Effect of Mammalian Gonadotropin Releasing Hormone Agonist, Human Chorionic Gonadotropin and Pituitary Homogenate on Spermiation in *Rana tigerina* and *Rana catesbeiana*

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ABSTRACT Frog pituitary extract, mammalian gonadotropin releasing hormone agonist(GnRHa) and human chorionic gonadotropin (hCG) were injected subcutaneously and intraperitoneally to determine the optimal doses required to elicit spermiation in adult male frogs, *Rana tigerina* and *R. catesbeiana*, during the breeding season. Pituitary extract showed the most pronounced effect in stimulating sperm release at dosages of 1 gland per frog in *R.tigerina* and 2 glands per frog in *R.catesbeiana*. GnRHa gave marked stimulating effect in both *R. tigerina* and *R. catesbeiana* at dosages of 10 and 25 µg/kg body weight, respectively.The induction of sperm release in both species of frogs could also be detected after the injection of hCG at the dosage of 50 and 200 IU per frog . The optimal doses of GnRHa were also tested for their stimulatory effect on spermiation during other seasons. *R. catesbeiana* could be stimulated to spermiate throughout the year, but with the reduction in sperm concentration during pre-, post- and non-breeding seasons. In contrast, *R. tigerina* could be induced to spermiate only during breeding season.

KEYWORDS: *Rana tigerina, Rana catesbeiana*, spermiation, gonadotropin, GnRHa.

INTRODUCTION

The reproductive cycles of amphibians have been studied by many investigators.[1,2] It is generally accepted that gonadotropic activity of pituitary gland which modulates the reproductive cycle is regulated by hypothalamus[3], through the release of gonadotropin- releasing hormone (GnRH).GnRH has been demonstrated to stimulate the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone(LH) in some species of frogs,such as *Rana catesbeiana*.[4] The testicular function is, in turn, controlled by LH and FSH[2], whose levels were markedly increased during the proliferation of secondary spermatogonia, primary and secondary spermatocytes in *Rana esculenta*.[5] In addition to native hormones , gonadotropin- releasing hormone agonist (GnRHA) could also stimulate primary spermatogonial multiplication and testicular androgen production in *R. esculenta*.[6]

There were many reports on the study of the effect of hormone priming on spermiation.[7,8] The stimulation of sperm release by GnRH administration could be demonstrated in *Hyla regilla*.[2] The effectiveness of various hormones, ie. frog pituitaries, human chorionic gonadotropin (hCG), LH, FSH and LH/FSH-RH on eliciting spermiation in bullfrogs, *R. catesbeiana* had also been reported.[8,9] Similarly, pituitary preparation, gonadotropins and hCG could stimulate the sperm release in *Rana pipiens*.[10,11]

Most anurans have exhibited a high spermatogenic activity in the breeding season as observed in *Rana temporaria*[12], *Bufo arenarum*[13] and *Bufo regularis*[14]. In contrast, Loumbourdis and Kyriakopoulou[15] studied the testicular activity in an Indian frog, *Rana ridibunda*, and found spermatogenesis to be continuous throughout the year. It remains to be seen whether frogs with discontinuous-type spermatogenesis could be induced to spermiate during various seasons.

In present study we investigate the effect of GnRHa, hCG and pituitary homogenate on stimulating sperm release in *R. tigerina* and *R. catesbeiana* during various phases of reproductive cycle. The results obtained may have application in controlling the reproduction of both species, which are indigenous (*R. tigerina*), and imported species (*R. catesbeiana*), for commercial culturing.

Materials and Methods

1. **The determination of effective dosages of various hormones on spermiation during breeding season**

R. tigerina aged more than 12 months old (weighing on the average 150-200 gm)and *R. catesbeiana* more than 18 months old (weighing on the average 350-400 gm) were obtained from the culture laboratory of the Faculty of Science, Mahidol University. The adult male frogs collected during breeding season (April-September) were used to study the effects of mammalian Gonadotropin releasing hormone agonist (Buserelin acetate), (GnRHa), hCG, and homoplastic pituitary homogenate on spermiation. Each hormone or pituitary extract was dissolved in distilled water and injected in a total volume of 0.1 ml. Ten frogs were used in each group of experiment.

1.1 GnRHa administration

In *R. tigerina*, the frogs were divided into five groups : group I were injected with distilled water to serve as control; groups II, III, IV and V received GnRHa 5 µg, 10 µg,25 µg and 50 µg/kg body weight (bw), respectively.

In *R. catesbeiana*, the frogs were divided into four groups : group I were injected with distilled water to serve as control; groups II, III and IV received GnRHa 10 µg, 25 µg and 50 µg/kg bw, respectively.

All the experimental frogs received a single subcutaneous injection of GnRHa into the dorsal lymph sac.

1.2 hCG administration

In *R. tigerina* there were four groups : group I was the control ; groups II, III and IV received hCG 25 IU, 50 IU and 100 IU per frog, respectively.

In *R. catesbeiana* there were five groups : group I was the control ; groups II, III, IV and V received hCG 50 IU, 100 IU, 200 IU and 300 IU per frog, respectively.

All the experimental frogs received a single intraperitoneal injection .

