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Effect of Luteinizing Hormone-Releasing Hormone Analogue on the Sexual Behavior of *Sacalia quadriocellata*

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Abstract Luteinizing hormone-releasing hormone (LHRH) is known to influence sexual behavior in many vertebrate taxa, but there have been no systematic studies on the role of LHRH in sexual behavior of turtles. We tested the hypotheses that exogenous LHRH analogues would induce sexual behavior of male Four-eyed turtle, *Sacalia quadriocellata*. We examined this by challenging males with intramuscular injections of mammalian luteinizing hormone-releasing hormone analogue (LHRH-A), human chorionic gonadotropin (HCG), or a combination of the two, and subsequently exposing them to sexually receptive females for behavioral observation. Our data show that the injection of only HCG could not, while that of only LHRH-A could, facilitate sexual behavior along with testicular recrudescence and spermatogenesis in *S. quadriocellata*. The injection of both LHRH-A and HCG would induce more drastic sexual behavior of the animals than that of LHRH-A alone, indicating HCG enhances the effects of LHRH-A induced sexual behavior. However, different pharmacological dosages of LHRH-A (0.5 μg, 1 μg, 2 μg per 100 g bodyweight) did not correspond to different activity levels. Though the mechanism of LHRH effect was not determined, this study may support that the sexual behavior of *S. quadriocellata* which occurs at the beginning of the injection despite regression of the gonads. This is the first report on the exogenous LHRH-A induced sexual behavior for this species.

Keywords Four-eyed turtle, *Sacalia quadriocellata*, luteinizing hormone-releasing hormone, sexual behavior

1. Introduction

Luteinizing hormone-releasing hormone (LHRH), sometimes called gonadotropin-releasing hormone (GnRH), is released from hypothalamus and is generally considered as the central control of reproductive function at multiple levels in some vertebrates (Sherwood et al., 1993; Everett, 1988). Pulses of LHRH stimulate episodic release of two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary, which control testicular recrudescence and steroidogenesis (Kalra, 1993). It is not well understood how GnRH is integrated in the hypothalamus-pituitary-gonadal (HPG) axis in turtles. Several discrepancies exist between *in vitro* and *in vivo* studies among different chelonian species in response to the treatment of GnRH (Licht *et al*., 1985; Tsai and Licht, 1993 a, b; Kuchling, 1999; Al-Kindi *et al*., 2001).

Little is known about the effect of those hormones on sexual behavior of turtles. It may be one of the least studied aspects of turtle endocrinology, partly because the attempts to find relationships between seasonal hormone levels and reproductive behavior have largely failed (Kuchling, 1999). However, several peptide hormones are now known to affect sexual responsiveness and behavior of male vertebrates, and Kuchling (1999) indicated that several neuroendocrine peptides might have roles as central mediators of sexual behavior of chelonians. GnRH peptide was one of the first hormonal peptides found to induce sexual behavior of many female vertebrates (Pfaff, 1973; Moss and McCann, 1973; Phillips et al., 1985; Barnett *et al*., 2005). However, much less is known about the role of GnRH in the expression of male sexual behavior (Propper and Dixon, 1997). Still, several studies have shown that GnRH stimulates male sexual behavior of a

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toad (*Bufo cognatus*), a dove (*Streptopelia risoria*), a stallion, and a marmoset monkey (*Callithrix jacchus*) (Cheng, 1977; Propper and Dixon, 1997; Sieme *et al*., 2004; Lunn *et al*., 2005). However, we could not find any published data on the effect of GnRH on turtles.

