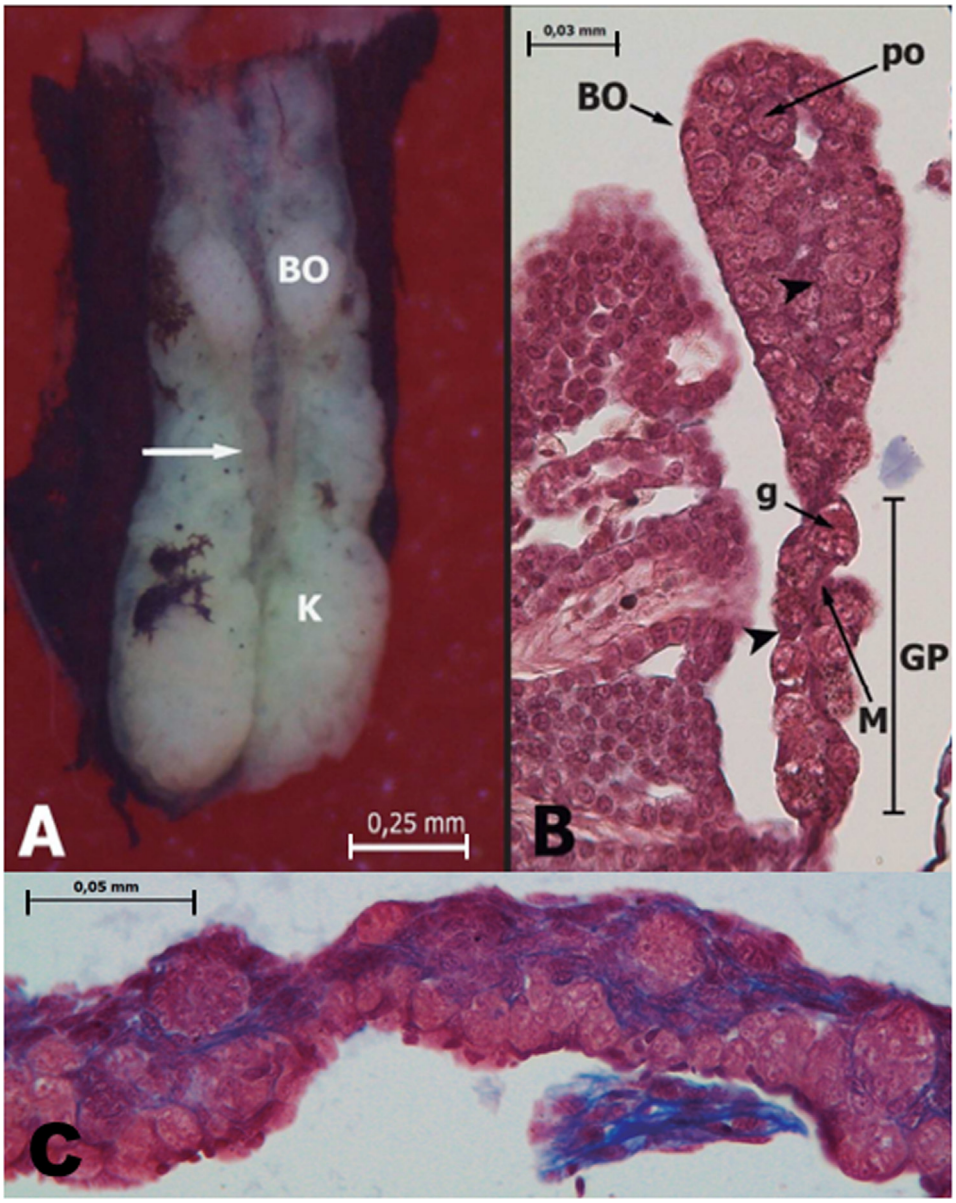

Fig. 1. Bidder's organ in *Rhinella arenarum* tadpoles in different stages. A. Genital ridges of tadpole in stage 26 located ventral to the kidneys (K). Bidder's organ (BO) and proper gonads (white arrow) are located in the cephalic and caudal portion of the genital ridge, respectively. B. Longitudinal section of BO and proper gonad (GP) of a tadpole in stage 26. C. Longitudinal section corresponding to the proper gonad of a tadpole in stage 46. Arrowhead: somatic cell; po: primary oogonia; g: germ cell; M: medullar layer. (Taken from Sassone et al., 2014).

are chemical compounds mimicking estrogenic action either by binding to ERs or by stimulating aromatase activity which in turn increases plasma E_2 levels. In amphibians, it has been suggested that E_2 and estrogenic compounds cause feminization of tadpoles (Lambert et al., 2015; Li et al., 2016). On the other hand, BOs and testes of *R. marina* show several abnormalities in animals captured in areas with intense agricultural practices (McCoy et al., 2008, 2017). Authors suggest that these malformations and highly developed BOs in animals from agricultural areas could be a consequence of a defect in testicular functions (e.g. testes not inhibiting BO development and vitellogenesis). Taking into account the aforementioned evidence, it is worth mentioning that the BO is not an atrophied organ in adult males, as it has been historically suggested: it is an important source of circulating E_2 and it has active follicular proliferation and oogenesis. This way, and considering the BO expresses aromatase and ERβ, it has also been suggested that highly developed BOs could also be the consequence of increased intra-oocyte E_2 levels and of the interaction of estrogenic agents with bidderian ER (Scaia et al., 2016). In this context, it is relevant to question about the importance of physiological concentrations of E_2 and its seasonal rhythm in the regulation of testicular steroidogenesis and spermatogenesis. As a consequence, studying bidderian steroidogenesis, oogenesis and endocrine regulation is essential because an abnormal development of this organ could highly alter physiological E_2 levels and could potentially affect normal male reproductive physiology in bufonids.