1.3 Pituitary homogenate administration

Pars distalis of pituitary glands were collected from the frogs during the breeding season from July to August.The glands were homogenized and lyophilized, and pituitary powder were kept at -20°C until use. The pituitary powder was completely dissolved in 0.1 ml distilled water and subcutaneously injected into the dorsal lymph sac. Each male *R. tigerina* received one homoplastic pituitary homogenate, whereas a male *R. catesbeiana* was given two homoplastic pituitary homogenate.

All the experimental frogs were examined for the spermiation responses by the cloacal aspiration carried out at interval beginning at 1 hour onwards after hormone treatment. The number of frog responding to the stimulation, the volume of semen released, and the concentration of semen were quantitated.

2. **The effect of the optimal dose of GnRHa on spermiation during various seasons**

The optimal doses of GnRHa that caused spermiation during the breeding season in both species were used to induce spermiation in adult *R. tigerina* and *R. catesbeiana* during the post-breeding (October-November), non-breeding (December-January) and pre-breeding (February-March) seasons. The observations and quantitations of spermiation response were done in the same manner as described above.

3. Statistical Analysis

The mean values obtained from each experimental group were compared with the normal control values. In addition, comparison among different dosages of each hormone as well as different kinds of hormones were statistically tested by using non-parametric equivalent, the Mann-Whitney test. A probability value less than or equal to 0.01 or 0.05 was chosen to indicate statistical significance.

Results

1. **The effects of GnRHa, hCG and pituitary homogenate on spermiation during breeding season**

1.1 GnRHa

In *R. tigerina*, GnRHa at 10 µg/kg bw could stimulate the sperm release in 100% of specimens at the first two hours. This effect was then decreased to 60% in the third hour. In contrast, the other doses of GnRHa (5, 25 and 50 µg/kg bw) showed less stimulating effect. The high concentration of GnRHa, particularly 50 µg/kg bw, could only stimulate 20% of frogs to spermiate at the beginning, while the proportion of positive response was gradually increased to 60% and 80% in the second and third hours, respectively (Fig 1A). The same trend of spermiation response could be observed in *R. catesbeiana*, where 10 µg/kg bw dose showed marked effect at the first two hours. The higher doses of 25

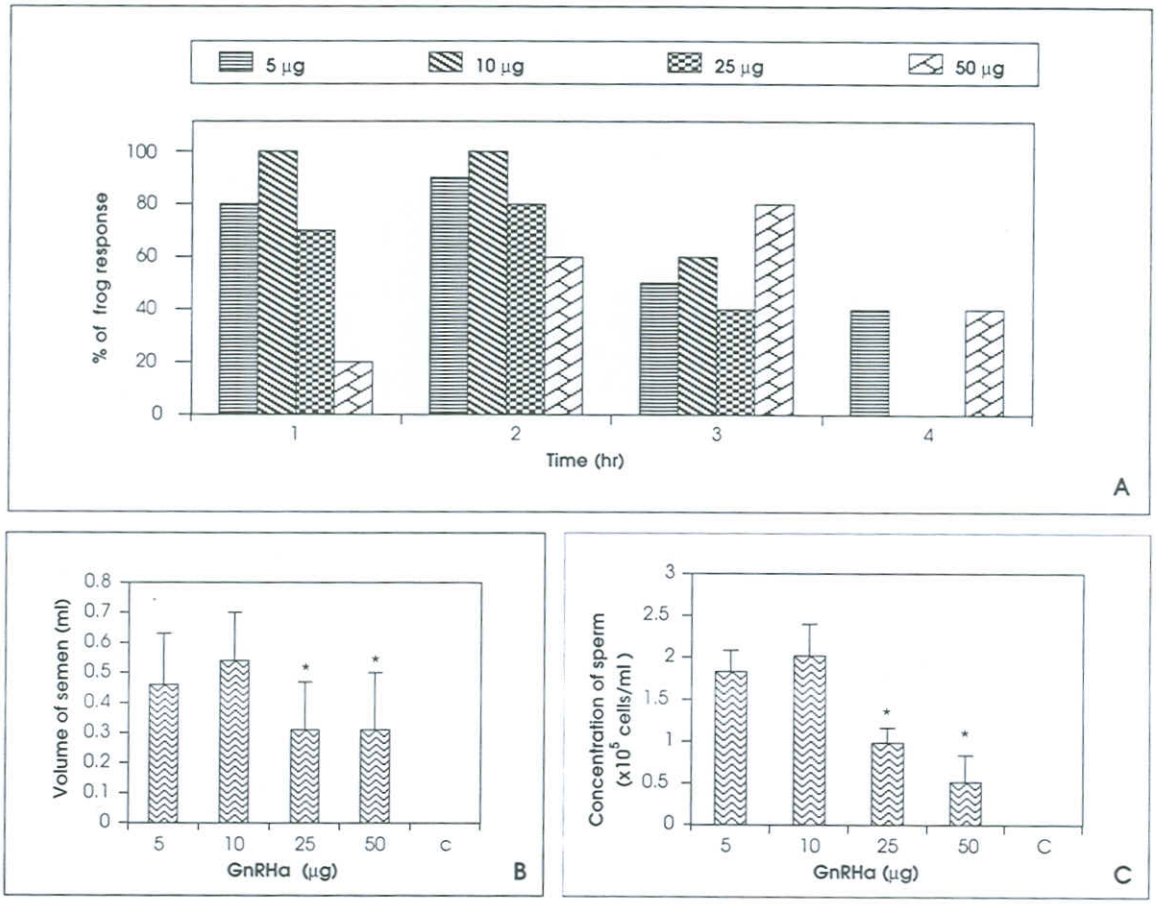

Fig 1. A: Histograms showing percentages of induced spermiation among male *R. tigerina* after being treated with various doses of GnRHa at 5, 10, 25 and 50 µg/kg bw

B,C: The changes in total volume of semen and concentration of sperm collected at the end of the experiment (4 hr, respectively) after the administration of 5, 10, 25 and 50 µg/kg bw of GnRHa.

