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Effect of Human Gonadotropins on Spermiation and Androgen Biosynthesis in the Testis of the Toad *Bufo arenarum* (Amphibia, Anura)

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ABSTRACT This paper analyzes, in the toad *Bufo arenarum*, the effect on spermiation and androgen secretion of two human recombinant gonadotropins, human recombinant LH (hrLH) and human recombinant FSH (hrFSH) as well as the well-known spermiation-inducing hormone, human chorionic gonadotropin (hCG). For this purpose, testes were incubated with different concentrations of hrLH (0.01–2.5 μg/ml) and hrFSH (0.05–5 μg/ml), and results were compared with those obtained with 2.5 μg/ml hCG. Spermiation was most efficiently stimulated by hrFSH, which elicited a higher response than either hrLH or hCG. Both hrFSH and hrLH produced a bell-shaped dose–response curve, with a 50% inhibition on spermiation at a concentration twice higher than that necessary to get the highest response. However, none of the gonadotropins yielded a biphasic response on androgen secretion, hrLH producing the highest response at a concentration that evoked a 70% inhibition in the spermiation test. Regarding steroidogenesis, hrLH and hrFSH were more active than hCG. Taken together, the results described in this paper suggest that, in *B. arenarum*, spermiation and androgen secretion are mediated by different receptors. After comparing the effects of recombinant hormones, we conclude that hrFSH has a greater effect on spermiation than hCG or hrLH. *J. Exp. Zool. 305A:96–102, 2006*.

In vertebrates, LH and FSH play a pivotal role in the regulation of all testicular functions. In mammals, the role of gonadotropins is clearly differentiated, LH and FSH possessing separate receptors with high specificity (De Kretser et al., '71; Means and Vaitukaitis, '72; Bhalla and Reichert, '74; Dufau et al., '76; Licht et al., '76; Ryan and Lee, '76). The situation is similar in birds, in which LH and FSH have different target cells (Ishii and Furuya, '75), with different receptors for each gonadotropin (Jenkins et al., '78; Bona-Gallo and Licht, '79). The biological activities of FSH and LH are directed to Sertoli and Leydig cells, respectively, by the restricted expression of FSH and LH receptors in each cellular type (McLachlan et al., '96). In contrast, in fish, amphibians, and reptiles the situation is still controversial. In the African catfish *Clarias gariepinus*, the FSH receptor appears to be less discriminatory for its species-specific LH than its avian and mammalian counterparts (Bogerd et al., 2001). In this species, human recombinant FSH (hrFSH) seems to be more potent than Human chorionic gonadotropin (hCG) in stimulating androgen secretion (Bogerd et al., 2001). In amphibians, pituitary gonadotropins were isolated and characterized as two different molecules (Licht and Papkoff, '74; Papkoff et al., '76). However, it is still unclear whether FSH and LH have separate functions. In *Rana esculenta*, both gonadotropins induce ovarian steroid production (Polzonetti-Magni et al., '98), while in *Rana catesbeiana* both hormones exhibit different actions (Licht and Papkoff, '74). In the bullfrog,

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FSH mainly controls spermiation, while ovulation is under LH control. However, the differentiation of LH and FSH receptors seems to be incomplete (Takada et al., '86; Yamanouchi and Ishii, '90). In *Xenopus laevis* and *Ambystoma tigrinum*, mammalian LH and FSH induced an increase in 3β-hydroxysteroid dehydrogenase activity (Wiebe, '70; Moore, '74). Furthermore, in *X. laevis* rat FSH binds specifically to the testis, its binding being displaced by rat LH but only at a very high concentration (Adachi et al., '79). In *Bufo arenarum* Hensel, hrFSH strongly reduced cytochrome P450, 17-hydroxylase, 17–20 lyase activity, suggesting that FSH could be involved in the regulation of the steroidogenic changes undergone by testis during the breeding season (Canosa and Ceballos, 2002).

