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Steroid production in toads☆

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Abstract

In *Bufo arenarum*, androgen biosynthesis occurs through a complete 5-ene pathway, including 5-androstane-3β,17β-diol as the immediate precursor of testosterone. Besides, steroidogenesis changes during the breeding period, turning from androgens to C_{21}-steroids such as 5α-pregnan-3α,20α-diol, 3α-hydroxy-5α-pregnan-20-one and 5α-pregnan-3,20-dione. In *B. arenarum*, steroid hormones are not involved in hCG-induced spermiation, suggesting that the steroidogenic shift to C_{21}-steroids during the breeding be not related to spermiation. The activity of 17-hydroxylase-$C_{17–20}$ lyase ($CypP450_{c17}$) decreases during the reproductive season, suggesting that this enzyme would represent a key enzyme in the regulation of seasonal changes. However, the increase in the affinity for pregnenolone of 3β-hydroxysteroid dehydrogenase (3αHSD)/isomerase could also be involved. Moreover, the reduction in $CypP450_{c17}$ leading to a reduction in C_{19}-steroids, among them dehydroepiandrosterone (DHE), would contribute to the conversion of pregnenolone into progesterone, avoiding the non-competitive inhibition exerted by DHE on this transformation. Additionally, $CypP450_{c17}$ possesses a higher affinity for pregnenolone than for progesterone, explaining the predominance of the 5-ene pathway for testosterone biosynthesis. Animals in reproductive condition showed a significant reduction in circulating androgens, enhancing the physiological relevance of all the in vitro results. The in vitro effects of mGnRH and hrFSH on testicular steroidogenesis revealed that both hormones inhibited $CypP450_{c17}$ activity. In summary, these results demonstrate that, in *B. arenarum*, the change in testicular steroidogenesis during the reproductive period could be partially due to an FSH and GnRH-induced decrease in $CypP450_{c17}$ activity.

Keywords: Testis; GnRH; Steroidogenic–enzymes; Amphibians

1. Introduction

There are several reports showing that, in amphibians, testosterone and 5α-dihydrotestosterone (DHT) represent the major androgens secreted by the testis [39,40,50,64]. Nevertheless, the production of C_{21}-steroids such as 5α-pregnan-3β,17,20α-triol, 17,20α/β-dihydroxy-4-pregnen-3-one (17,20α/βP4), and 20α-hydroxy-4-pregnen-3-one (20αP4) has also been detected [40]. However, the role of testicular C_{21}-steroids in amphibian reproduction has received little attention. Kobayashi et al. [41] have demonstrated the biosynthesis of 17,20αP4 in *Rana nigromaculata*, suggesting that this steroid is involved in hCG-induced spermiation, although the seasonal variation of this steroid has not been determined.

Plasmatic concentration of sexual steroids through the reproductive cycle has been determined by RIA, in several amphibian species [33,34,45,66] and a relationship between androgens and the development of secondary sexual characters has been clearly established [16,37,59,61]. The relevance of androgens in sexual behaviour remains more controversial. In species such as *Xenopus laevis* [65], *Pachymedusa dacnicolor* [60], *Rana catesbeiana* [45] and *Taricha granulosa* [61], the reproductive behaviour seems to be associated with high levels of plasmatic androgens. However, other studies failed to determine a correlation between androgens and behaviour [49,64]. In several amphibian species, it has been shown that plasmatic androgens decline in spring, when the reproduction takes place, reaching the lowest values in summer [30,37,60]. Besides, gonadotropins are believed to be involved in the regulation of steroidogenesis by stimulating testosterone production. However, both LH and FSH rise during the reproductive season even if androgen levels are declining [36–38,42,55]. Thus, several aspects of the regulation of steroidogenesis as well as steroid function in amphibian reproduction require more research.