4. Hormonal regulation of reproduction

4.1. Gonadotropins

As in other vertebrates, the overall functions of amphibian testes are controlled by two pituitary gonadotropins, follicle-stimulating

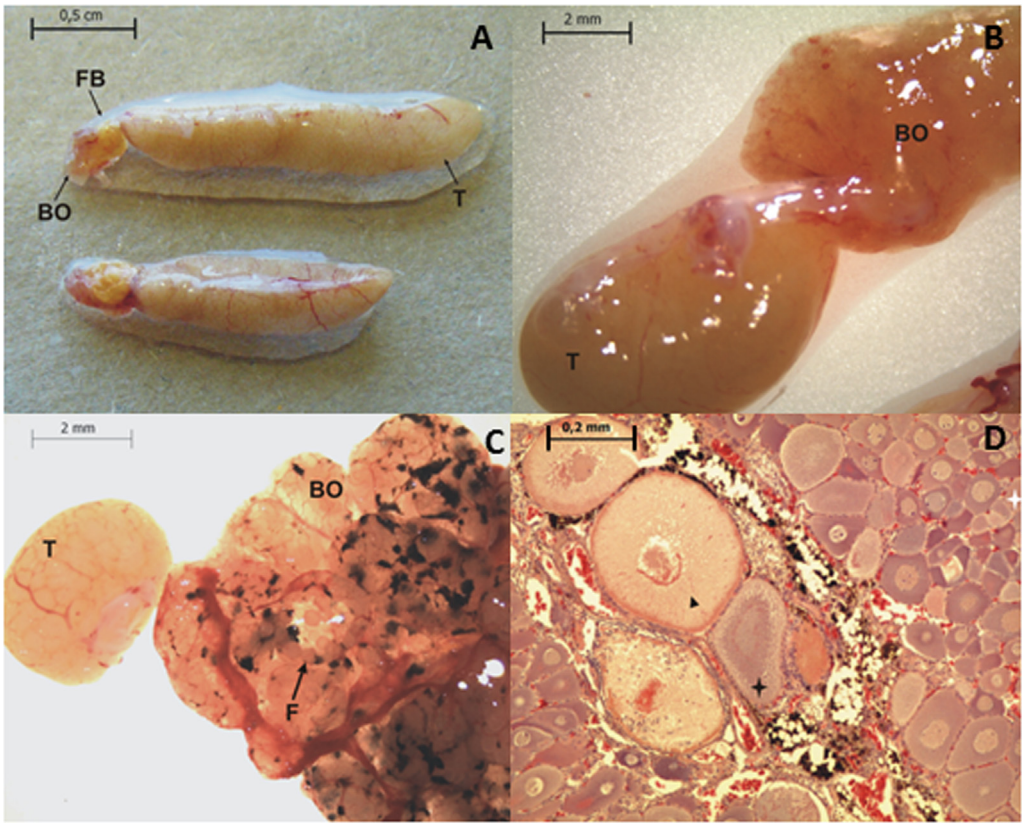

Fig. 2. Bidder's organ in *Rhinella arenarum* adults. A. Well-developed testis (T) with small Bidder's organs (BO) and fat bodies (FB). Both testes of different sizes correspond to an animal collected during the pre reproductive period (PreR). B. In this animal, BO has similar size than testis. C. Pigmented BO belonging to an animal collected during the PreR period. In this case, reduced testes are attached to a highly developed and irrigated BO, presenting follicles (F) of different sizes. D. Cross section of Bidderian follicles in different stages. Previtellogenic follicles (white star), early vitellogenic follicles (black star) and late vitellogenic follicles (head of arrow). (Taken from Scaia et al., 2011).

hormone (FSH) and luteinizing hormone (LH) (Kawauchi and Sower, 2006; Norris, 2007), among other non-pituitary factors. Hormonal fluctuations strongly depend on hypothalamic GnRH which in some species, such as *P. esculentus*, is stored and released to sustain gonadotropin discharge (Cobellis et al., 1999b; Fasano et al., 1993; Meccariello et al., 2004; Polzonetti-Magni et al., 1998).

In anurans, many papers have reported that bullfrog pituitary has two chemically distinct gonadotropins similar to mammalian LH and FSH (Hayashi et al., 1992a,b; Takada and Ishii, 1984). In addition, two types of gonadotropins were described in *Lithobates pipiens* (Zhang et al., 2007), *Xenopus laevis* (Huang et al., 2001), *B. japonicus* (Itoh and Ishii, 1990; Itoh et al., 1990; Komoike and Ishii, 2003), *Rana dybowskii* and *P. nigromaculatus* (Kim et al., 1998), *P. esculentus* (Pinelli et al., 1996) and *R. arenarum* (Volonteri et al., 2013). In later species, β subunits from both gonadotropins are similar to those of another member of the Bufonidae family and they are more distant from β subunits of members of Ranidae and Pipidae families. In addition, the study of the seasonal changes in the expression of LHβ and FSHβ mRNA indicates that transcript levels have seasonal variations associated with the reproductive cycle of this species (Volonteri et al., 2013).