In amphibians, similarly to mammals, FSH is related to spermatogenesis with mammalian FSH involved in several actions relating to this process. In the newt *Cynops pyrrhogaster*, FSH from mammalian sources maintains spermatogonial viability, stimulates spermatogonial proliferation (Abé and Ji, '94; Ji and Abé, '94), and also inhibits prolactin- and cold-induced spermatogonial cell death, both in vivo and in vitro (Mazzi and Vellano, '68; Yazawa et al., 2001). Yu et al. ('96) suggested that the binding sites of tetrapodian FSHs, from amphibian to mammalian, share a relatively high degree of homology. In the newt, the cloned FSH receptor shares approximately 70% homology with the mammalian receptor and binds specifically to human FSH (Nakayama et al., 2000). This receptor is highly expressed in Sertoli cells, suggesting that the effect of human FSH on newt spermatogenesis is evoked through the FSH receptor (Ji et al., '95; Ito and Abe, '99).

In several amphibian species, the importance of pituitary gonadotropins in inducing spermiation is largely accepted (Licht, '73; Nagahama, '86; Parvinen et al., '86). In *Rana pipiens*, *Hyla regilla*, and *Eleutherodactylus coqui*, extremely pure preparations of ovine FSH and LH, with no detectable cross-contamination induced spermiation in an equipotent manner (Licht, '73). In *B. arenarum*, incubation of testicular fragments with hCG induced spermatozoa release in the incubation media, which showed that spermiation does not depend on steroid biosynthesis, although steroid secretion is stimulated (Pozzi and Ceballos, 2000).

During amplexus in *Bufo japonicus*, plasmatic concentration of LH and FSH is increased (Ishii and Itoh, '92). However, it is difficult to ascertain which of the two gonadotropins is responsible for inducing spermiation. In *R. catesbeiana*, LH and FSH are also secreted simultaneously, with a higher concentration of plasmatic FSH than of LH (Licht et al., '83).

Because of the limited availability of homologous gonadotropic hormones, our understanding of gonadal control in non-mammalian vertebrates is largely based on experiments carried out with mammalian gonadotropins purified from pituitary glands. Frequently, preparations containing one gonadotropin are contaminated with the other, and, in those cases, the effect on spermiation cannot be clearly attributed to one or the other.

To avoid this problem, human recombinant LH (hrLH) and hrFSH were used in this study. Experiments performed with porcine Leydig cell showed that hrLH increases steroidogenesis, as does LH (Lejeune et al., '98), and hrFSH displays all the functions of FSH purified from human pituitary (Hakola et al., '98).

The present work examines the effect of hrLH and hrFSH on spermiation and androgen production in the toad *B. arenarum*.

MATERIALS AND METHODS

Materials

Testosterone was supplied by Sigma Chemical Co. (St. Louis, MO). [^{3}H]testosterone (3,418.8 GBq/mmol) was acquired from NEN (Boston, MA). hCG was from Elea Laboratory (Buenos Aires, Argentina) and hrLH and FSH were provided by Serono Laboratory (Madrid, Spain). All other chemicals were of reagent grade. Testosterone antibody was from Colorado State University (Fort Collins, CO).

Tissue preparation

Testes of adult males of *B. arenarum* were used. Toads were kept at 25° for 1 week prior to the study. Animals were euthanized with MS222 in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of The Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina. Testes were rapidly excised; placed in ice-cold saline; and fat bodies, mesorchia, and Bidder's organ were removed. Testes were cut with scissors into $1 \times 1 \times 2\,mm^3$ pieces weighing approximately 20 mg each.

Incubation system

Testicular fragments were transferred to plastic dishes and pre-incubated for 1 hr with 1 ml of incubation medium to remove unbound spermatozoa (Pozzi and Ceballos, 2000). The incubation medium was Krebs–Ringer glucose solution containing 10 mM Hepes, pH 7.4 (KRGH). After pre-incubation, medium was discarded and tissue incubated with different hormones as described below.

Effect of human gonadotropins

Fragments were incubated with or without hCG (0.1–25 μg/ml), hrLH (0.01–2.5 μg/ml), or hrFSH (0.05–5 μg/ml) in KRGH, for 2 hr at 28°C. After incubation, 100 μl medium was separated to evaluate spermiation by counting spermatozoa with a Neubauer chamber. The results were expressed as the total number of spermatozoa per ml incubation medium. The effect of the gonadotropins on steroid biosynthesis was analyzed by assaying androgens (testosterone plus 5α-dihydrotestosterone) by RIA. The cross-reactivity of testosterone antibody with 5α-dihydrotestosterone was 35%. The sensitivity of the assay was 6 pg/ml. Steroids were assayed in triplicate. Intra- and interassay coefficients of variation were under 8% and 12%, respectively. Androgen production was expressed as media contents per ml. Scintillation counting was carried out with Wallac 1409 DSA equipment (Wallac Co, Turku, Finland), in which quenching is corrected individually for each sample through automated optimal energy-window opening. The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co, Turku, Finland).