* indicates $p < 0.05$ vs 10 ug GnRHa injected group.

and 50 µg /kg bw showed less effect at the first two hours, but later the effect increased to reach the peak at only 80-90% at 4-5 hours (Fig 2A).

The total volume of pooled semen of *R. tigerina* collected after the fourth hour in the group receiving the dosage 10 µg/kg bw gave the highest value (0.54±0.16 ml), whereas the total volume obtained from other doses remained relatively low (Fig 1B). The mean difference of the total volume in the 10 µg/kg bw GnRHa group is statistically significant ($P \leq 0.05$) when compared to the groups receiving 25 and 50 µg/kg bw GnRHa. In *R. catesbeiana*, after the end of the experiment at seventh hour the dosage of 25 µg/kg bw GnRHa gave the highest value of semen volume (1.86±0.4 ml, Fig 2B). This mean value, however, did not show any significant difference from 10 and 50 µg/kg bw GnRHa injected group.

The average concentration of *R. tigerina*'s sperm obtained from 10 µg /kg bw GnRHa injection showed the highest value (2.02±0.38 x 10^5 cell/ml), whereas the mean concentrations of the other groups remained relatively low (1.83 ± 0.25 , 0.98±0.18 and 0.51±0.32 x 10^5 cells/ml) (Fig 1C). The high concentration of sperm collected from 10 µg/kg bw GnRHa injected group is significantly different ($P \leq 0.05$) from those of the other two dosages (25 and 50 µg/kg bw GnRHa groups). In *R. catesbeiana*, the dose of 25 µg/kg bw of GnRHa gave the maximal concentration of sperm (2.98 ± 0.36 x 10^5 cells/ml) (Fig 2C), while the doses of 10 µg and 50 µg/kg bw of GnRHa showed less effect. The mean concentrations of sperm from these two groups are 2.01±0.32 and 1.90±0.25 x10^5 cells/ml, respectively. These mean differences of the average concentration are statistically significant ($P \leq 0.001$) when compared to the value of 25 µg /kg bw injected group . None of the semen from the control group showed any spermatozoa.