We investigated the effect of GnRH on chelonians by injecting male *S. quadriocellata* with a synthetic mammalian luteinizing hormone-releasing hormone analogue (LHRH-A). Hazum and Conn (1988) indicated that desensitization occurred after repeated large doses of LHRH-A, through the result of receptor down-regulation and loss of receptor-associated ion channels. However, Wang and Wang (1988) found that the injection of human chorionic gonadotropin (HCG) synchronously in *Bufo gargarizans* resolved the desensitization to LHRH-A. Therefore, in this investigation we injected LHRH-A in conjunction with a resolving injection of HCG synchronized to induce sexual behavior. The studies of *S. quadriocellata* were concentrated on anatomy (Hong *et al*., 2004), histology (Fu *et al*., 2006; 2007), endocrinology (Fu *et al*., 2008) and field ecology (Shi *et al*., 2002). Analysis of courtship behavior was conducted by Liu *et al*. (2008). Mean plasma level of testosterone (T) rose significantly in a period from August to October, which also made the testicles achieve their maximum weight. Spermatogenesis began in March, with transforming spermatids appearing in August (Fu *et al.*, 2007). The exogenous hormones injection was done during the normal development of testicles of this species, to observe whether the treatment with LHRH-A had any effect on sexual behavior.

2. Material and Methods

Matured individuals of *S. quadriocellata* were collected from Qiongzhong in Hainan, China and maintained in the laboratory for 3–4 years. The turtles were kept in indoor cement pools (70×80 cm) with a shelter. Sex was determined by the eye spots on the dorsum of the head. The eye spots of females were yellow and those of males olive green (Shi *et al*., 2002). No reproductive behavior was observed in captivity for over three years, and we therefore concluded that the gonads were regressed.

In all experiments, two kinds of hormones, LHRH-A and HCG (Ningbo Second Hormone Factory, Ningbo, Zhejiang) were used. All the hormones were dissolved in physiological saline and injected into the hind leg muscle of male *S. quadriocellata*. Experiments were performed from 01 August to 10 November 2005. The injections were made every 10 days for a total of 10 times. After the injections, male turtles were subsequently exposed to sexually receptive females. All behaviors were observed in a period from August to December, once every month, at least on the day after hormonal injections. The observation continuously lasted for 24 h. Each individual was used in a maximum of five trials. All the observations were conducted using video cameras and the ethogram described by Liu *et al*. (2008). All-occurrence recording was used to record the beginning and ending time of sexual behaviors including courtship and copulation.

Experiment 1 Animals were divided into four groups: (1) injected with LHRH-A 1 μg/100 g BW, HCG 200IU (n=6); (2) injected with LHRH-A 1 μg/100 g BW only (n=5); (3) injected with HCG 200IU only (n=5); and (4) control group injected with saline only (n=6).

Experiment 2 Animals were divided into three groups, six individuals in each group: (1) injected with LHRH-A 0.5 μg/100 g BW, HCG 200IU; (2) injected with LHRH-A 1 μg/100 g BW, HCG 200IU; and (3) injected with LHRH-A 2 μg/100 g BW, HCG 200IU.

Statistical analysis Chi-square tests were used to determine the effects of different hormones and dosages used. A one way analysis of variance (ANOVA) was used to determine the significant differences in the time budgets among the groups. A post-hoc test using Dunn's multiple comparison procedure was performed to detect the significance in sexual behavior between each group. The differences were considered significant at $P<0.05$.

3. Results

Experiment 1 The sexual behavior of the animals injected with LHRH-A in conjunction with HCG was significantly stronger than that of the control group ($\chi^2=5.486$, $df=1$, $P<0.05$). Also, individuals treated with only LHRH-A were found having significantly stronger sexual behavior than those of the control group ($\chi^2=4.481$, $df=1$, $P<0.05$). No significant difference between HCG treated and control individuals was found ($\chi^2=0.009$, $df=1$, $P>0.05$). There were significant differences in the time budgets of sexual behavior among the four groups ($F_{3, 18}=3.952$, $P<0.05$) (Table 1).

Experiment 2 There was no difference in the proportions of animals exhibiting sexual behavior among the three groups ($\chi^2=0.643$, $df=2$, $P>0.05$). Obvious and complete courtship behavior was observed for the males and ejaculate was found on an individual in a low level group after mating in October, which indicates the recur-

Table 1 Effect of LHRH-A and HCG on sexual behavior of *S. quadriocellata*

Treatment	No. of individuals	No. of individuals with sexual behavior	Sexual behavior time budget (%)
LHRH-A & HCG	6	5	2.78±1.84[a]
LHRH-A	5	4	0.89±0.66[b]
HCG	5	1	0.06±0.14[c]
Saline	6	0	0[c]

[a] The time budget of sexual behavior with LHRH-A + HCG treated more than other groups.
[b] The time budget of sexual behavior with LHRH-A treated only more than HCG and saline treatment.
[c] Significant difference between HCG only and saline treated.