Statistical analysis

Results are expressed as means ± SE and were analyzed and compared using a randomized block ANOVA test (Steel and Torrie, '80).

RESULTS

In a previous study, the mechanism of hCG-induced spermiation in *B. arenarum* was studied using an in vitro system (Pozzi and Ceballos, 2000). In the present work, both spermiation and androgen secretion were analyzed using human recombinant gonadotropins (hrLH and hrFSH) and compared with a well-known stimulator of toad spermiation, hCG. Testes fragments were incubated for 2 hr with different concentrations of hCG (0.1–25 μg/ml) and results normalized to 2.5 μg/ml hCG, which evoked the highest response in both spermiation and androgen secretion.

When the effect of hCG on spermiation was assayed, a well-defined dose–response curve was obtained, with a maximal stimulation approximately 20 times greater than in the control (Fig. 1A).

In order to analyze whether hrFSH is able to induce spermiation as hCG, testes were incubated with different concentrations of hrFSH (0.05–5 μg/ml). As a positive control, 2.5 μg/ml of hCG was used in each experiment, and the results related to these data. Figure 2B shows that hrFSH stimulated spermatozoa release, with stimulation reaching the highest level at 0.5 μg/ml, a concentration 5 times lower than that necessary to get the highest stimulation with hCG. Also, hrFSH was approximately 60% more potent than hCG (Figs. 1A and B). However, hrFSH provoked a biphasic response (Fig. 1B), with a dose of 1 μg/ml of hrFSH producing approximately 50% inhibition (0.5 μg/ml vs. 1 μg/ml). However, 25 μg/ml of hCG, a dose 10 times higher than that for the maximal response, did not inhibit spermiation (Fig. 1A).

Experiments performed with hCG and hrFSH demonstrated that the two gonadotropins exhibit different behavior. In order to investigate whether the effect of hCG on spermiation is more closely related to hrLH than to hrFSH, testes were incubated with different concentrations of hrLH (0.01–2.5 μg/ml) and normalized, as mentioned before, to 2.5 μg/ml hCG (Fig. 1C). hrLH evoked a maximal response slightly lower (90%) than hCG, although with a concentration 10 times lower than 2.5 μg/ml hCG. As hrFSH, hrLH produced a biphasic effect, although the highest response was approximately 50% of the maximal response achieved with hrFSH (Figs. 1B and C).

Regarding androgen secretion, hCG increased two-fold androgen production only, at the same concentration that evoked the highest response on spermiation (Fig. 2A). Furthermore, both hrFSH (Fig. 2B) and hrLH (Fig. 2C) were more efficient than hCG on steroidogenesis, both gonadotropins increasing androgen production approximately three-fold.

In order to determine the effect of hCG on the biphasic response elicited by hrFSH, two concentrations of hCG (2.5 and 25 μg/ml) were added to 5 μg/ml of hrFSH. Figure 3 shows that neither 2.5 nor 25 μg/ml of hCG were able to revert the inhibitory effect produced by hrFSH.