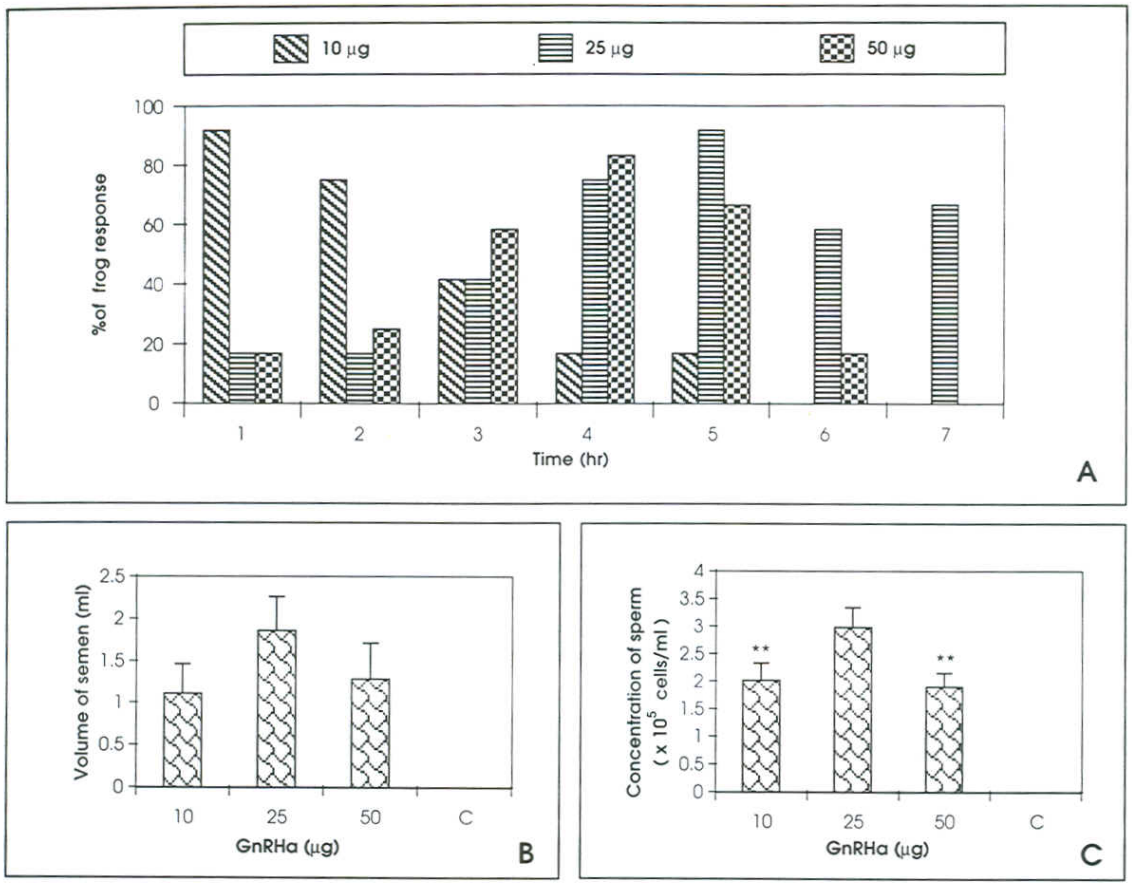

Fig 2. A: Histograms showing percentages of induced spermiation among male *R. catesbeiana* after being treated with various doses of GnRHa at 10, 25 and 50 µg/kg bw
B,C: The changes in total volume of semen and concentration of sperm collected at the end of the experiment (7 hr., respectively) after the administration of 10, 25 and 50 µg/kg bw of GnRHa.
** indicates p< 0.001 vs 25 ug GnRHa injected group.

1.2 hCG

The induction of sperm release in *R. tigerina* can also be detected after the hCG administration. The number of frogs responded to the dose at 50 IU/frog is 100% at the first two hours, and then abruptly decreased to 20% at the third hour (Fig 3A). In contrast, there is a noticeable lower percentage of frog response at the dosage of 25 and 100 IU/frog (57% and 50%, respectively). No positive responses was observed at any time after the treatment in the control group.

In *R.catesbeiana*, hCG at 50 IU/frog could not elicit spermiation while hCG at 200 IU/frog gave the highest response (88%) at the first hour (Fig 4A). The other two doses of hCG at 100 IU and 300 IU/frog showed less effect (33% and 38%, respectively).

In *R. tigerina*, the mean value of the total semen volume collected over the entire period of the experiment is highest in the 100 IU/frog group (Fig 3B). This mean total volume (0.64±0.14 ml) is significant different (P≤0.001) from those injected with 25 and 50 IU hCG/frog . In *R. catesbeiana*, the highest total volume of semen could be obtained in the group treated with 200 IU hCG/frog (Fig 4B). However, this value is not significantly different from the other two groups (100 and 300 IU/frog of hCG).

In *R. tigerina*, the average concentration of semen collected in 100 IU/frog group is slightly higher (11.9± 2.2 x10^4 cells/ml) than in other groups (7.6±1.5, 10.8± 2.3 x10^4 cells/ml). However, statistically the sperm concentration of this group is not significantly different from the groups injected with 25 and 50 IU/frog (Fig 3C). In *R. catesbeiana*, the average concentration of sperm following administration of 200 IU hCG/frog was less (6 ± 2 x10^3 cells/ml) than those injected with 100 and 300 IU hCG/frog (10 ± 3 and 9 ± 2 x 10^3 cells/ml, respectively) (Fig 4C). Moreover, sperm concentration obtained from the group injected with 200 IU/frog is significantly different (P≤0.05) from the others.

