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Changes in the Oscillatory Electric Potential on the Olfactory Epithelium and in Reproductive Hormone Levels during the Breeding Season in the Toad (*Bufo japonicus*)

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ABSTRACT—A slow transient electric potential change (electro-olfactogram, EOG) can be recorded through an Ag-AgCl electrode placed on the olfactory epithelium in response to stimulation with an air stream to the tissue in toads (*Bufo japonicus*). During the breeding season, oscillatory potential changes (OSC) superimpose on the EOG. In the present study the OSC amplitude was found to be highly correlated with the migratory behavior. Since toads track the route to and from the breeding pond using olfactory cues along the migration route, the enhanced OSC should be responsible for the breeding migration.

A significant positive correlation was found between plasma gonadotropin levels and the OSC amplitude in males captured during the breeding migration. There was no significant relationship between plasma gonadotropin levels and the OSC amplitude in female toads during the breeding season, but there was a significant correlation between plasma progesterone levels and the OSC amplitude. In males, hypophysectomy just before the breeding season decreased the OSC amplitude. And testis weight was also positively correlated with the OSC amplitude in January. These results suggest that the appearance of the OSC is related to the timing of the activation of the reproductive system. However, treatment of toads with hCG (human chorionic gonadotropin), testosterone, estradiol or progesterone in the non-breeding season did not induce a significant change in the OSC amplitude. Other factor or factors may be required in activation of the olfactory system of the toad in a non-reproductive stage together with the hormones of the gonadal axis.

INTRODUCTION

Olfactory cues are used for orientation during migration in many vertebrate species (Able, 1980). The most famous example is the breeding migration to the home river in salmonid fish (Hasler and Scholz, 1983). Homing behavior using olfaction has also been suggested in the tortoise (Chelazzi and Delfino, 1986) and in the pigeon (Papi, 1991).

In amphibians, the involvement of olfaction in the breeding migration has been investigated in several species (newt, Grant *et al*., 1968; frog and toad, Grubb, 1973; toad, Sinsch, 1990). Ishii *et al*. (1995) reported that adult toads of *Bufo japonicus* track the route to and from the breeding site by catching local olfactory cues presumably originating from the migration route itself, and not from the destination (for example, the breeding pond).

Based on these behavioral studies, we expected that the olfactory system should be activated during the migration period and hence that some electrophysiological change should occur in the olfactory system during the breeding period in the toad.

A slow transient electric potential change can be evoked on the olfactory epithelium by stimulating it with a stream of air or with air containing some organic substances in a number of vertebrates (catfish, Byrd and Caprio, 1982; frog, Senf *et al*., 1980; box turtle, Tonosaki, 1993; rat, Edwards *et al*., 1988). The recording of this potential change has been referred to as the electro-olfactogram (EOG). Nakazawa *et al*. (2000) measured the EOG of *Bufo japonicus* at regular intervals throughout a year and found that toads in the breeding season showed a series of oscillatory electric potential changes (OSC) that were superimposed on the EOG in about 60% of animals examined. A similar electric activity was first described by Ottoson (1956) in the frog, *Rana temporaria*.

The pattern of monthly changes in mean OSC amplitude (Nakazawa *et al*., 2000) was similar to the patterns of monthly changes in mean pituitary contents of luteinizing hormone (LH) and follicle stimulating hormone (FSH) reported by Itoh *et al*., (1990). All these variables were highest in the breeding period, quickly decreased after the breeding migration, were low from May through September, and then increased higher in the hibernation period.

In the present study we conducted a more precise examination of changes in the amplitude of the EOG and OSC in relation to the breeding migration in the toad, *Bufo japonicus*. We also examined correlation of hormones and the electrophysiological parameters of the olfactory activity by measur-

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ing plasma levels of gonadotropin and gonadal sex steroid hormones, administering these hormones to toads and removing the hypophysis surgically.

MATERIALS AND METHODS

Animals

For the studies during the breeding migration, wild adult male and female toads of *Bufo japonicus* were captured in parks and fields in Tokyo and surrounding areas from March 8th to April 4th 1994. Immediately after capture, 1 ml of blood was collected from each toad into a heparinized syringe by heart puncture. Blood samples were chilled on ice during transportation to the laboratory. Plasma was separated by centrifugation in the laboratory and kept at –20°C until hormone assay was performed. The toads were taken to the laboratory in cool boxes. For the electrophysiological study, toads were kept in a low temperature room (6°C) in order to maintain physiological condition at the time of capture and were used for experiments within two weeks. Under natural conditions, toads temporarily stop the breeding migration and bury themselves under the ground when the ambient temperature falls below 6°C.