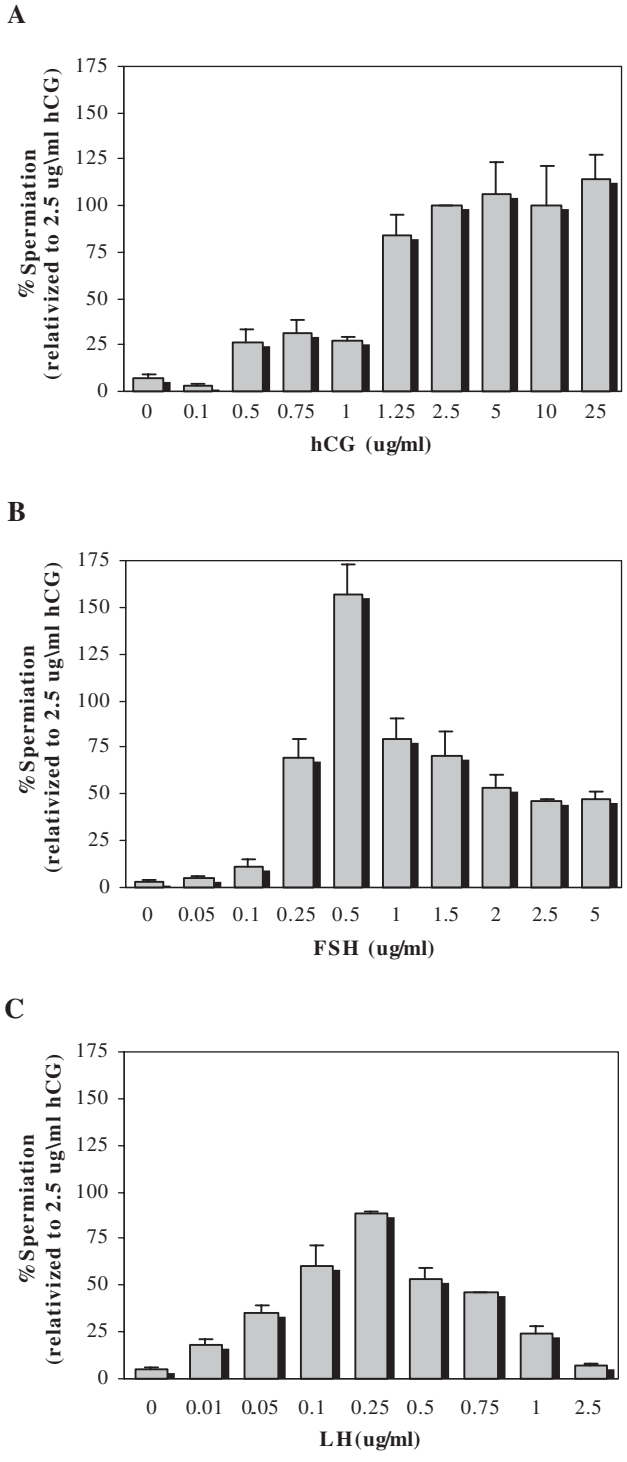

Fig. 1. Effect of increasing amount of gonadotropins on spermiation. Testis fragments were incubated with or without different concentrations of hCG (**A**) hrFSH (**B**), and hrLH (**C**) in Krebs–Ringer-glucose solution containing 10 mM Hepes, pH 7.4, for 2 hr at 28°C. Results were normalized to hCG 2.5 μg/ml and expressed as means of 10 duplicate experiments ±SE.