As in mammals, amphibian gonadotropins are involved in the regulation of testicular steroidogenesis (Kobayashi et al., 1993; Licht et al., 1983). In particular, in *R. arenarum* both LH and FSH from mammalian origin stimulate androgen production after 2 h incubation in a dose-dependent manner (Pozzi and Ceballos, 2000; Pozzi et al., 2006). However, in several anuran species plasma androgens decline in spring, when reproduction takes place, reaching the lowest values during the summer (Itoh et al., 1990; Rastogi et al., 1986), even though LH and FSH rise during the reproductive season (Kim et al., 1998; Polzonetti-Magni et al., 1998). The fact that plasma gonadotropins increase when androgens decline suggest that some aspects of the regulation of steroidogenesis and gonadotropin synthesis and secretion remain to be explored. Despite the fact that usually the expression of mRNA of LHβ and FSHβ not necessary represents the amount of plasma gonadotropins, in *R. arenarum* the annual profile of pituitary mRNA correlates with plasma levels of gonadotropins in *L. catesbeianus* (Licht et al., 1983), with minimum concentrations in autumn and winter seasons while the maximum was measured in warm months (Volonteri et al., 2013). In addition, plasma gonadotropins in *L. catesbeianus* and mRNA expression in *R. arenarum* get to the highest concentration in months corresponding to spring and early summer, when mating occurs. However, in *R. arenarum* both mRNA exhibit a second increase in the post reproductive season. In contrast, in *B. japonicus* the annual profile of both gonadotropins differs from *R. arenarum* and *L. catesbeianus*. In males of the Japanese toad, plasma LH and FSH levels are dissociated with LH concentration increasing during March, just prior the breeding. On the other hand, the higher levels of FSH occur between June and December, months corresponding to summer and autumn (Itoh et al., 1990).

The annual change in gonadotropins mRNA is associated with testicular steroidogenesis since, as mentioned before, during the reproductive season of *R. arenarum* there is a fall in androgens synthesis and an increase in 5α-reduced C21-steroids (Canosa and Ceballos, 2002b). However, in *B. japonicus* plasma levels of LH are associated with plasma androgens, with both hormones increasing just prior breeding (Itoh et al., 1990), similarly that it was reported in the newt (Kano et al., 2005; Tanaka et al., 2004). On the other hand, in *R. arenarum*, the profile of androgens is opposite to that

of both gonadotropins mRNA, with the lowest concentration of androgens associated with an increase in LHβ and FSHβ mRNA. Additionally, the increase in LHβ mRNA seems to be associated with the increase in 5α-reduced C21-steroids previously described and probably linked to the inhibition of androgen production elicited by FSH. Moreover, *R. arenarum* has been defined as having an androgen dissociated reproductive pattern with FSH proposed as the gonadotropin responsible of the inhibition of androgen production after 48 h incubation (Canosa and Ceballos, 2002a,b; Fernández Solari et al., 2002). The difference between *R. arenarum* and *B. japonicus* could be linked to the fact that the latter species seems to express an androgen-associated reproductive pattern (Itoh et al., 1990; Itoh and Ishii, 1990).

On the other hand, in *P. esculentus* FSH and E_2 reach the highest concentrations at the same time of the year, proposing that FSH could induce the biosynthesis of E_2 by increasing the expression of aromatase (Polzonetti-Magni et al., 1998) which in turn could induce spermatogonial proliferation (Chieffi et al., 2000a,b). Consequently, it is possible that in *R. arenarum* seasonal variations of FSH are associated with spermatogenesis (see below).

4.2. Glucocorticoids and reproduction

In amphibians, glucocorticoids (GCs) are synthesized in the interrenal gland and exert several biological actions through the interaction with at least two different intracellular receptors, mineralocorticoid (MR), glucocorticoid receptors (GR) and plasma membrane-associated receptor have been proposed (Orchinik et al., 2000). Intracellular receptors for GCs have been described in liver, kidney, skin and testis (Denari and Ceballos, 2006; Lange and Hanke, 1988; Lange et al., 1989; Orchinik et al., 2000; Regueira et al., 2013a). In *R. arenarum*, GR is activated by corticosterone, the most important GC synthesized by the interrenal gland of this species (Ceballos et al., 1983; Regueira et al., 2013b).

Several papers have described that GCs inhibit testosterone production in rat Leydig cells through their interaction with intracellular GR (Hardy et al., 2005; Hu et al., 2008; Monder et al., 1994). However, intracellular levels of GCs in *R. arenarum*, as in other vertebrates (Witorsch, 2016), are regulated by the expression in Leydig cell of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) with a predominantly oxidative activity (Denari and Ceballos, 2005). This enzyme could modulate the amount of active corticosterone in Leydig cells, protecting these cells from the potentially negative effect of GCs on steroidogenesis. As mentioned before, among the great variety of actions, GCs potently disrupt mammalian reproductive physiology through a number of mechanisms. Several studies suggest that basal concentrations of GCs are insufficient to disrupt reproductive physiology but GCs-mediated stress is thought to inhibit reproduction at several levels not only in mammals (Witorsch, 2016) but also in amphibians (Moore and Zoeller, 1985; Moore et al., 2005). However, it is evident that during breeding many amphibians display a significant elevation of GCs levels with no suppression of reproductive behavior or physiology (Denari and Ceballos, 2005; Romero, 2002). Therefore, it is possible that elevated plasma levels of GCs facilitate several aspects of reproduction. Moreover, even if studies in amphibians report a positive correlation between corticosterone and reproduction (Moore and Jessop, 2003; Romero, 2002), there is a lack of information regarding the effect of endogenous GCs on amphibians testicular function (Tesone et al., 2012).