For the study of the effect of hypophysectomy on the electric activity of the olfactory system, eighteen male adult toads captured on January 10th 1999 were divided into two groups of nine toads. From January 14th to 20th, the toads were hypophysectomized (pars distalis extirpated) or sham operated and kept for a month at a temperature of 10°C. The hypophysectomized toads were injected with 2 I.U. of ACTH (Sigma chemical Co.) every other day to maintain the animals in a healthy condition (cf., Jorgensen and Larsen, 1963; Middler *et al.*, 1969). Control animals were injected with vehicle only (0.2 ml saline).

For treatment with hCG, sixteen male adult toads were collected in August 1999. For the experiment of treatment with steroid hormones in the non-breeding season, adult male toads were collected from parks in Tokyo in July (for testosterone), December (for progesterone) and January (for estradiol).

Recording of electric potential change

In the studies during the breeding season, toads were acclimatized to the room temperature in the laboratory for at least 30 min until they become active. After immobilization by pithing, the dorsal part of the olfactory cavity was cut and the olfactory epithelium was exposed. The local field potential was amplified through an Ag-AgCl electrode (0.2 mm diameter) by using a commercial amplifier (Nihon Kohden, AVB-21). Waveforms were recorded in a thermal array recorder.

As a stimulus, an air puff was delivered to the surface of the olfactory epithelium through a glass pipette. The diameter of the opening of the pipette was 1 mm. The airflow from a pump was controlled with an electromagnetic bulb. The duration of the stimulation was 4 sec and the rate of the airflow was 8.0 ml/sec.

In studies of the effect of hypophysectomy and hCG treatment, the potential changes of the olfactory nerve were recorded simultaneously. The olfactory nerve was exposed by cutting a part of the skull. For recording electric activity from the olfactory nerve, a tungsten electrode was placed on the nerve and another indifferent electrode was placed on the head skin.

OSC was induced by stimulation with odorized air for studies of the effect of hypophysectomy and hormonal treatments. Odorized air was prepared by passing air through an aqueous suspension of Isoamyl acetate (50 µl in 50 ml of distilled water) in a 100 ml bottle. Patterns of OSC induced by odor stimulation were similar to those induced by stimulation with air alone.

Electric potential analysis

The maximal depth of the EOG from the resting level (0 mV) during the stimulating period of 4 seconds was referred to as the EOG amplitude (Fig. 1a) and the maximal amplitude of OSC during the stimulation period was referred to as the OSC amplitude (Fig. 1b). The maximal amplitude of potential change in the olfactory nerve during the stimulation period was referred to as the amplitude of olfactory nerve potential change. In the recordings from the olfactory