Bufo arenarum is a species that, as a consequence of its wide geographic distribution, possesses an extensive reproductive season (September to February), its reproduction being highly dependent on the local weather conditions. Thus,

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this species has been classified as an opportunistic or explosive breeder whose breeding behaviour correlates with the heavy rains of spring and summer [25]. For those populations surrounding Buenos Aires City, the breeding season is restricted to the period between September and December.

It has been previously described that in *B. arenarum* spermatozoa can be found all year long [56]. These results could be interpreted as an adaptation to the unpredictability of the environment. Moreover, the spermiation can be stimulated by gonadotropin injection at any time of the year [26,27,56]. As consequence, this species can be characterised as a potentially continuous breeder. Additionally, experiments performed in our laboratory have demonstrated that hCG-induced spermiation is not mediated by steroid biosynthesis [56], these results being opposite to those from Kobayashi et al. [41]. However, they raise the speculation that C_{21}-steroids could play a role in other processes associated or simultaneous with spermiation.

The present article summarises our results on steroid biosynthesis capability and its regulation in *B. arenarum* and discusses them in relationship to the present knowledge in amphibian reproductive endocrinology. We hope that this article will serve as a stimulus for further research in steroid hormone biosynthesis and function in amphibian gonads.

2. Steroid biosynthesis

Steroidogenic studies in *B. arenarum* [8,11] showed that pregnenolone is efficiently transformed into several C_{19}-steroids such as dehydroepiandrosterone (DHE), 5-androsten,3β,17β-diol, testosterone, DHT and other C_{19}-reduced steroids (Table 1). Besides, C_{21}-steroids such as progesterone, 5α-pregnan-3,20-dione (5α-pregnanedione), 3α-hydroxy-5α-pregnan-20-one, 5α-pregnan-3α,20-diol and 17-hydroxy-4-pregnen-3,20-dione are described, although they were scarcely isolated in the non-reproductive condition (Table 1). Even the presence of high plasmatic and testicular levels of estradiol has been described in other anuran amphibian [20,54], in *B. arenarum* no oestrogen biosynthesis could be detected. This difference could be due to the fact that male toads possess rudimentary ovaries attached to the testes, called Bidder's organs. Estradiol could be synthesised in this organ as previously suggested by Ghosh [28].

Additionally, the combined interpretation of double-labelled experiments and isotopic dilution experiments [11] as well as biosynthetic results suggests the predominance of a complete 5-ene pathway for androgen biosynthesis, including 5-androsten-3β,17β-diol as the immediate precursor of testosterone. Although testosterone comes from that precursor, a small proportion is also produced from androstenedione [8,11]. However, androstenedione is synthesised from dehydroepiandrosterone and not from progesterone, suggesting that this species completely bypasses progesterone for androgen biosynthesis. A similar "full 5-ene pathway" has also been described for aldosterone biosynthesis in the interrenal of the same species [13]. A 4-ene pathway, pathway not conducing to androgen biosynthesis, is also described. This pathway is important for the production of 5α-pregnanedione and other progesterone-reduced derivatives. Besides, these results also show that toad testes possess, as other amphibians, a high 5α-reductase (5αRed) activity, this enzyme being important for DHT and 5α-pregnanedione biosynthesis. Moreover, toad testis also expresses 3α-hydroxysteroid dehydrogenase (3αHSD) activity. The production of 5α and 3α-reduced steroids was also demonstrated in other anuran species [38,39,50,64], suggesting that dehydrogenation in positions C3 and C5 is a common feature in amphibian testes.

In addition, the ability of toad testes in shifting the steroid production from androgens to C_{21}-steroids during the breeding season is described (Fig. 1) [10]. Testes from non-reproductive toads synthesise androgens through a complete 5-ene pathway. Under this condition, progesterone is scarcely isolated, probably because it is completely transformed into 5α-pregnanedione. On the contrary, during the breeding season the recovery of 5-ene steroids and androgens is significantly reduced while progesterone and its 3α/5α-reduced derivatives increase. A similar shift in steroidogenesis has been described in fish [51] but not in other amphibian.