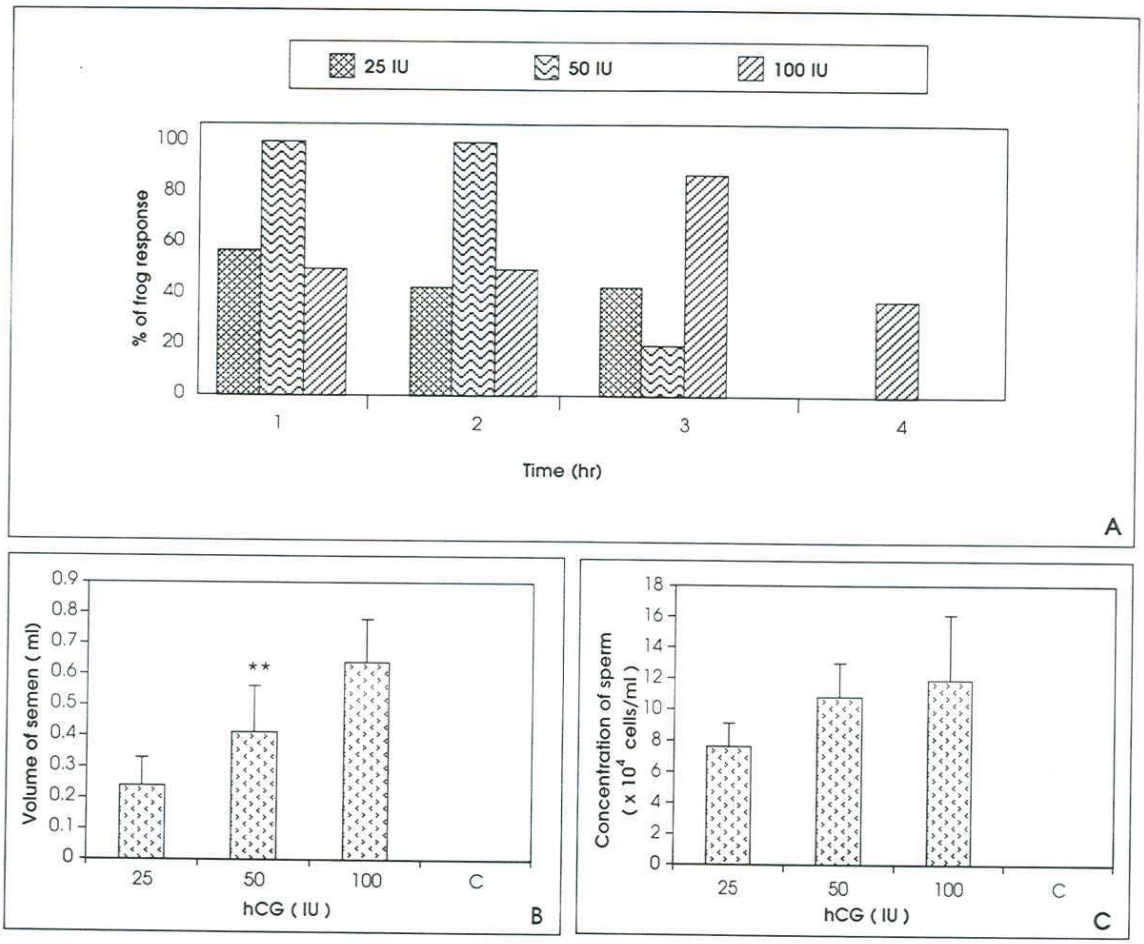

Fig 3. A: Histograms showing percentages of induced spermiation among adult male *R. tigerina* after being treated with hCG at 25, 50 and 100 IU.

B,C: The changes in total volume of semen and concentration of sperm collected at the end of the experiment (4 hr., respectively) after 25, 50 and 100 IU hCG administration.

** indicates $p < 0.001$ vs 100 IU hCG injected group.

1.3 Pituitary homogenate

Pituitary homogenates have very pronounced effect on spermiation. All the experimental *R. tigerina* (100%) responded to this treatment within the first two hours. Total response could also be detected in *R. castesbeiana* at the second and fourth hours, respectively. The total volume and concentration of semen of *R. tigerina* collected at one hour after injection are 0.7 ml and 3.09 $x10^5$ cells/ml, respectively. The volume of semen collected from *R. castesbeiana* rose to 0.48 ml with the concentration of 2.16 x 10^5 cells/ml in the first hour.

2. Spermiation response to the optimal doses of GnRHa administered outside the breeding season

The dose of 10 µg/kg bw GnRHa has a remarkable effect on *R. tigerina's* spermiation in both pre-breeding and breeding seasons. All the experimental frogs (100%) can release the sperm after this hormone administration. As the frogs progressed to post-breeding season, the percentage of response is reduced to 25%. None of them could spermiate in non-breeding season (Fig 5A). The average volumes of semen collected from pre-breeding, breeding and post-breeding seasons are 0.24±0.17, 0.51± 0.2 and 0.14±0.05 ml, respectively (Fig 5B). The concentration of sperm obtained during pre-breeding, breeding and post-breeding are 0.12± 0.05,1.86±0.58 and 0.06± 0.02 $x10^5$ cells/ml, respectively (Fig 5C). Thus, there is a remarkable reduction in the number of sperm released when compared to those obtained in breeding season. The differences of mean values are statistically significant ($P \leq 0.05$).

The dose of 25 µg/kg bw GnRHa can stimulate spermiation in *R. castesbeiana* throughout the year