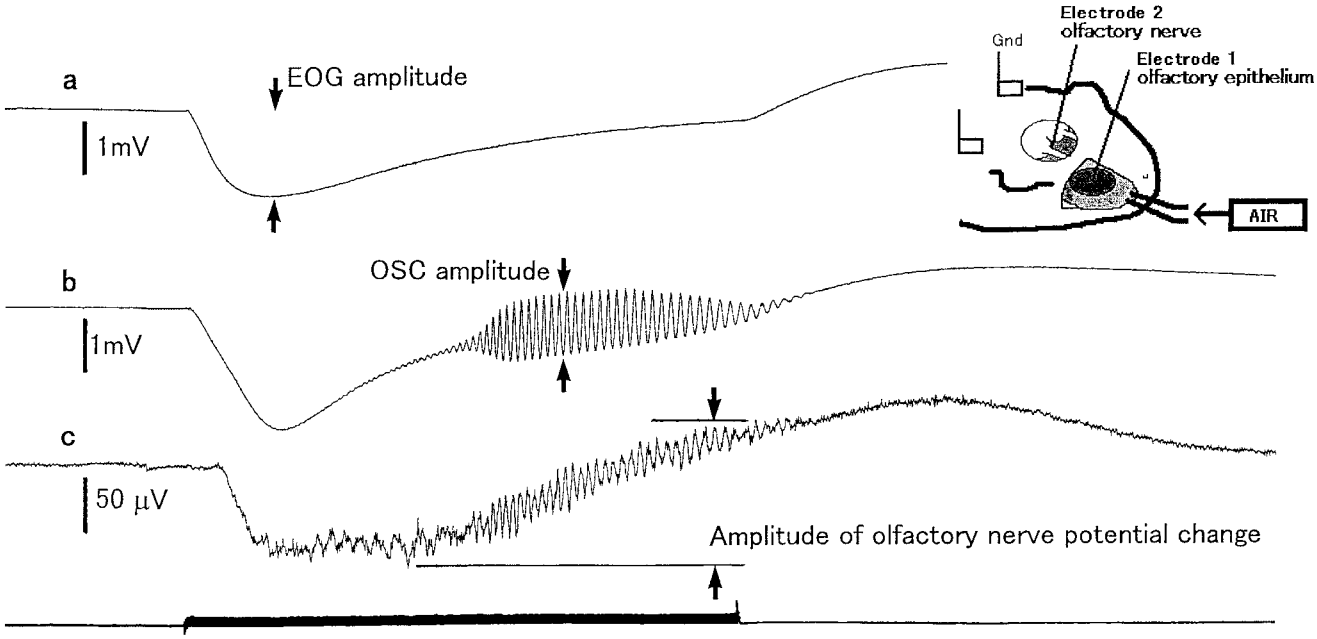


Fig. 1. Example of recordings of the EOG (a) and the OSC (b) in the olfactory epithelium and the olfactory nerve potential change (c). The trace in the bottom indicates air stimuli for 4 seconds. The maximal depth of the EOG from the resting level (0 mV) during the stimulating period was referred to as the EOG amplitude (a) and the maximal amplitude of OSC during the stimulation period was referred to as the OSC amplitude (b). The maximal amplitude of potential change in the olfactory nerve during the stimulation period was referred to as the amplitude of olfactory nerve potential change (c). Example (b) and (c) were recorded simultaneously.

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nerve, the variance in the amplitude between animals was large and depended on the condition of electrode on the surface of the olfactory nerve. The activity of the olfactory nerve was indicated with the ratio of mean amplitude of potential change induced by odorized air to the mean amplitude of potential change induced by air puff containing no odor (control stimuli) in each animal.

Radioimmunoassay (RIA) of toad gonadotropin

Gonadotropin levels in plasma were determined by means of a homologous RIA. An anti-toad LH serum (Takada *et al*., 1989) and I^{125}-labeled toad LH (B1D) were used in this RIA. The cross-reactivity with toad FSH in this RIA was 29.4% and hence we referred to the hormone measured by this RIA as gonadotropin and not LH or FSH. The minimal detectable level of gonadotropin was 0.05 ng/ml and intra-assay and inter-assay variations were 2.8% and 4.2%, respectively.

Solid phase enzyme immunoassay for progesterone

A full automatic Enzyme Immuno Assay system (AIA-600) of TOSOH Corporation was used for determination of progesterone levels in plasma. This system employs a sandwich method using alkaline phosphatase (ALP) as the enzyme and 4-methyl umbelliferil phosphate as the substrate. The minimal detectable level of progesterone is 0.1 ng/ml and intra-assay and inter-assay variations are less than 9.2% and 9.1%, respectively, according to the maker's information.

Treatments of toads with various hormones

OSC induction by hormonal treatments was attempted in the non-breeding season of July, August, December and January. Only adult male toads were used in these hormonal treatment experiments. Steroid hormones were obtained from Sigma.

In July testosterone was injected into 4 animals intra-peritoneally every day for two weeks. The dose of testosterone was 1 mg/0.05 ml propylene glycol. In August hCG was injected into 8 animals. The toads were injected with 2000 I.U. of hCG (Teikoku Hormone Mfg. Co., Ltd.)/0.2 ml saline for 4 days intra-peritoneally. In December four animals were injected with progesterone (1 mg/0.2 ml sesame oil) intra-peritoneally for a week. In January seven animals were injected with 17beta-estradiol intra-peritoneally every day for two weeks. The dose was 0.1 mg/0.05 ml propylene glycol. The same numbers of control animals for each experimental group were injected with vehicle only.

Statistical analysis

Overall differences in the EOG and OSC amplitude values among migration stages were analyzed by the Kruskal-Wallis test. Paired comparisons between two different migration stages were analyzed by Dunn's test, which is a non-parametric equivalency of the multiple range test. The correlation between the hormone concentration in plasma and the OSC amplitude was analyzed using Pearson's correlation coefficient (r). The significance level of r was evaluated by the t-test. When the relation between variables was not linear judging from the scatter-diagram, Kendall's rank correlation method (Campbell, 1974) was also used. Student's t-test was employed for the evaluation of differences in mean values between two treatment groups.