5. Apoptosis and cellular proliferation in the testis

The progression of amphibian spermatogenesis is regulated by several factors, and the dynamic balance between proliferation and death of the germ cells also define the rate in which spermatogenesis occurs. Apoptosis is a physiological process of programmed cell death responsible of germ cells removal in all spermatogenic stages (Shaha et al., 2010).

In mammals, hormones such as FSH, LH, testosterone and E_2 are essential to regulate germ cell apoptosis and survival during spermatogenesis (Shaha, 2008; Zhou et al., 2001). Particularly, E_2 regulates the equilibrium between cell survival, apoptosis and cell proliferation in several tissues, but the reason why estrogens change the balance towards survival or towards cell death is still not totally understood (Vasconsuelo et al., 2011). In mammals, it has been suggested that hormonal regulation of apoptosis is a complex mechanism involving not only individual effect of each hormone but also synergistic action of different hormones (for a revision, see Schlatt and Ehmcke, 2014).

In amphibians, the balance between the total number of germ cells and the capacity of Sertoli cells to support them is also regulated by hormones, particularly by seasonal variations of several steroids. However, only few studies examine the molecular mechanisms of testicular apoptosis in this group, and most of them focus on seasonal variations of apoptosis and spermatogenesis. Molecular mechanisms have been studied in *Hypselotriton orientalis* and *P. nigromaculatus*, and evidence suggest that apoptosis can occur by caspase-3 activation through extrinsic and intrinsic pathways (Jin et al., 2016; Wang et al., 2012; Zhang et al., 2013). Temperature is one of the environmental factors that could also regulate several processes in amphibians, among them apoptosis seasonality. In *Cynops pyrrhogaster*, an urodele from template areas, low temperatures induce cell death of spermatogonia II due to an increase in plasma levels of prolactin, being the testes devoid of spermatocytes during winter, which is the quiescent season (Yazawa et al., 1999). However, in *Triturus marmoratus*, another urodele, temperature has no influence on testicular cell death (Ricote et al., 2002). Regarding evidence in apodes, in *Ichthyophis tricolor*, during testicular regression germ cells show several apoptotic characteristics, such as heterochromatic nuclei, nuclear fragmentation, apoptotic bodies and nuclear margination of chromatin (Smita et al., 2005).

Among anurans, in *L. catesbeianus* from tropical areas there is high production of spermatocytes during winter, meiosis is completed during spring and eventually spermiogenesis and spermiation occur in summer (Sasso-Cerri et al., 2004; Sasso-Cerri and Miraglia, 2002). Moreover, in this species high apoptosis occurs in testicular cysts during the reproductive season at the end of spring and beginning of summer, when there is a long photoperiod and high temperatures (Sasso-Cerri et al., 2006). However, this seasonal variation in testicular apoptosis is not observed in *R. arenarum*. In this species cysts of spermatocytes and spermatids undergo apoptosis during the post-reproductive season instead of the reproductive one, suggesting that cysts that do not form spermatozoa are removed from testes by apoptosis after breeding and before the next spermatogenic wave (Scaia et al., 2016).

As mentioned before, the progression of spermatogenesis is a balance between apoptosis and proliferation of the germ line. Proliferation of spermatogonia has been studied in *C. pyrrhogaster* (Yuwen et al., 2008), *R. arenarum* (Scaia et al., 2015, 2016) and, in more detail, in *P. esculentus* (Ferrara et al., 2004). Spermatogonial proliferation in *R. arenarum* and *P. esculentus* occurs during the reproductive season (Ferrara et al., 2004; Scaia et al., 2016). In *P esculentus* seasonal variation of proliferations seems to be associated to changes in the proliferating cell nuclear antigen (PCNA), suggesting high levels of this marker in spermatogonia I in March and in spermatogonia II in July, October and November (Chieffi et al., 2000b). Seasonal variations of PCNA were also studied together with the expression of c-kit, a proto-oncogene essential for proliferation and spermatogonial differentiation (Sette et al.,

2000). In *P. esculentus* c-kit is expressed in all spermatogenic stages, and both c-kit and PCNA increase significantly during breeding season (Raucci and Di Fiore, 2007). As a consequence, maximum cell proliferation during the reproductive season is associated with high levels of PCNA and c-kit activation.

In view of this evidence, both apoptosis and cellular proliferation are very complex processes involving seasonal variations in different molecular mechanisms and protein expression. Considering that differential activation in each spermatogenic stage will determine the balance between apoptosis and cellular proliferation and the rate in which the spermatogenic wave will ocurr, it is necessary to deepen the study of seasonal variations of both processes in order to broaden our knowledge on amphibian spermatogenesis and reproduction.