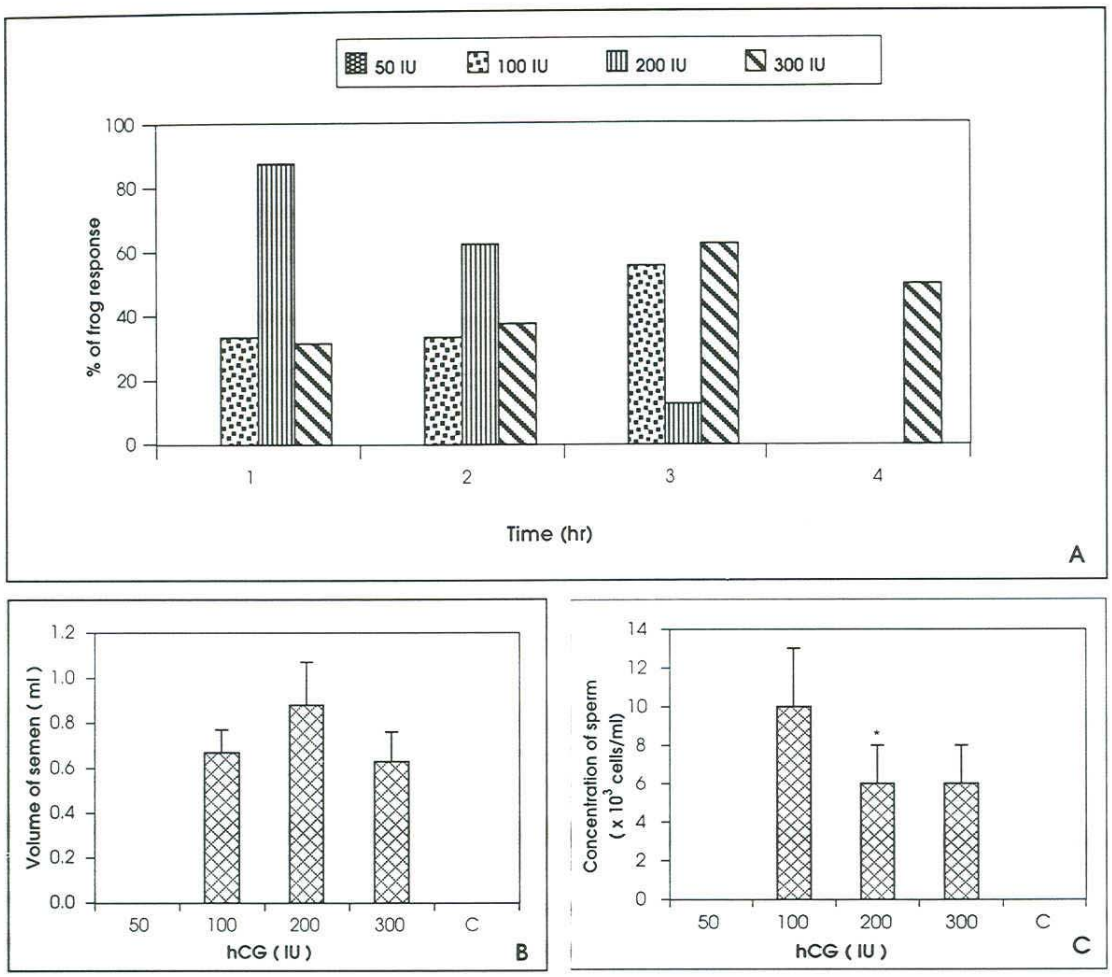

Fig 4. A: Histograms showing percentages of induced spermiation among adult male *R. catesbeiana* after being treated with hCG at 50, 100, 200 and 300 IU.
B,C: The changes in total volume of semen and concentration of sperm collected at the end of the experiment (4 hr., respectively) after 50, 100, 200 and 300 IU hCG administration.
* indicates $p < 0.05$ vs 100 IU hCG injected group.

(Fig 6A). The volumes of semen collected from different periods are indistinguishable from those obtained from breeding season (Fig 6B). In contrast to the volume of semen, the concentration of sperm collected in pre-, post-and non-breeding seasons are significantly lower than the value recorded in the breeding season, with $P \leq 0.05$ (Fig 6C).

Discussion

Effects of administration of GnRHa, hCG and pituitary homogenate on spermiation

The present study revealed that GnRH agonist (GnRHa) and hCG could be used to elicit the sperm release in *R. tigerina*, a native rice field frog of Thailand. There was a significant difference among various doses of GnRHa in their effectiveness: the 10 µg/kg bw of GnRHa gave a better response than the lower and higher three doses. In *R. catesbeiana*, the frogs that have been imported from north America for commercial culturing in Thailand, the highest of both volume and sperm concentration could be obtained from the administration of GnRHa at 25 µg/kg bw Similar effect was also reported in bullfrogs cultured in the temperate region[8] and in another species of frogs, *Hyla regilla*[7]. In contrast to these optimal doses, the higher doses of GnRHa up to 50 µg/kg bw could not elicit any substantial spermiation within the first hour in both species of frogs, and the peak response occurred at three hours after treatment. The GnRH agonist may act through the anterior pituitary gland which, in turn, induces