RESULTS

Changes in the EOG and the OSC amplitudes during the breeding migration

Toads migrating toward the pond for breeding were divided into the following stages according to the distance to the breeding pond: stage A) more than 50 m to the pond, stage B) 50 to 25 m to the pond, stage C) 0 to 25 m to the pond, stage D) swimming in the pond. Toads leaving the pond after breeding were also divided into the following three stages according to the distance from the pond: stage E) 0 to –25 m from the pond, stage F) 25 to 50 m from the pond, stage G) more than 50 m from the pond. Data from males and females were pooled in this analysis, because there were no sexual differences in the amplitudes of EOG and OSC during the breeding season (Nakazawa *et al*., 2000).

The mean EOG amplitude was calculated for each migration stage (Fig. 2a). There was no significant difference in the mean EOG amplitude among the seven stages of the migration (p=0.196 by Kruskal-Wallis test).

The mean OSC amplitude in each migration stage was calculated for all toads and also toads that showed OSC. There were significant differences in the both means among the seven stages of the migration (Fig. 2b and 2c). The mean amplitude of OSC for all toads in each migration stage was smallest (0.2±0.1 mV) in stage A when toads started the migration (Fig. 2b). It gradually increased until toads entered the pond. The highest mean amplitude (3.2±1.0 mV) was observed in post-breeding toads just after landing (stage E). The mean level decreased to the initial level of 0.4±0.2 mV when toads had moved far away from the pond (stage G). We divided the seven migration stages into two subgroups of the earlier half of migration (stage A to C) and the later half of migration (stage E to G). When the mean OSC amplitudes were compared between these two groups, the difference was highly significant (p=0.002 by Dunn's test).

The smallest mean OSC amplitude for individuals that showed OSC (Fig. 2c) was 0.7±0.5 mV in stage A. The amplitude increased as the toad moved to the pond and reached

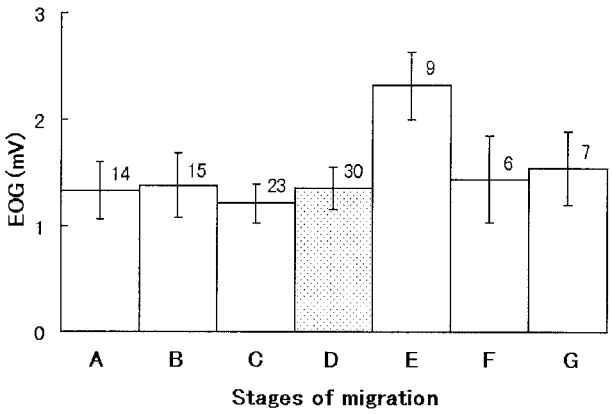


Fig. 2a. Mean EOG amplitudes at various stages of the breeding migration

Stage A) migrating to the pond at a distance >50 m. Stage B) approaching the pond in the middle range (50−25 m away from the pond). Stage C) arriving an area close to the pond (0−25 m), Stage D) swimming in the pond. Stage E) just landed soon after breeding (0−25 m). Stage F) leaving the pond in the middle range (25−50 m). Stage G) having moved far away from the pond (50 m<). Data are indicated as means±SEM. The number of animals used for calculation is indicated above each column (n=14, 15, 23, 30, 9, 6, 7 for stage A to G). There was no significant difference in the mean EOG amplitude among the seven stages of the migration by Kruskal-Wallis test (p=0.196).

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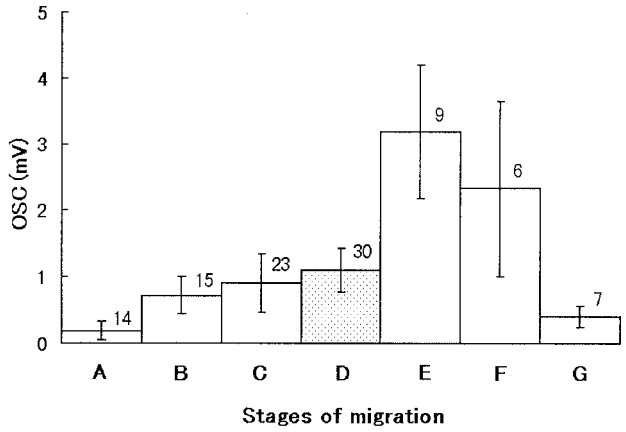


Fig. 2b. Mean OSC amplitudes for all the toads collected at various stages of the breeding migration
Data are indicated as means±SEM. The number of animals used for calculation is indicated above each column (n=14, 15, 23, 30, 9, 6, 7 for stage A to G). OSC amplitudes of stage A to C are statistically different from those of the stages of E to G by Kruskal-Wallis test of Dunn's method (p=0.002).