6. Hormonal regulation of spermatogenesis

The progression of spermatogenesis is regulated by a delicate balance between different hormones promoting proliferation or apoptosis. In mammals, FSH, LH, E_2 and testosterone regulate the progression of germ line, and an excess or the absence of any of these hormones can stimulate testicular apoptosis. For instance, testosterone and FSH, in combination with E_2 promote cell survival while E_2 alone has a pro-apoptotic effect. As a consequence, a drop in levels of FSH and testosterone in mammals activates apoptosis, suggesting that both hormones are essential to maintain spermatogenesis (Pareek et al., 2007). In mammals, FSH regulates spermatogenesis, although its role regulating testicular physiology varies according to the species (Siegel et al., 2013; Tarulli et al., 2006).

The hormonal regulation of spermatogenesis in amphibians has been reviewed by Pierantoni et al. (2002). In the first place, the role of GnRH in regulating mammalian reproduction was established several years ago (for a revision see Plant, 2015). Moreover, GnRH plays also an essential role regulating reproduction in amphibians (Meccariello et al., 2014). In *P. esculentus*, a complete GnRH system with two ligands (GnRH1 and GnRH2) and three receptors (GnRH-R1, GnRH-R2, GnRH-R3) has been characterized in testis (Chianese et al., 2012). Also in this species, GnRH directly stimulates androgen production and spermatogonial proliferation, while androgens induce both spermatids formation and spermatogonial proliferation (Minucci et al., 1992). In *R. arenarum*, GnRH also has a direct effect on testis, since it reduces testosterone production by inhibiting CypP450c17 (Canosa et al., 2002). Whether GnRH has any direct effect on spermatogenesis in this species still remains unknown.

Kisspeptins are also important for spermatogenesis by regulating GnRH or gonadotropins (Oakley et al., 2009) and also by participating in intra-gonadal regulation of spermatogenesis (Chianese et al., 2015). Moreover, kisspeptins regulate not only spermatogenesis, but also other testicular functions. For instance, in several experimental models kisspeptins are involved in estrogen related activities controlling testicular physiology, Leydig cells, germ cell progression and sperm functions (reviewed by Chianese et al., 2016). In this respect, in frogs kisspeptins induce the progression of spermatogenesis at the end of the winter stasis (Chianese et al., 2015), modulates sperm release (Chianese et al., 2017), steroid signaling (Chianese et al., 2013; Chianese et al., 2015; Chianese et al., 2017), and the expression of steroidogenic enzymes (Chianese et al., 2017). This way, kisspeptins are key elements in the control of testosterone/estradiol ratio and their related testicular functions (Chianese et al., 2017).

In amphibians, spermatogenesis is also controlled by gonadotropins and sexual steroids. In urodeles, in particular in *Ambystoma tigrinum*, administration of mammalian FSH to hypophysectomized animals stimulates spermatogenesis (Moore, 1975). Moreover, it has been suggested that in *P. esculentus* pituitary hormones induce testicular morphological changes related to sperm release (Cobellis et al., 2008) and in *C. pyrrhogaster*, porcine FSH induces spermatogonial proliferation and their differentiation into spermatocytes I (Ito and Abé, 1999; Ji et al., 1992).

Androgens have also a central role in regulating spermatogenesis, although their actions vary among species and the stage of spermatogenesis. For instance, in *L. pipiens*, 5α-DHT induces an increase in the differentiation of spermatogonia into spermatocytes I, while inhibits the maturation of these cells to spermatocytes II (Tsai et al., 2003). In some species, such as *P. perezi* and *Rana italica*, plasma testosterone concentrations are maximal during the season with high spermatogonial proliferation, but the concentration decreases even if spermatogenesis continues (Delgado et al., 1989, Guarino et al., 1993). In the desert frogs, variations in plasma testosterone during summer correlate with variations in seminiferous lobule diameter and with gonadosomatic index, and authors suggest that this androgen is important for regulating testicular activity (Shalan et al., 2004). Furthermore, as mention before, during the reproductive season of *P. esculentus* there is a positive association between high levels of testosterone, mitotic activity of germinal epithelium and PCNA and c-kit expression, which are essential for spermatogenesis and spermatogonial proliferation (Raucci and Di Fiore, 2007). In *R. arenarum*, androgens increase testicular size (Penhos, 1956) but there are no studies regarding spermatogenesis. For an extensive revision on this topic see Propper (2011).

Glucocorticoids are also involved in the regulation of spermatogenesis. An increase in plasma levels of corticosterone as a consequence of stressing situations could affect spermatogenesis. However there are few studies regarding the role of these steroids in amphibian spermatogenesis. In *D. melanostictus, in vivo* treatment with corticosterone during short or long periods suppress spermatogenesis (Biswas et al., 2000). Similarly, administration of corticosterone to *L. pipiens* significantly inhibits formation of spermatocytes II and spermatids (Tsai et al., 2003).

Taken together, this evidence suggests that spermatogenesis in amphibians is a complex process in which each cellular stage can be regulated by different steroids, and the role of each factor could also differ among species.

7. Role of estradiol in spermatogenesis

In mammals, estrogens and their receptors (ERα and ERβ) are also important factors regulating spermatogenesis (O'Shaughnessy, 2014; Shaha, 2008). In this group, it has been demonstrated that depending on their concentration, estrogens are detrimental for spermatozoa production and maturation (Dostalova et al., 2017) probably due to the pro-apoptotic effect of these hormones on germ cells (Mishra and Shaha, 2005; Nair and Shaha, 2003).