The molecular mechanism involved in the regulation of steroid production was studied by analysing the percentage of contribution of each steroidogenic enzyme to the total metabolism in both periods. As shown in Table 2, a significant reduction of 17-hydroxylase-$C_{17–20}$ lyase ($Cyp450_{c17}$)

Table 1
Steroids isolated from incubations of testis fragments with [^{3}H]pregnenolone

Steroid	Non-reproductive	Reproductive
C_{19}		
Dehydroepiandrosterone	4.9 ± 0.8	N.D.
5-Androsten-3β,17β-diol	24.8 ± 3.0	N.D.
Testosterone	9.9 ± 1.1	1.3 ± 0.6*
5α-Dihydrotestosterone	26.0 ± 1.8	N.D.
Androstenedione	2.2 ± 0.3	0.4 ± 0.2*
5α-Androstan-3,17-dione	4.8 ± 0.9	N.D.
5α-Androstan-3α,17β-diol	2.9 ± 1.5	4.0 ± 1.1
3α-Hydroxy-5α-androstan-17-one	2.5 ± 0.9	2.1 ± 1.0
C_{21}		
Pregnenolone	10.3 ± 1.6	36.5 ± 3.9*
Progesterone	0.1 ± 0.1	10.3 ± 1.1*
5α-Pregnan-3,20-dione	2.7 ± 0.5	13.8 ± 2.6*
3α-Hydroxy-5α-pregnan-20-one	0.8 ± 0.3	13.4 ± 1.2*
5α-Pregnan-3α,20α-diol	N.D.	13.5 ± 1.2
17-Hydroxy-5-pregnen-3,20 dione	2.5 ± 1.0	N.D.
20-Hydroxy-5α-pregnan-3-one	0.8 ± 0.3	13.4 ± 1.3*

Yield in percentage of isolated steroid from incubations of testis fragments from animals in non-reproductive and reproductive period, in the presence of [^{3}H]pregnenolone. Each value represents media ± S.E. of nine (non-reproductive) or seven (reproductive) independent experiments.

* Significant difference ($P \leq 0.01$) between periods.

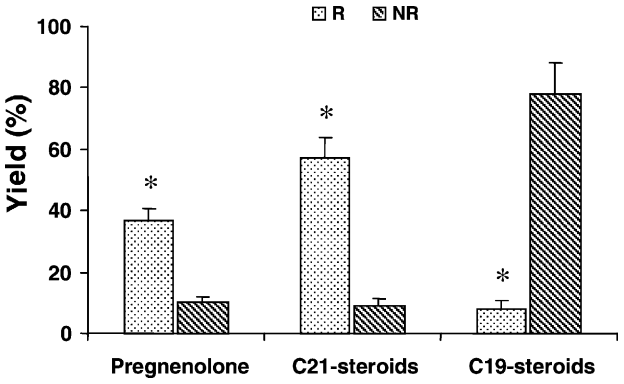

Fig. 1. C_{19} and C_{21}-steroids in reproductive (R) and non-reproductive (NR) periods. Yield are expressed as percentage of isolated steroid from incubations of testis fragments with [^{3}H]pregnenolone. Each value represents media ± S.E. of nine (NR) or seven (R) independent experiments. Asterisks show significant differences ($P \leq 0.01$) between periods.

Table 2
Contribution in percentage of steroid metabolising enzymes during non-reproductive and reproductive periods

Enzyme activity	Non-reproductive	Reproductive
3βHSD	54.66 ± 1.46	59.62 ± 4.55
$Cyp450_{c17}$	78.04 ± 2.76	7.87 ± 2.19*
17βHSD	63.67 ± 4.93	5.29 ± 1.38*
5αRed	34.17 ± 1.56	39.36 ± 5.77
3αHSD	8.11 ± 1.12	20.61 ± 3.48*
20HSD	0.82 ± 0.35	29.95 ± 3.46*

3βHSD: 3β-hydroxysteroid dehydrogenase/isomerase; $Cyp450_{c17}$: 17-hydroxylase-$C_{17–20}$ lyase; 17βHSD: 17β-hydroxysteroid dehydrogenase; 5αRed: 5α-reductase: 3αHSD: 3α-hydroxysteroid dehydrogenase; 20HSD: 20-hydroxysteroid dehydrogenase.