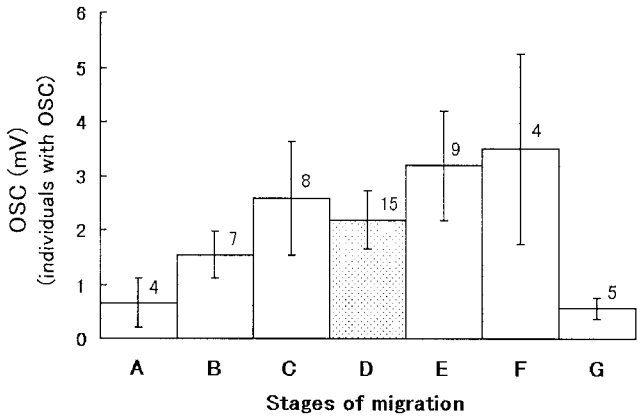


Fig. 2c. Mean OSC amplitudes for toads with OSC at various stages of the breeding migration
Only individuals with detectable OSC (with the amplitude over 0.1 mV) were used in the analysis. Data are indicated as means±SEM. The number of animals used for calculation is indicated above each column (n=4, 7, 8, 15, 9, 4, 5 for stage A to G). Amplitudes of stage A and G are statistically different from those of the stages of B to F by Kruskal-Wallis test of Dunn's method (p=0.03).

2.6±1 mV when toads arrived at the pond area (stage C). This high level was maintained in the pond and even after landing following mating (stage E and F), but the level abruptly decreased to 0.6±0.2 mV when toads had migrated far away from the pond (stage G). There was no significant difference in the OSC amplitude between any two of the five stages, except between the first and the last stages of the migration. The mean amplitudes of stage A and G were statistically different from those of the breeding stages of B to F by Dunn's method (p=0.03) after the Kruskal-Wallis test. There was no significant difference in the frequency (Hz) of the OSC between the 7 stages of the migration (data not shown).

Correlation between plasma gonadotropin concentration and the OSC amplitude

Eight males and 9 females were randomly selected from migrating toads (stage A to D) showing the OSC amplitude

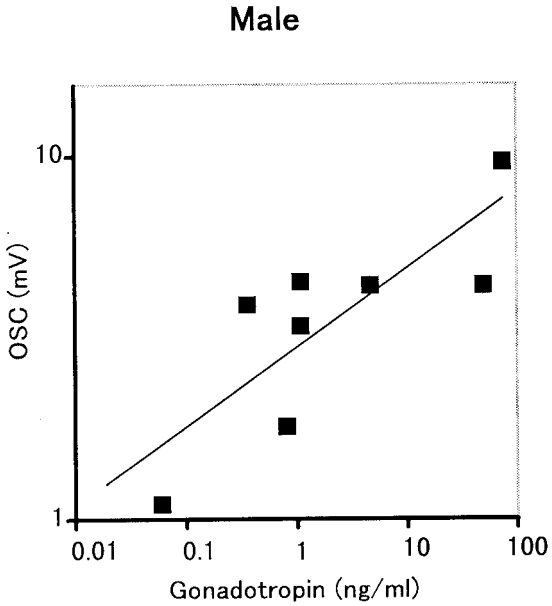


Fig. 3a. Correlation between plasma gonadotropin concentrations and the OSC amplitudes in 8 male toads during the breeding season
Plasma gonadotropin concentrations and the OSC amplitude were both plotted on logarithmic scales. There was a significant correlation (r=0.81, p=0.01, n=8) between plasma gonadotropin concentrations and OSC amplitudes. The solid line is the linear regression.