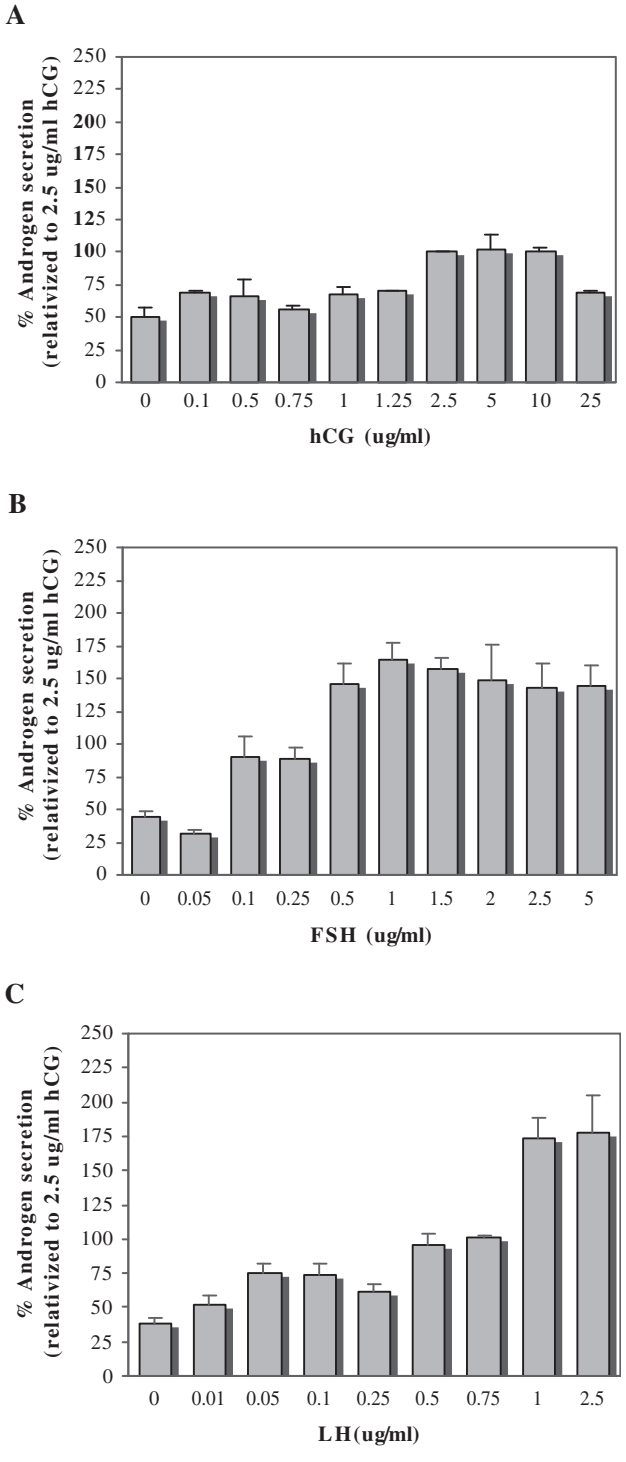

Fig. 2. Effect of increasing amount of gonadotropins on androgen secretion. Testis fragments were incubated with or without different concentrations of hCG (**A**) hrFSH (**B**), and hrLH (**C**) in Krebs–Ringer-glucose solution containing 10 mM Hepes, pH 7.4, for 2 hr at 28°C. Androgen production was quantified by RIA and normalized to hCG 2.5 μg/ml. Results are expressed as means of 10 duplicate experiments ±SE.

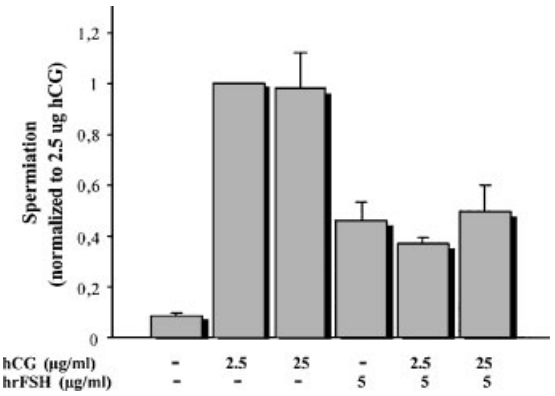

Fig. 3. Effect of hrFSH on spermiation induced by hCG. Testis fragments were incubated in the presence of 2.5 and 25 µg/ml hCG with or without 5 µg/ml hrFSH. Results were normalized to hCG 2.5 µg/ml. Testicular tissue was incubated for 2 hr at 28°C in Krebs–Ringer-glucose solution containing 10 mM Hepes, pH 7.4. Results are expressed as means of three duplicate experiments ± SE.

DISCUSSION

The present paper analyzes, in the toad *B. arenarum*, the effect on spermiation and androgen secretion of three human gonadotropins, hrLH, hrFSH, and the well-known spermiation-inducing hormone, hCG. In mammals, hCG has been largely recognized as a LH-mimetic hormone, which suggests that all its actions are mediated by the LH receptor. This assumption has also been applied to amphibian spermiation. Kobayashi et al. ('93) have proposed that hCG-induced spermiation in *Rana nigromaculata* is mediated by steroids. However, in *B. arenarum*, spermiation elicited by hCG does not depend on steroid biosynthesis (Pozzi and Ceballos, 2000).