* Significant difference ($P \leq 0.01$) between periods.

and 17β-hydroxysteroid dehydrogenase (17βHSD) during the breeding is detected while the contribution of 3αHSD and 20-hydroxysteroid dehydrogenase (20HSD) increases. However, the contribution of 3β-hydroxysteroid dehydrogenase/isomerase and 5αRed does not change during the reproductive cycle, suggesting that in the toad, the contribution of $Cyp450_{c17}$ would be a key factor to explain the steroidogenic change mentioned above. Nevertheless, the increase in the activity of 3αHSD could also be involved. Considering that products of $Cyp450_{c17}$ are substrates of 17βHSD, a reduction in the activity of the cytochrome could be responsible for the decrease in the contribution of 17βHSD. As a consequence, it is possible that the change in the contribution of 17βHSD detected in the reproductive season does not necessarily represent changes in its activity.

Results concerning $Cyp450_{c17}$ contribution were confirmed by the study of kinetic parameters of the enzyme in both reproductive conditions (Table 3). Pregnenolone is the preferred substrate in both seasons [21], supporting our previous data regarding the predominance of the 5-ene pathway for testosterone biosynthesis. The high affinity of toad 17-hydroxylase for pregnenolone is similar to that described for progesterone in *N. maculosus* [7]. However, the predominance of the 5-ene pathway for androgen biosynthesis in this species has not been assessed yet. Moreover, animals in reproductive condition show a significant reduction in V_{max} of $Cyp450_{c17}$. Consequently, the reduction in the activity of the cytochrome is probably the most important factor involved in the reduction of androgen biosynthesis. However, other enzymes like 3βHSD could be involved as well. During the reproductive season, the affinity of 3βHSD for pregnenolone as well as the biological active enzyme increase while the affinity for DHE decreases (Table 3) [58]. These modifications in 3βHSD adjust the enzyme activity to the decrease in DHE availability, representing an adaptation to a new biochemical situation. Besides, DHE produces a strong non-competitive inhibition of pregnenolone conversion while pregnenolone a slight competitive inhibition of DHE transformation [57]. Consequently, the reduction in $Cyp450_{c17}$ that conduces to a decrease in C_{19}-steroids, among them DHE, would contribute to the conversion of pregnenolone to progesterone, avoiding the non-competitive inhibition exerted by DHE on this transformation.

As animals in reproductive condition exhibit a significant decrease in circulating androgens while 5α-pregnanedione increases, it is possible to conclude that the in vitro experiments highly correlate with the in vivo results (Fig. 2). Even if low levels of plasmatic androgen during the reproductive season have been also found in other amphibian species

Table 3
K_m and V_{max} of microsomal 3βHSD and $Cyp450_{c17}$ activities

Enzyme	Substrate	K_m		V_{max}	
		NR	R	NR	R
$Cyp450_{c17}$	Pregnenolone	43.76 ± 4.63	37.46 ± 4.19	0.29 ± 0.01	0.06 ± 0.01*
	Progesterone	217 ± 63	306 ± 19	0.28 ± 0.02	0.05 ± 0.02*
3βHSD	Pregnenolone	2900 ± 400	860 ± 190*	0.19 ± 0.08	0.60 ± 0.02
	DHE	220 ± 60	1040 ± 160*	0.28 ± 0.09	0.21 ± 0.06

K_m and V_{max} of microsomal 3βHSD were measured with subsaturating concentrations of pregnenolone (0.2–5 μM) or DHE (0.05–3 μM) in the presence of 0.5 mM NAD^+. K_m and V_{max} of $Cyp450_{c17}$ were determined with subsaturating concentrations of pregnenolone (2.5 nM to 1 μM) and progesterone (25 nM to 10 μM) in microsomal fraction and 0.5 mM NADPH. Kinetic parameters were calculated by Wilkinson linearisation. Values represent means of eight independent experiments ± S.D. NR: non-reproductive period; R: reproductive period. V_{max}: nmol/min mg protein; K_m: nM.