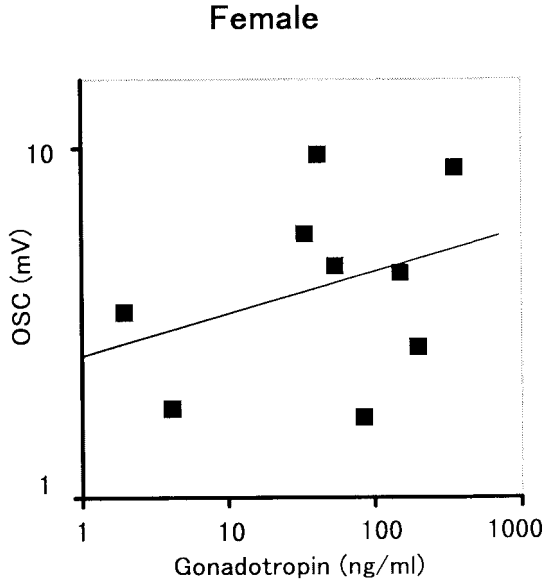


Fig. 3b. Correlation between plasma gonadotropin concentrations and the OSC amplitudes in 9 female toads during the breeding season
In females, the correlation between plasma gonadotropin concentrations and OSC amplitudes was not significant (r=0.36, p=0.35, n=9). Plasma gonadotropin concentrations and the OSC amplitude were both plotted on logarithmic scales.

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larger than 1 mV and their gonadotropin levels were measured. In the 8 randomly selected males, a significant positive correlation (r=0.81, p=0.01) was detected between the two parameters (Fig. 3a). In the 9 similarly selected females, no significant correlation (r=0.36, p=0.35) was observed, although a positive coefficient value was obtained (Fig. 3b).

There was no significant correlation between plasma gonadotropin concentrations and EOG amplitudes in both male and female sexes (in males: r=0.031, p>0.05, n=8, in females: r=−0.162, p>0.05, n=9).

Correlation between plasma progesterone concentration and OSC amplitude

We analyzed the correlation between the progesterone concentration and OSC amplitude in both males and females migrating to the breeding pond (stage A to D). A statistically significant positive correlation (r=0.89, p≦0.05, n=6) was found in females (Fig. 4a), while an insignificant positive correlation (r=0.59, p>0.05, n=8) was found in males (Fig. 4b). However, when we applied the rank correlation analysis of Kendall, the association in males became statistically significant (tau=0.64, p<0.05). The correlation was also significant in females (tau=0.73, p<0.05).

Positive but insignificant correlation coefficients were obtained between plasma progesterone concentrations and the EOG amplitude in both males and females (in females r=0.736, p>0.05, n=6; in males r=0.512, p>0.05, n=8).

Effect of hypophysectomy on the electric activity of the olfactory system in the breeding season

Hypophysectomized toads had a smaller mean OSC amplitude than did sham operated animals. This difference was significant by the t-test (p=0.045) (Table 1). There was no significant difference in the amplitude of EOG between the two treatment groups. The mean of "the ratio of the olfactory nerve potential change" was significantly smaller in hypophysectomized animals (p<0.05).

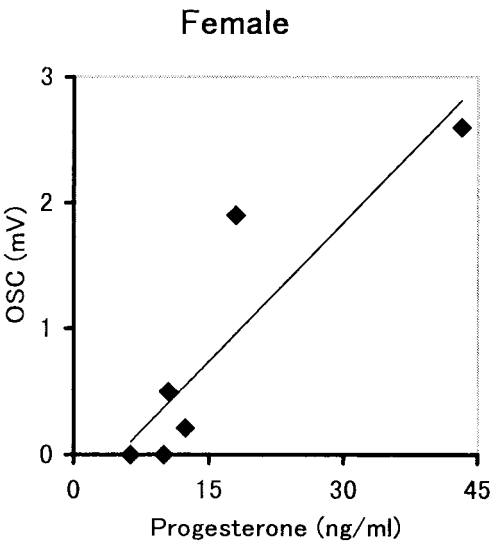


Fig. 4a. Correlation between plasma progesterone concentrations and the OSC amplitudes in 6 female toads during the breeding season
Raw data of plasma progesterone concentrations and the OSC amplitude were both plotted. The correlation was significant (r=0.89, p≦0.05, n=6).

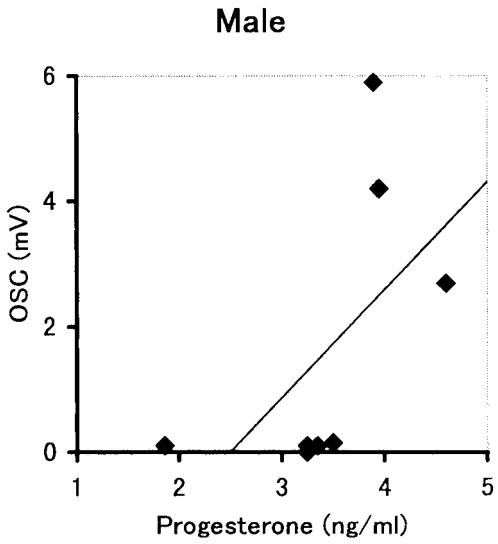


Fig. 4b. Correlation between plasma progesterone concentrations and the OSC amplitudes in 8 male toads during the breeding season
Raw data of plasma progesterone concentration and the OSC amplitude were both plotted.
The correlation was not significant (r= 0.59, p>0.05, n=8). In the rank correlation analysis of Kendall, the association between the two parameters was statistically significant (tau=0.64, p< 0.05).