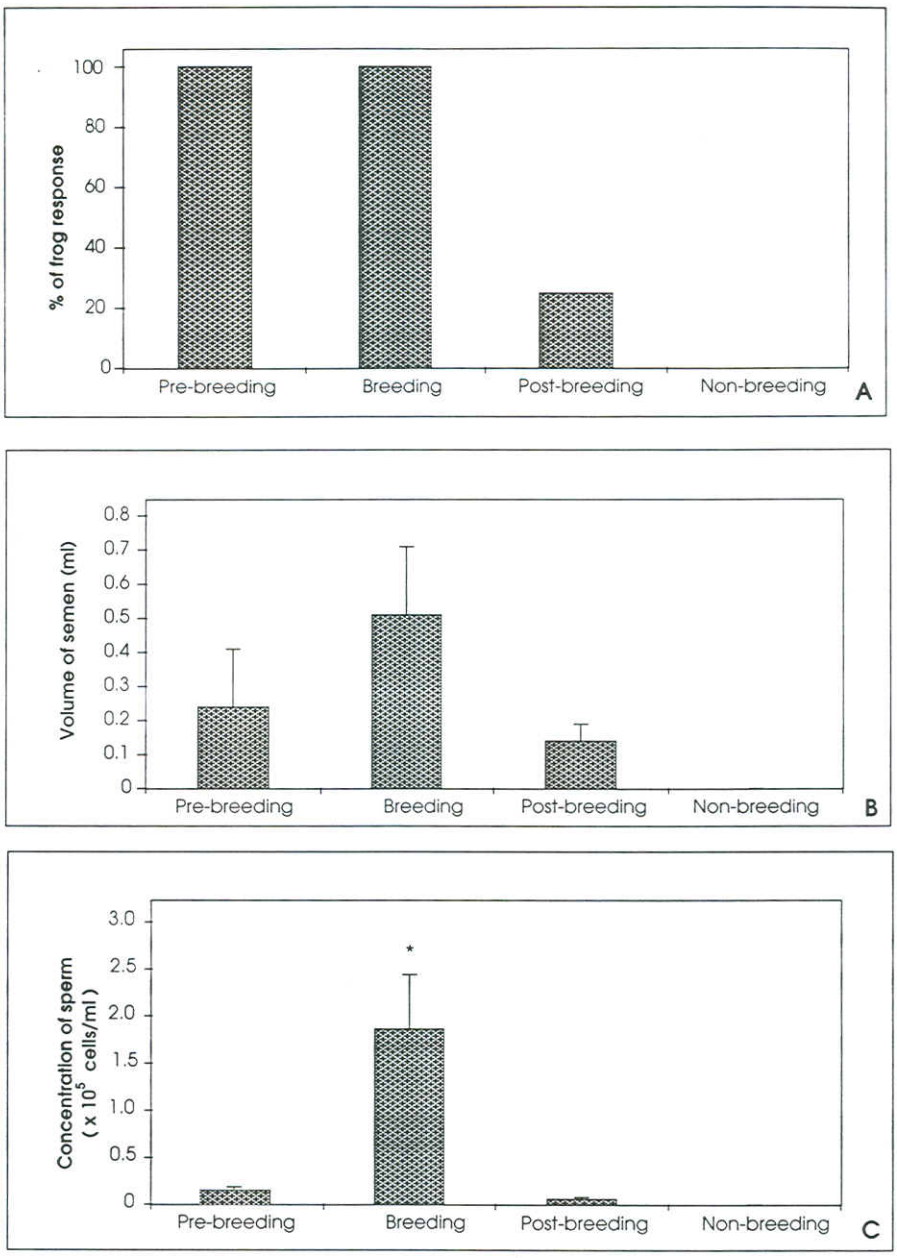

Fig 5. A: Histograms showing changes in the percentages of spermiation of *R. tigerina* in different seasons after administration of the most effective doses of GnRHa (10 µg/kg bw).

B,C: The changes in volume of semen and concentration of sperm collected from different seasons after injections with the most effective doses of GnRHa.

* indicates $p < 0.05$ vs other seasonal periods.