The response of *B. arenarum* testes to hCG, hrLH, and hrFSH showed that spermiation is most efficiently stimulated by hrFSH. This gonadotropin elicited a response higher than either hrLH or hCG. Although hrLH induced spermiation with a lower concentration than hrFSH, this response never reached that obtained with hrFSH. These results disagree with those previously described for three anuran species, *R. pipiens*, *H. Regilla*, and *E. coqui* (Licht, '73), in which extremely pure preparations of ovine LH and FSH were equally potent in inducing in vivo spermiation. This discrepancy could be due to species-specific differences, or it may indicate that, in amphibians, ovine gonadotropins behave differently from human ones. Another possibility that cannot be excluded is that in the in vitro studies carried out in *B. arenarum*, variables such as the hormone's half-life, important in the in vivo conditions employed by Licht ('73), have not been taken into consideration. The fact that both gonadotropins employed in the present paper were recombinant hormones leads to the conclusion that FSH effect is not due to a LH contamination, as was suggested in other in vivo studies (Licht, '73). It thus seems reasonable to conclude that spermiation in *B. arenarum* is more specific for human FSH than for human LH and hCG, and could be elicited via FSH receptor. These results agree with those previously described in *C. pyrrhogaster*, in which FSH receptor had a higher affinity for human FSH than for human LH (Nakayama et al., 2000). Moreover, in the toad, hrFSH could elicit spermiation directly in Sertoli cells, since in a previous work it was demonstrated that iodinated hrFSH binds to a population-cell resembling Sertoli cells (Pozzi et al., 2001). In other amphibian species as well, it has been proposed that mammalian FSH mainly exerts its actions on Sertoli cells (Ji et al., '95; Ito and Abe, '99; Yamamoto et al., 2001; Yazawa et al., 2001).

It is remarkable that even though hrFSH is more efficient than hrLH, both recombinant hormones produced a bell-shaped dose–response curve. Recombinant gonadotropins evoked a 50% inhibition on spermiation with a concentration twice higher than that necessary to get the highest response, suggesting that both hormones are acting through the same receptor. Clearly, this inhibitory effect is not due to downregulation but it is not possible to discern whether it is evoked by a low-affinity second population of the same receptor or to a failure in coupling to the second messenger system (Fuh et al., '93; Zarkesh-Esfahani et al., 2000). Otherwise, hCG always showed a non-biphasic response, even at the highest concentrations assayed. However, high concentrations of hCG were not able to prevent the inhibitory effect of hrFSH, probably because of the higher affinity of hrFSH for the receptor.

These results clearly demonstrate that the receptor associated with spermiation does not discriminate between human gonadotropins, and probably not between homologous ones. This conclusion is in agreement with the results previously described in *R. catesbeiana*, using homologous gonadotropins (Takada et al., '86). In Takada's study, it was described how FSH displaces the binding of LH only partially and in a non-parallel manner. However, binding of FSH was completely displaced by LH as well as by FSH, although a higher concentration of LH was

required. These authors also demonstrated that hCG is able to displace the binding of homologous LH and FSH, although with a lower potency than homologous hormones. These results suggested that hCG could reproduce the effect of both homologous LH and FSH. The situation seems to be similar in the sea turtle, in which FSH binding sites in the ovary have a similar affinity for both turtle LH and FSH (Licht et al., '77).

Regarding androgen secretion, none of the gonadotropins employed yielded a biphasic response. More evident is the effect of hrLH, which produced the highest response on androgen secretion at a concentration that evoked a 70% inhibition in the spermiation test. Similar non-biphasic effect was evoked by hrFSH. Surprisingly, hCG stimulated androgen secretion in a lower magnitude than both recombinant hormones. Similar results with hCG were described in the African catfish *C. gariepinus*, in which hrFSH was more potent than hCG in stimulating androgen secretion (Bogerd et al., 2001). In this species, the FSH receptor appears to be less discriminatory for its species-specific LH than its avian and mammalian counterparts (Bogerd et al., 2001).

In light of the results described in this paper, it is possible to conclude that testes of *B. arenarum* respond with a different sensitivity to each human gonadotropin. Also, both spermiation and androgen secretion seem to be mediated by different receptors. Moreover, this conclusion agrees with the steroid-independent hCG-induced spermiation model previously described in *B. arenarum* (Pozzi and Ceballos, 2000). The biphasic effect on spermiation described for hrLH and hrFSH could be mediated by a receptor different from that involved in the regulation of androgen secretion, with a response showing a saturation dose–effect curve. From the comparison of recombinant hormone effects, it is possible to conclude that, in *B. arenarum*, hrLH possesses a steroidogenic effect similar to that of hrFSH, while the latter gonadotropin seems to be more important in the spermiation test.

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