Table 1. Effect of hypophysectomy on electric activities of the olfactory epithelium and nerve in the breeding season in the toad, *Bufo japonicus*

Treatment group (n=9 for each)	EOG amplitude (mV)	OSC amplitude (mV)	Olfactory nerve potential ratio	Number of toads showing the OSC
Hypophysectomized	1.70±0.16	0.08±0.04	1.36±0.15	5
Sham operated	1.67±0.17	0.38±0.15	2.17±0.26	7
	p =0.441	p =0.045*	p =0.016 *	

Values given are mean±standard error of the mean (SEM).
Number of animals (n) is 9 for each treatment group.
*p<0.05 by the single sided Student t-test.

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Table 2. Effect of hCG treatment on electric activities of the olfactory epithelium and nerve in the non-breeding season in the toad, *Bufo japonicus*.

Treatment group (*n*=8 for each)	EOG amplitude (mV)	OSC amplitude (mV)	Olfactory nerve potential ratio
hCG injected	2.39±0.47	0.38±0.25	2.27±0.29
Control	1.25±0.18	0.21±0.14	1.61±0.16
	p =0.025*	p =0.273	p =0.032*

Values given are mean ± SEM.
Number of animals (*n*) is 8 for each treatment group.
*p<0.05 by *t*-test, single side.

Table 3. Effect of treatments with steroid hormones on the EOG amplitude in the toad, *Bufo japonicus*.

Treatment group	Testosterone (mV)	17β-Estradiol (mV)	Progesterone (mV)
Treated	3.11±0.53	1.25±0.17	1.91±0.46
Control	2.63±0.28	1.09±0.11	2.01±0.61
(Number of animals)	4	7	4

The data are mean±SEM (mV).
The same number of animals were used for treated and control groups in each experiment.
There was no significant difference between the treated and the control groups by *t*-test.

Table 4. Effect of treatments with steroid hormones on the OSC amplitude in the toad, *Bufo japonicus*

Treatment group	Testosterone (mV)	17β-Estradiol (mV)	Progesterone (mV)
Treated	2.20±0.84	0.09±0.04	0.14±0.07
Control	2.88±0.86	0.18±0.16	0.66±0.63
(Number of animals)	4	7	4

The data are mean ± SEM (mV).
There was no significant difference in the mean OSC amplitudes between the treated and the control groups by *t*-test.

Effect of treatment with hCG on the electric activity of the olfactory system in the non-breeding season

The mean amplitude of the EOG was significantly larger in hCG injected animals than in control animals (p<0.05) (Table 2). By stimulation with odorized air OSC was induced in 3 animals among 8 animals tested in each group. The difference in the mean amplitude of OSC between treatment groups was not significant (p=0.273 by *t*-test). The mean ratio of the olfactory nerve potential change was significantly larger in hCG injected animals (p<0.05).

Effect of sex steroid hormone treatments on the EOG and OSC amplitudes

In July, testosterone injections for two weeks to male toads did not cause a significant change in the EOG amplitude (p=0.45, Table 3) or the OSC amplitude (p=0.59, Table 4) in comparison with those of the control animals.

Treatment with progesterone for a week in December did not cause any significant differences in the EOG amplitude (p=0.91) and the OSC amplitude (p=0.47).

In January, treatment with estradiol for two weeks was performed but there was no significant difference in the EOG amplitude (p=0.43) nor in the OSC amplitude (p=0.61).