Estradiol has a critical role in male gametogenesis in vertebrates (Chianese et al., 2016). Regarding the role of estrogens in amphibians, the presence of ERβ was detected in testis by immunohistochemistry in *L. catesbeianus, R. arenarum* and in *P. esculentus* (Caneguim et al., 2013a,b; Scaia et al., 2016; Stabile et al., 2006). In *R. arenarum* ERβ was mostly detected in the interstitial compartment, in Sertoli cells associated to spermatozoa and in Sertoli cytoplasmic projections surrounding cysts (Scaia et al., 2016). Moreover, in this species spermatogonial proliferation is maximal during the reproductive season, which corresponds with high plasma levels of E_2, suggesting that estrogens could participate in the regulation of proliferation (Scaia et al., 2013, 2016). However, no spermatogonial proliferation could be detected during the post-reproductive season, when plasma levels of E_2 are the highest in males of this species, suggesting that other factors participate in the control of the germ cells mitosis (Scaia et al., 2016). In addition, *in vitro* experiments with physiological concentrations of E_2 had no effect on testicular proliferation (Scaia et al.,

2015) and an *in vivo* approach would be necessary to clarify the role of estrogens in the proliferation of germ cells in this species.

In other amphibians, like salamanders, it has been suggested that estrogens act early during spermatogenesis regulating premeiotic stages (Callard, 1992). In the anuran *L. catesbeianus*, the presence of PCNA, ERβ and aromatase were detected by immunohistochemistry in primordial germ cells (PGCs), suggesting that estrogens have a role in regulating spermatogenesis. The PGCs proliferative activity was lower during the winter than in the summer, suggesting that the seasonal progression of spermatogenesis depends on the activity of these cells (Caneguim et al., 2013a,b). In *L. pipiens*, *in vivo* studies with surgical implants suggest that E_2 could have both inhibitory and stimulatory effects on spermatogenesis depending on the cyst stage, since treatment with estrogens during twenty days delays the formation of spermatogonia II and spermatocytes but accelerates the progression of other stages (Tsai et al., 2003). Furthermore, in *P. esculentus*, plasma concentrations of E_2 reach the highest values during April and May, months corresponding to the reproductive season (Fasano et al., 1989). During this season there is also maximal expression of ERβ (Stabile et al., 2006), and mitotic activity and PCNA expression in spermatogonia I (Chieffi et al., 2000a), indirectly suggesting a possible relationship between E_2 and spermatogonial proliferation. The effect of E_2 was confirmed by *in vitro* experiments (Minucci et al., 1997) and involves c-fos activation and protein kinase induction (Chieffi et al., 2000a; Cobellis et al., 1999a; Cobellis et al., 2002) as well as the activation of Akt-1 (Stabile et al., 2006) that in turn could inactivate pro-apoptotic (Kandel and Hay, 1999).

Seasonal variations in testicular cells apoptosis have been studied in *C. pyrrhogaster* and *L. catesbeianus* (Ricote et al., 2002; Sasso-Cerri et al., 2004; Yazawa et al., 1999) but, unfortunately, there are only few studies analyzing the effect of estrogens on apoptosis in amphibian testes. In *R. arenarum*, recent *in vitro* studies suggested that physiological concentration of E_2 during the breeding season induces apoptosis of the germ cells, while no induced apoptosis was detected in all treatment conditions during the post-reproductive and pre-reproductive season, respectively (Scaia et al., 2015). Considering that the removal of germ cells by apoptosis could determine the progression of spermatogenesis, it is essential to distinguish which kind of cell undergoes apoptosis. In the newt *C. pyrrhogaster*, spermatogenesis is arrested during winter as a consequence of cell death of spermatogonia before meiosis (Yazawa et al., 1999, 2002) while in *C. orientalis* apoptosis was detected in early spermatids and mature spermatozoa (Wang et al., 2012). In *R. arenarum* apoptosis is mostly detected in spermatocytes and spermatids during the post-reproductive season, since the end of the summer until autumn (Scaia et al., 2016). Moreover, in the toad apoptosis induced by *in vitro* treatment with E_2 was mostly detected in spermatocytes during the breeding and in spermatids during the post-reproductive season, suggesting that cysts that do not reach spermatozoa undergo apoptosis via an E_2-mediated mechanism (Scaia et al., 2016). Nevertheless, further studies are necessary to determine if E_2 induces apoptosis of the germ line in other amphibian species.

The above aforementioned evidence suggests that the mechanism of action of E_2 on amphibian spermatogenesis is complex and more studies with *in vitro* and *in vivo* approaches in other species are necessary to fully understand the role of estrogens regulating the balance between cellular proliferation and apoptosis.