Table 2 Effect of different dosages of LHRH-A in conjunction with HCG on sexual behavior of *S. quadriocellata*

Dosage of LHRH-A	No. of individuals	No. of individuals with sexual behavior	Sexual behavior time budget (%)
0.5 μg/100 g BW	6	4	1.14±1.12
1 μg/100 g BW	6	5	2.78±1.84
2 μg/100 g BW	6	5	1.30±0.88

descence of the testes and spermatogenesis. The time budget of sexual behavior among the groups of the three levels (LHRH-A 0.5, 1, 2 μg/100 g BW) was not significant ($F_{2, 15}$=0.2.718, P>0.05) (Table 2). Thus, there was no significant difference in sexual behavior among the groups treated with the different dosages.

4. Discussion

In experiment 1, the treatment with only LHRH-A was found to significantly induce sexual behavior. To our knowledge, this study demonstrates that LHRH or its analogues induces the sexual behavior of *S. quadriocellata* for the first time. A physiological role for the peptide is supported by its ability to further increase the sexual behavior of this species. In previous studies, GnRH-induced desensitization occurred in many other species of turtles (Licht *et al*., 1985; Tsai and Licht, 1993). This could be the reason why the animals treated with LHRH-A and HCG exhibit more drastic sexual behavior than those with LHRH-A only. This seems to indicate that HCG enhances the action of LHRH-A induced sexual behavior. Furthermore, HCG stimulates testicular LHRH-like activity in rats (Sharpe and Fraser, 1980), and HCG has no effect on *S. quadriocellata*.

In the LHRH-A dosage experiment, an attempt to investigate which dosage of LHRH-A was most effective in inducing male *S. quadriocellata* for drastic sexual behavior, we found no significant difference among the three treated groups in terms of number of individuals mating and time budget of sexual behaviors. We believe that the behavioral effects of LHRH-A that we document here are not the result of a pharmacological dosage of the hormone in the range. Even when injected with very high doses of GnRH (more than 50 μg per individual), most turtles failed to respond to the dosage (Tsai and Licht, 1993a). We are confident that we would have seen a more traditional dose-dependent response if we had chosen dosage levels lower than LHRH-A 0.5 μg/100 g BW. The responses of individuals to LHRH-A were not the same, potentially resulting from the difference in sensitivity to LHRH-A. This difference could be due to LHRH receptors or inhibition of postreceptor mechanism (Tsai and Licht, 1993b).

This study shows the effects of LHRH-A injections on sexual behavior. However, the mechanism by which LHRH induces courtship behavior is still rudimentary. Previous reports have indicated that *in vivo* injections of GnRH can elicit reproductive behavior in a castrated, estrogen-primed anoline lizard, *Anolis carolinensis*, suggesting that GnRH may affect the behavior through a pathway that does not involve the gonad (Alderete *et al*., 1980). Though the ultrastructural changes of the testes after in injection were not investigated, we believe that the LHRH-A induces sexual behavior through a pathway that does not involve the testicular recrudescence, since the courtship behavior was displayed in a threshold manner. The behavioral effect of LHRH along to testes has been found in other species (Boyd and Moore, 1985; Sieme *et al*., 2004).

In conclusion, in this study we found that only exogenous LHRH-A can induce, either directly or indirectly, the sexual behavior of *S. quadriocellata*. A physiological role for the peptide is supported by its ability to further increase sexual behavior of this species. The individuals treated with LHRH-A and HCG are found induced with more drastic sexual behavior than those with LHRH-A only, indicating that HCG enhances the action of the LHRH-A induced sexual behavior, but HCG alone dose does not induce sexual behavior. The mechanism of LHRH

facilitating the display of sexual behavior was not determined, but this study seems to support that sexual behavior of *S. quadriocellata* occurs despite regressed gonads.

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