Relation between testicular weight and OSC amplitude

There was a significant correlation (r=0.8, p<0.01, n=14) between the testes/body weight ratio and the OSC amplitude in animals used in an estradiol injection experiment conducted in January (Fig. 5). However, no significant correlations were

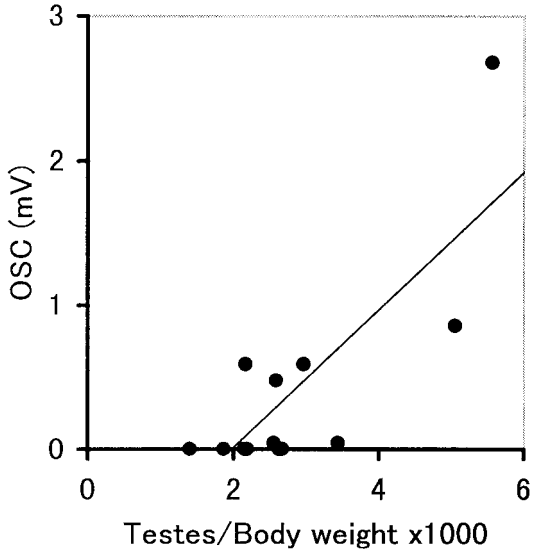


Fig. 5. Correlation between the ratio of testes weight to body weight and the OSC amplitude in 14 male toads in January. (r=0.8, p<0.01, n=14).

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observed in animals used in the non-breeding season: i.e. animals from a testosterone injection experiment, conducted in July (p=0.68) and animals from a progesterone injection experiment conducted in December (p=0.30).

DISCUSSION

We have previously described the properties of the OSC and suggested a correlation of the OSC and the breeding migration in the toad, *Bufo japonicus* (Nakazawa *et al*., 2000). In the present study, we showed that the OSC became inducible in a large proportion of toads when they started the breeding migration and became uninducible when they stopped the migration. The amplitude of the OSC was highest in the migratory period or when they land to leave the pond for migration. These results clearly support our previous suggestion on the relation of the OSC and the breeding migration. Ishii *et al*., (1995) found in the same species that the olfactory sense but not the visual sense is indispensable for orientation to the breeding pond and also that the cue for the orientation derives from the route of migration and not from the breeding pond. The close association of the OSC and the breeding migration found in the present study supports the important role of the olfactory sense for the orientation to the breeding pond in the toad.

In the present study, we demonstrated significantly positive correlations between the OSC and some reproductive hormone levels in blood plasma; i.e. the gonadotropin level in males and the progesterone level in females. The OSC also positively correlated with the relative testicular weight in males. However, in females, the correlation between gonadotropin and the OSC was not so clear. A surge of luteinizing hormone with extremely high levels occurs in all females when they approach the pond in the toad (Itoh and Ishii, 1990). This transitory and extremely high gonadotropin level may have masked a relationship between gonadotropin levels and the OSC in female toads.

These findings suggest that the pituitary-gonadal endocrine system stimulates the olfactory system represented by the OSC. However, disappointedly, all the hormone administration experiments have provided negative results. The hCG injections influenced the EOG level and electric activity of the olfactory nerve but showed no effect on the OSC. However, hypophysectomy reduced the mean OSC amplitude. Careful examination of data showed that some toads showed the OSC even after hypophysectomy that completely diminishes secretion of gonadotropin and hence secretion of sex steroid hormones from the gonads. This may indicate that some hormone secreted from the pituitary gland plays a subsidiary role in the olfactory activity.

To explain the present results, we may assume that an unknown factor activates both the reproductive endocrine system and the olfactory system. This factor should be activated in the breeding season. A hypothalamic neurosecretory hormone such as gonadotropin-releasing hormone could be the factor, although a possibility of gonadal steroid which we have not examined still remains as a candidate that induces the OSC. It was reported in males of *Rana nigromaculata* that 17 alpha, 20 alpha-dihydroxy-4-pregnen-3-one induced spermiation but progesterone and 17 alpha-hydroxy progesterone did not (Kobayashi *et al*., 1993). Furthermore, it has been found in *Bufo japonicus* that plasma levels of thyroid hormones (Tasaki *et al*., 1986) and adrenocortical hormones (Jolivet-Jaudet *et al*., 1984) were elevated during the breeding season and prolactin was elevated when toads moved into the pond (Ishii *et al*., 1989). Accordingly, one or more of these hormones could be related to the induction of OSC in some way.

The EOG response in the vomeronasal epithelium to peptide pheromone "sodefrin" was increased in ovariectomized female newt treated with prolactin and estrogen (Kikuyama *et al*., 1999), and Arechiga and Alcocer (1969) reported that adrenaline and noradrenaline enhanced the amplitude of the EOG in anurans. However, in the present study, we could not find clear change in the EOG during various stages of migration and under different hormonal conditions. Further study to find a main humoral factor that induces the OSC is needed.

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