* Significant differences between periods with $P \leq 0.01$.

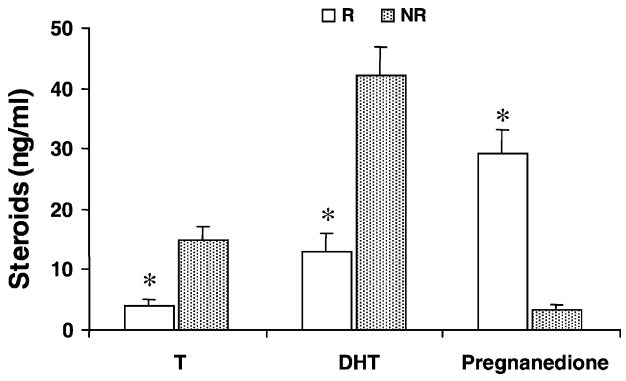

Fig. 2. Plasmatic concentration of immunoreactive testosterone (T), DHT and 5α-pregnanedione (pregnanedione) in animals collected during non-reproductive (NR) and reproductive (R) periods. Results represent means of 10 independent determinations for each period ± S.E. Asterisks symbolise significant differences compared with NR period ($P \leq 0.01$).

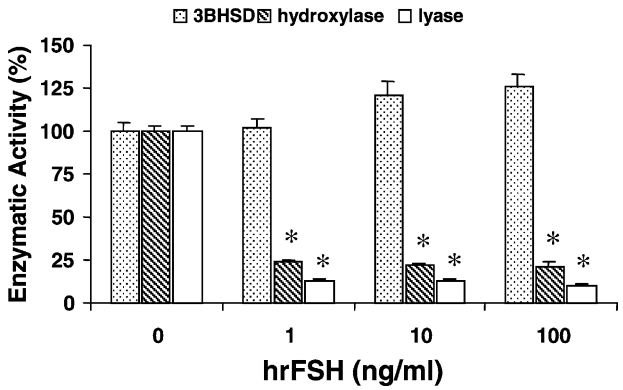

Fig. 3. Effects of hrFSH on 3βHSD and $Cyp450_{c17}$ activities. Testis fragments were stabilised for 2 days in L-15 medium with 10% FBS. Medium was replaced by serum-free medium containing the indicated amounts of hrFSH and incubated for 2 more days. After that, tissue was homogenised and the enzymatic activities were assayed. Bars represent average ± S.E. of 12 replicates. Hydroxylase: 17-hydroxylase activity of $Cyp450_{c17}$; lyase: 17–20 lyase activity of $Cyp450_{c17}$. Asterisks symbolise significant differences between treatments and control (without hrFSH) with $P \leq 0.01$.

[16,37,61], this is the first time that this decrease is associated to an increase in C_{21}-steroids.

3. Regulation of steroid production

Although the role of androgens in amphibian reproduction has been studied [44], the regulation of its secretion remains still obscure. The available antiserum to testosterone possesses, in general, a high cross-reactivity with DHT and the results obtained are generally expressed as androgens. However, in this way, changes in 5αRed activity could be masked while information on the production of different steroid hormones such as 5α-pregnanedione or other progesterone derivatives would be missed. Besides, the study of seasonal changes in in vitro androgen release or plasma androgen concentration provides little information about the mechanism involved in the control of steroid biosynthesis. Therefore, the study of the regulation of steroidogenic enzyme activity can overcome these limitations.