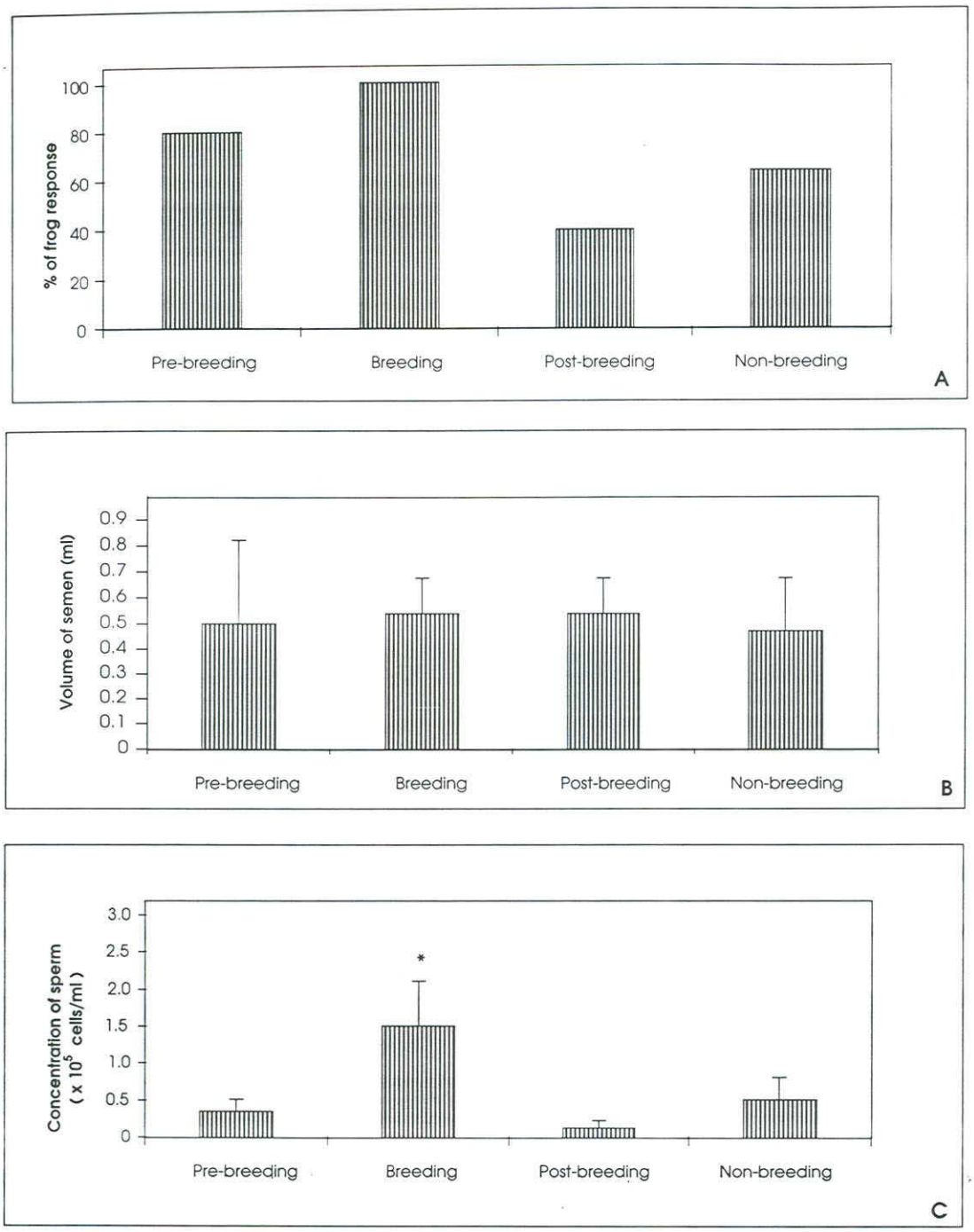

Fig 6. A: Histograms showing changes in the percentages of spermiation of *R. catesbeiana* in different seasons after administration of the most effective doses of GnRHa (25μg/kg bw).
B,C: The changes in volume of semen and concentration of sperm collected from different seasons after injections with the most effective doses of GnRHa.
* indicates $p < 0.05$ vs other seasonal periods.

the sperm release in the experimental frogs. The direct effect of GnRH on testicular activity has also been reported in *R. esculenta* and *R. pipiens*.[6,16,17,18]

The volume of semen and concentration of sperm in *R. tigerina* after the administration of GnRHa at dosage 50 µg/kg bw were also remarkably decreased in comparison with the results from the dosages of 10 or 25 µg/kg bw The high dose of GnRHa may lead to the desensitization of the pituitary gland which, in turn, could not maintain the level of circulating gonadotropins. There was an evidence done in rhesus monkey which indicated that the constant infusion of exogenous GnRH could reduce pituitary response in a phenomenon generally known as "down regulation".[19] The restoration of gonadotropin secretion, however, was achieved in the same animals by the intermittent administration of GnRH. In the present study, a single injection of high dose of GnRHa to the frogs would probably create the condition of prolonged exposure to this hormone. Therefore, it might result in the decrease in the response of pituitary gland at the beginning of the experiment. Furthermore, the development of pituitary refractoriness after continuous infusion of GnRH for a short duration was also found in rat[20] and sheep[21]. This phenomenon may also be resulted in part from a reduction in the available receptors for GnRH.

The administration of pituitary homogenate showed significant effect on spermiation in both species of frogs. This may be due mainly to the stimulatory effects of LH and FSH. It was reported that mammalian LH and FSH could induce spermiation in frogs.[8,10] The sperm release could also be induced by hCG, in which the doses of 50 and 100 IU/frog could induce 85-100% response in *R. tigerina* and *R. catesbeiana*, respectively. Similar effect was also reported in *R. catesbeiana* after receiving 200 IU hCG.[8] The activity of hCG in inducing spermiation in frogs is probably due to its LH-like action which could act directly at the gonadal level.[22] There was an evidence which showed that the hCG receptors existed in the testicular tissue.[23]

The effect of high doses of hCG (100 and 300 IU/frog), however, appeared to delay the maximal response, especially in *R. catesbeiana* which showed only 60% of response after three hours of injection. The delayed effect of high dose of hCG might occur from the loss of receptors in the gonads and the phenomenon of "down regulation" as discussed earlier. Conti *et al*,[24] who studied rat ovary, had found that there was a receptor loss after hCG administration, and there seemed to be an inverse correlation between the number of receptors and the dose of hCG.

Effect of the optimal dose of GnRHa on spermiation outside the breeding season

The present study also showed that the exogenous GnRHa could have the effect on spermiation outside the breeding season in *R. catesbeiana*. However, the percentage of frogs responding to GnRHa decreased during the post-and non-breeding periods. The testicular activities of bullfrogs were well correlated with the level of gonadotropin throughout the year.[25] Histological investigation of testes of *R. catesbeiana* from natural habitat in the state of Missouri,USA, revealed that spermatozoa in seminiferous tubules were reduced in number after breeding period but they never reached zero level.[26] Similar testicular picture was found in bullfrogs cultured in Thailand.[27] The spermiation response in *R. catesbeiana* throughout the year may be due to their continuous type of spermatogenic cycle.

In contrast, the spermiation could not be successfully induced in *R. tigerina* outside breeding season. The negative response to GnRHa in *R. tigerina* could be explained by the work of van Oordt,[28] who have shown that the sensitivity of the germinal cells to gonadotropic hormones were least sensitive during autumn and winter. Our earlier observation also showed that in *R.tigerina*, the testicular tissue usually break down during the non-breeding periods, and late stages germ cells were completely absent.[29] Similarly, the seminiferous tubules of a tropical frogs, *R. tigrina* Daud, in the period apart from the breeding season contained very few cell nests and most of which were spermatogonia.[30] These frogs are, hence, considered to have discontinuous spermatogenic cycle.

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