8. Effect of estradiol on testicular steroidogenesis

The role of estrogens regulating testicular steroidogenesis in amphibians is even less studied than its effect on spermatogenesis. *In vivo* studies in *Xenopus laevis* adults exposed to 0.1 μg/L of E_2 in laboratory freshwater during 49 days resulted in a significant decrease in testosterone plasma levels, even though whether this decrease was due to a reduction in testicular steroidogenic enzymes or in plasma levels of gonadotropins was not clarified (Hecker et al., 2005). In *R. arenarum* there is an inverse relationship between plasma levels of E_2 and testosterone, since E_2 increases in the reproductive season, being highest during the post-reproductive season, while plasma testosterone levels are very low (Denari and Ceballos, 2005). Similarly, in males of *P. esculentus* there is a negative correlation between high plasma levels of E_2, in early summer, and low plasma levels of testosterone (Polzonetti-Magni et al., 1984). Based on this indirect evidence, authors suggest that the decrease of androgens could be due to the inhibition of GnRH and LH secretion by E_2 (Polzonetti-Magni et al., 1984). Moreover, in the same species, *in vitro* treatment of testis with E_2 (10^{-6} M) caused a decrease in testicular androgen production (Pierantoni et al., 1986) because estrogens inhibit 17-hydroxilase activity of testicular CypP450c17 (Fasano et al., 1989, 1991). However, in *R. arenarum*, *in vitro* studies suggested that physiological concentrations of E_2 have no effect on CypP450c17 protein levels or enzymatic activity, while they reduce 3β-HSD/I activity during the post reproductive season (Scaia et al., 2015). As a consequence, even though there seems to be species-specific differences in which enzyme is affected, E_2 reduces testicular androgen production in amphibians by inhibiting testicular steroidogenesis. However, it is essential to study more species and analyze if this is a conserved effect in amphibians or if it is a species-dependent effect.

9. Effect of estradiol on gonadotropins

Taking into account that estrogens seem to regulate testicular physiology by modulating directly testicular steroidogenesis, it is essential to analyze whether besides this direct effect, estrogens could also affect upstream targets of the hypothalamic-pituitary-gonadal axis.

The annual change in gonadotropins mRNA in *R. arenarum* is associated with testicular steroidogenesis. As already mentioned, during the reproductive season of *R. arenarum* there is a fall in testicular biosynthesis of androgens and an increase in 5α-reduced C21-steroids (Canosa and Ceballos, 2002a) mainly due to a decrease in the activity of the enzyme CypP450c17 (Fernández Solari et al., 2002). The fact that androgens are low during the breeding when LHβ mRNA increases suggests that androgens could regulate LHβ mRNA expression by negative feedback and that LHβ mRNA reaches the minimum expression in the pre reproductive period, when androgens are elevated. However, there are many studies in anurans that show that LH has a similar pattern than androgens, suggesting that LH could be associated to androgen production (Itoh et al., 1990; Licht et al., 1983; Polzonetti-Magni et al., 1998).

On the other hand, Polzonetti-Magni et al. (1998) showed that in *P. esculentus* FSH and E_2 reach the highest values at the same time of the year, proposing that FSH could induce E_2 biosynthesis, which could contribute to spermatogonial proliferation (Chieffi et al., 2000a). Therefore, it is possible to speculate that in *R. arenarum* FSH is associated with spermatogenesis since FSHβ mRNA is mainly elevated in the post reproductive season, when spermatogonia develop and proliferate (Burgos and Mancini, 1948). In addition, there is an increase of FSHβ mRNA in October, when spermatogonia are also proliferating. In the red-belied newt, mammalian FSH stimulates the proliferation of secondary spermatogonia and their differentiation into elongated spermatids (Abé and Ji, 1994; Yazawa et al., 2002), suggesting that this seems to be a common mechanism in amphibians. However, in *B. japonicus* the

highest concentration of FSH could be measured between the early and late spermatogenesis, indicating that FSH could be associated with proliferation and differentiation.

In vitro studies in *R. arenarum* demonstrated that 24 h treatment with physiological concentrations of E_2 significantly diminish FSHβ mRNA expression all over the year suggesting a down regulation process carried out by this steroid (personnel communication). This negative feedback exerted by E_2 was also reported for *L. pipiens* (Pavgi and Licht, 1989; Tsai et al., 2005). On the other hand, in *P. esculentus* E_2 has been suggested as a positive regulator of FSH and annual profiles of FSH and E_2 are quite similar (Polzonetti-Magni et al., 1998). Thereby, it is not absolutely clear why in *R. arenarum* the profiles of both hormones are also comparable (Scaia et al., 2013; Volonteri et al., 2013). Regarding the effect of E_2 on LHβ mRNA, this steroid has a dual effect since physiological concentrations of E_2 increase the expression of LHβ mRNA during the reproductive season, suggesting a positive effect in the non-reproductive season it has no effect (personnel communication). In contrast with *R. arenarum*, in *L. pipiens* E_2 treatment down regulates the secretion of LH (Pavgi and Licht, 1989) and the expression of LHβ mRNA during the reproductive season (Tsai et al., 2005). Zhang et al. (2007) also showed a down regulation in LHβ mRNA but they do not analyze seasonality of the effect. Taken together these results suggest that in *R. arenarum* the expression of β subunits of both gonadotropins is independently regulated by E_2, being the positive effect of E_2 over LHβ mRNA active only during the reproductive season.