The effect of hrFSH and hCG on steroidogenic enzymes in a long-term incubation system was studied [9]. After 48 h incubation, hrFSH strongly decreases $Cyp450_{c17}$-associated activities at all concentrations used (Fig. 3). However, hrFSH does not modify 3βHSD activity (Fig. 3). Moreover, hCG fails to decrease both $Cyp450_{c17}$ activities except when a very high concentration is used [9]. These results allow the conclusion that FSH would be involved in the change of the steroidogenic pathway. This effect seems to be specific for $Cyp450_{c17}$ since no effect on 3βHSD is observed. Furthermore, the effect of hrFSH on $Cyp450_{c17}$ is specific of this gonadotropin since hCG has no effect on $Cyp450_{c17}$-associated activities [9].

In *Rana*, an increase in plasmatic FSH has been described during the reproductive period [38,42] and although there is no information about the plasmatic profile of gonadotropins in *B. arenarum*, it is possible to assume that, during the reproductive season, a similar increase in FSH could occur. Burgos and Mancini [6] have demonstrated that, in this species, the spermatogenic wave starts in October and November, when the reproduction takes place, and it is reasonable to presume that FSH would be involved in this process. In this sense, the action of FSH on $Cyp450_{c17}$ activities could explain the decrease in androgen production, the concomitant increase in C_{21}-steroids biosynthesis, and the low level of plasmatic androgens as well as the increase in plasmatic 5α-pregnanedione during the reproductive period. However, the participation of other factors could not be excluded.

In the rat, GnRH treatments provoke a reduction of $Cyp450_{c17}$, which could account for its antigonadal effects [4]. If a similar inhibitory effect did exist in *B. arenarum* it would represent a component of the regulatory mechanism of steroid biosynthesis. Testicular GnRH binding sites of *B. arenarum* have been characterised as a single low-affinity high-capacity one [12]. The K_d (34 nM) is similar to the low-affinity high-capacity receptor characterised in the pituitary of goldfish [32] and a little higher than the constant determined for the testicular GnRH receptor of *Rana esculenta* using cGnRH-II as radioligand [19]. As in mammals, acute in vitro treatment with mGnRH blocks hCG-induced androgen secretion at all the concentrations used (Table 4). However, chronic in vitro treatment with mGnRH inhibits the activity of $Cyp450_{c17}$ at all doses assayed (Fig. 4), indicating that this decapeptide could produce a strong reduction in androgen biosynthesis [12]. These results are in agreement with those previously described in rat and human [3–5], however, they disagree with those reported for other amphibians, in which GnRH stimulates androgen production [14,18,29]. This discrepancy could be due to species-specific differences or it may represent animals captured at different reproductive or physiological conditions

Table 4
Effects of mGnRH on the in vitro testosterone release

Treatments		Androgens (ng/ml)
hCG (nM)	mGnRH (μM)	
0	0	401.3 ± 37.4
50	0	663.7 ± 60.1*
	0.1	432.2 ± 23.5
	1	462.2 ± 45.1
	10	440.5 ± 45.4

Testicular fragments were incubated for 2 h in 2 ml of incubation medium in absence or presence of 50 nM hCG and/or the indicated amounts of mGnRH. The basal and hCG-stimulated testosterone release were determined by RIA.

* Significant differences with $P \leq 0.05$.

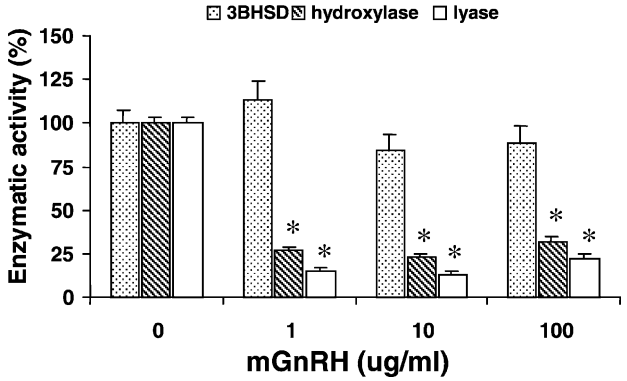

Fig. 4. Effects of mGnRH on 3βHSD and $Cyp450_{c17}$ activities. For experimental conditions see Fig. 3. Bars represent average ± S.E. of 12 replicates. Hydroxylase: 17-hydroxylase activity of $Cyp450_{c17}$; lyase: 17–20 lyase activity of $Cyp450_{c17}$. Asterisks symbolise significant differences between treatments and control (without GnRH) with $P \leq 0.01$.

[31]. Other possibility is that the in vitro studies carried out in ranids for a short-term period were not long enough to reveal some inhibitory effect [29]. As mentioned before, in the rat GnRH treatments provoke a reduction of $Cyp450_{c17}$, which could account, at least in part, of its antigonadal effects [4]. In *B. arenarum*, however, that inhibition would represent part of the regulatory mechanism of steroid biosynthesis that, during the reproductive period, shifts from androgen to C_{21}-steroid production.

In summary, a shift in testicular steroidogenesis during the reproductive season has been demonstrated for the first time in an amphibian species, this shift mainly depending on changes in $Cyp450_{c17}$. Moreover, it has also been shown that GnRH and FSH have a regulatory effect on $Cyp450_{c17}$ activities, influencing testicular steroidogenesis.

4. Future directions

Having in mind that both GnRH and FSH have a similar action on testicular steroidogenesis, the study of the regulation and/or interaction between them could increase the knowledge of the regulation of testicular function. In this regard, it is important to establish the source of GnRH involved in the regulation of testicular steroidogenesis. GnRH-like materials have been shown in gonads of *Rana esculenta* [2,15], goldfish [43,53] and mammals [1,17,35,52]. However, our preliminary studies on testicular production of GnRH, using a combination of HPLC and RIA with a poly-specific GnRH-variant antiserum have failed to demonstrate the presence of GnRH in testis of *B. arenarum*. Immunohistochemistry employing antibodies against mGnRH and cGnRH-II have given also negative results. Nevertheless, GnRH could be originated in the hypothalamus reaching the gonads through the blood stream. In *B. arenarum*, mGnRH fibres in the neural lobe of the pituitary gland have been described [48] and the authors hypothesised that GnRH could be released to the general circulation. If that hypothesis were accurate GnRH plasma levels would increase during the reproductive period having effects on testicular steroidogenesis. Alternatively, GnRH could control testicular function via direct innervation.

Another interesting area of research is referred to the function of progesterone-reduced derivatives. The participation of a C_{21}-steroid in hCG-induced spermiation in *Rana nigromaculata* has been suggested [41]. However, in *B. arenarum* it has been demonstrated that steroid hormones are not involved in hCG-induced in vitro spermiation [56], suggesting that the steroidogenic shift to progesterone derivatives during the breeding season is not related to spermiation. Although the participation of C_{21}-steroids in amphibian spermatogenesis remains to be determined, their role in spermatogenesis seems to be very improbable. Such a role has not been shown in either fish or mammals.

In the goldfish, it has been found that 17,20β-dihydroxy-4-pregnen-20-one, acting as a pheromone, is released into the water to induce sexual behaviour [62]. Moreover, several 3α, 5α-reduced steroids have been described as neuroactive-steroids, producing behavioural effects in mammals [22–24]. In amphibian brain, both the production of neurosteroids and the expression of steroidogenic enzymes have been described [46,47]. Moreover, seasonal changes in brain steroidogenic enzymes have also been described, suggesting a role of neurosteroids in the breeding cycle [63]. Consequently, it is possible that the reduced steroids produced during the breeding season of *B. arenarum* exert their actions on the central nervous system, inducing the expression of reproductive behaviour or even steroidogenic enzymes.

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revised 1985 of the National Institute of Health and also with the argentine laws.

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