In conclusion, the effect of E_2 on gonadotropins and testicular function cannot be generalized to different amphibian species and the effect exerted by E_2 is a complex, not clearly understood process. This revision focuses on bufonids, mainly *R. arenarum*, because BO is characteristic of this family. However, in cases where there is no evidence on bufonids, results on other amphibian species were included. Fig. 3 summarizes what it is known about the regulation of testicular function, Bidder's organ and hypothalamo-pituitary axis. It has been historically suggested that testicular physiology maintains BO's development inhibited in anuran adults. Bidderian oogenesis and steroidogenesis in different species is inhibited by testicular functions and probably because of testicular products, such as androgens (*Bufo vulgaris, A. woodhousii, D. melanostictus, R. schneideri*). Moreover, recent studies suggest that the BO also exerts a regulatory function on testicular physiology in *R. arenarum* adults. Estradiol, which is mainly produced by the BO, regulates spermatogenesis by increasing apoptosis of cysts in late stages, and also steroidogenesis by reducing testicular 3βHSD/I activity during the post reproductive season. This regulation of estradiol over testis is possible because the interstitial and in the germ compartment in testis express estrogen receptor β (ERβ). Moreover, ERβ were also detected in both compartments in the BO, suggesting the possibility that estradiol could have an

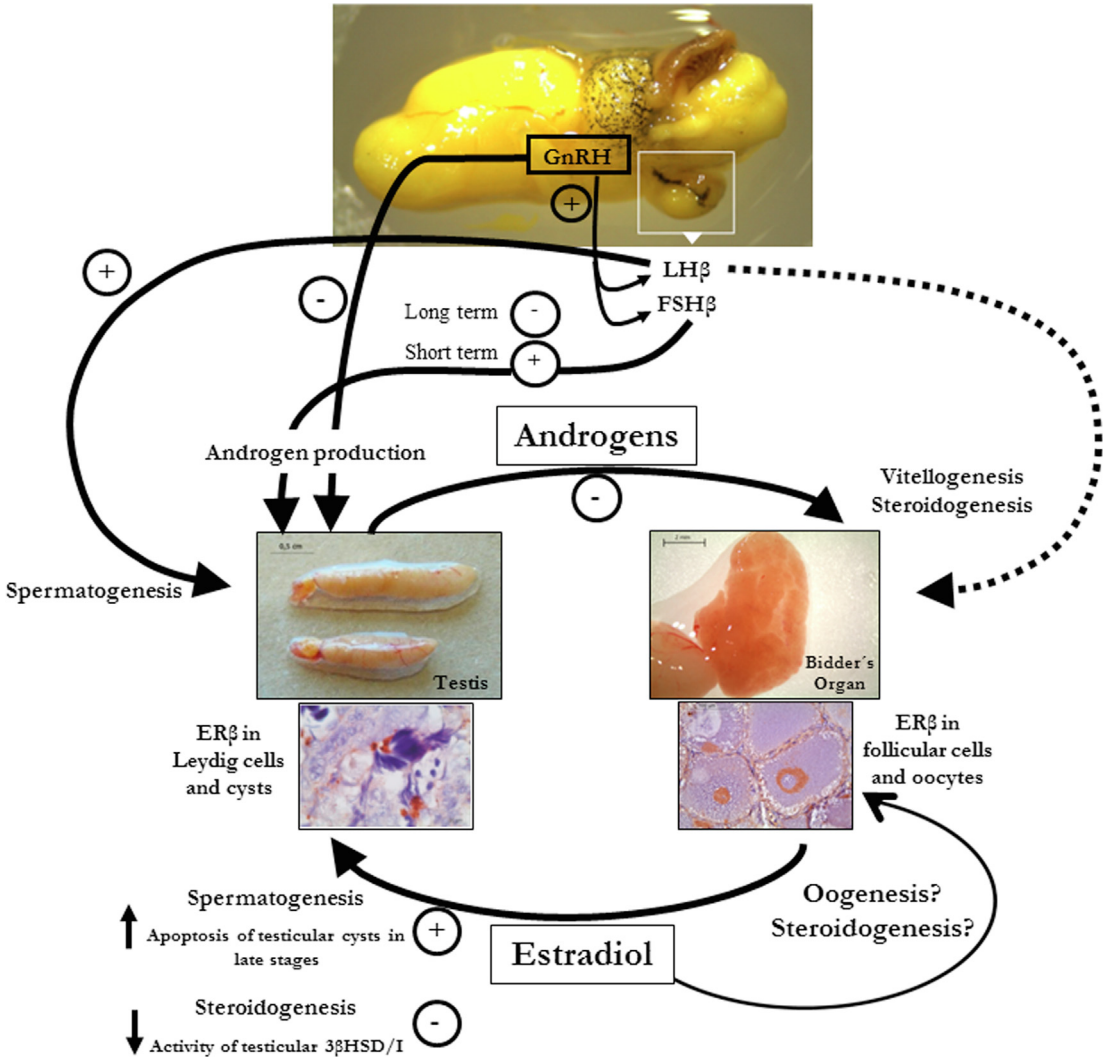

Fig. 3. Summary of inter-regulation between Bidder's organ (BO), testis and hypothalamo-pituitary axis in males.

autocrine/paracrine action on bidderian steroidogenesis and oogenesis. In turn, both gonads are under the regulation of GnRH, either directly or with gonadotropins' effect. In some species, GnRH directly stimulates androgen production (*P. esculentus*), while in others it reduces testosterone production by inhibiting CypP450c17 (*R. arenarum*). Pituitary hormones in *R. arenarum* stimulate androgen production in a dose-dependent manner in short term incubations, while they have a negative effect in long-term treatments. Moreover, pituitary hormones also stimulate spermatogenesis (*A. tigrinum, P. esculentus, C. pyrrhogaster*). Regarding gonadotropins' effect on the Bidder's Organ, there is only evidence in *A. woodhousii* suggesting that the inhibition of oogenesis is not due to the lack of circulating gonadotropins but rather due to testicular inhibition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.03.018.

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