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Oscillatory Electric Potential on the Olfactory Epithelium Observed during the Breeding Migration Period in the Japanese Toad, *Bufo japonicus*

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ABSTRACT—Japanese toad (*Bufo japonicus*) tracks the route to and from the breeding sites using the olfactory cues from the migration route and not from the destination (Ishii *et al*., 1995). We recorded a slow extracellular potential change (electro-olfactogram or EOG) evoked on the olfactory epithelium by applying an olfactory stimulus with an air stream. In September toads, only a simple typical EOG that is common in various vertebrate species was observed. Oscillatory potential changes (OSC) superimposed on the typical EOG were observed in the breeding season when studied throughout a year. There were no sexual differences in the occurrence and the amplitude of the OSC. Oscillatory potentials were observed also from the olfactory nerve of the brain. The OSC in the olfactory epithelium remained even after denervation. In addition, it was suggested that there are multiple sites of OSC initiation in the olfactory epithelium. These results suggest an intimate relationship between OSC appearance and the breeding migration in the toad.

INTRODUCTION

Olfactory cues are used for the orientation during migration in a number of vertebrate species (see Able, 1980). The most popular example is salmonid fish that use the olfactory sense in the migration to the home river (Hasler *et al*., 1978; Dittman and Quinn, 1996). More recently, the importance of olfaction has been reported in the homing behavior in a turtle (Grassman, 1993) and a bird (Papi, 1991; Bingman and Benvenuti, 1996).

In anuran amphibians, Grubb (1973), Sinsch (1987) and Ishii *et al*. (1995) reported the importance of the olfactory sense for the migration. Among them, the first two investigators assumed that a chemical cue comes from the destination of the migration such as the breeding pond. In contrast, 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.

Based on these behavioral studies, we expected that the olfactory system should be activated during the migration period and hence 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 with a stream of air or vapor of some organic substances in a number of vertebrates (Byrd and Caprio, 1982 in catfish; Senf *et al*., 1980 in frog; Tonosaki, 1993 in box turtle; Edwards *et al*., 1988 in rat). The recording of this potential change has been referred to the electro-olfactogram (EOG). Nakazawa *et al*. (1994) observed EOG throughout a year in *Bufo japonicus* and found no clear difference in the EOG between breeding and non-breeding seasons. They also observed that some toads in the breeding season showed a series of oscillatory electric potential changes (OSC) that were superimposed on the EOG. A similar electric activity was first described by Ottoson (1956) in the frog, *Rana temporaria*. In 1961, Ai had observed OSC in the toad and found that it disappeared after the spring breeding season (personal communication and unpublished). Since these studies, no further attention has been paid to the OSC by physiologists.

In this study, we investigated the OSC in the toad, *Bufo japonicus*, with special emphasis on its site of generation and seasonal change of its occurrence.

MATERIALS AND METHODS

Animals

Wild adult toads of *Bufo japonicus* were used in two series of observations in the present study. First, 28 toads were captured during the breeding migration in three different places in Yamanashi, Saitama and Tochigi Prefectures in 1996. They were used to study the electrophysiological properties of OSC, Second, 5 to 10 toads were captured every month between 3rd March 1994 and 20th February 1995 in one of three parks near the main campus of Waseda

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University. They were used to study annual changes in the EOG and OSC amplitudes.

For the electrophysiological study, all the toads were carried to our laboratory in Waseda University, and kept at low temperature (4 to 6°C) condition for 1 to 14 days in order to maintain physiological condition at the time of capture. Under the natural condition, toads temporarily stop the breeding migration and bury themselves under the ground when the ambient temperature falls below 6°C. They restart the breeding migration, when the ambient temperature becomes higher, although the humidity is also another important environmental factor that initiates the migration.

Electric potential measurement

Just before electrophysiological study, toads were acclimatized to the room temperature in the laboratory at least for 30 min until toads become active. Following immobilization by pithing, the olfactory epithelium was exposed by cutting the dorsal wall of the nasal cavity. In some studies, the olfactory nerve was also exposed by cutting a part of the skull. A recording Ag-AgCl electrode (0.2 mm of the diameter) was placed on the eminence of the olfactory epithelium and an indifferent electrode was placed on the head skin. 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.

Potential changes were differentially amplified with a preamplifier (Nihon Kohden, AVB-21) and the waveforms were recorded on a thermal array recorder. As a stimulus, an air puff or airflow containing no odor was delivered through a glass tube nozzle (with an aperture of 1 mm) placed at 1 cm distance from the epithelium. Duration of the air puff stimulation and speed of the airflow were kept constant by controlling the airflow from a pump with an electromagnetic bulb and an electric timer. The duration of the stimulation was 4 sec and the speed of the airflow was 8.0 ml/sec. To each toad, the air puff stimulation was delivered repeatedly 7 times with 3 min intervals. The EOG and OSC induced in this experiment was identical to those induced by odorized air.

In experiments to determine the origin of the OSC, outputs from the amplifier (of potential changes of the epithelium and nerve) were recorded in a computer through an AD converter board (CONTEC, AD12-16TA) at sampling rate of 5 msec for analysis of frequency. The frequency of OSC was analyzed by the periodogram method of Brockwell *et al.* (1987) with 200 data (sampling rate of 5 msec) measured for 1 sec between 0.5 sec before and 0.5 sec after the time when the OSC amplitude was at its maximum.

In some animals that did not show the OSC when the airflow without odor was applied, OSC was induced by stimulation with odorized air. 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 did not differ significantly from that induced by stimulation with air alone (data not shown). Odorized air was used only in experiments to determine the origin of the OSC.

Statistical analysis

The Kruskal-Wallis test was applied to examine overall difference in the EOG or OSC amplitude values among different months. Then, paired comparisons between two different months were made using Dunn's test, a non-parametric equivalency of the multiple range test.

RESULTS

Description of OSC.

By stimulating the olfactory epithelium with the airflow for 4 seconds, a typical EOG (i.e. a slow transitory decrease in the electric potential) was recorded from the surface of the epithelium in all toads examined. The maximal depth of the EOG from the resting level (0 mV) was referred to as "the amplitude of the EOG" (Fig. 1).

In some toads, especially in the breeding season, a series of oscillatory electric potential changes (OSC) were superimposed on the EOG (Fig. 2). The oscillation occurred soon after the EOG was evoked, increased in its amplitude over a short period, then declined. The duration of OSC varied from 1 to 10 sec. The maximum value of the potential difference between positive and negative peaks of the oscillation (arrows in Fig. 2a) was referred to as "the amplitude of OSC". The frequency and the amplitude of OSC ranged from 10 to 25 Hz and from 0.1 to 9.3 mV, respectively. The mean frequency and its standard error for 54 toads that showed OSC were 16.5 and 0.5 Hz, respectively. The mean amplitude of OSC and its standard error were 2.2 and 0.3 mV, respectively.

Classification of OSC

Three different types of OSC were observed in the present study (Fig. 2): a simple OSC (2a), a composite OSC without notch (2b) and a composite OSC with notch (2c). In the latter two composite types, the amplitude of OSC changed periodically with a certain interval (0.2 to 1.4 sec), just like waves of the sonic beat. The simple OSC was observed more frequently (61%) than the composite types (without notch, 32%; with notch, 7%) (Table 1).

Effect of Sectioning of the Olfactory Nerve on OSC

We may propose two possible origins of the OSC. One is

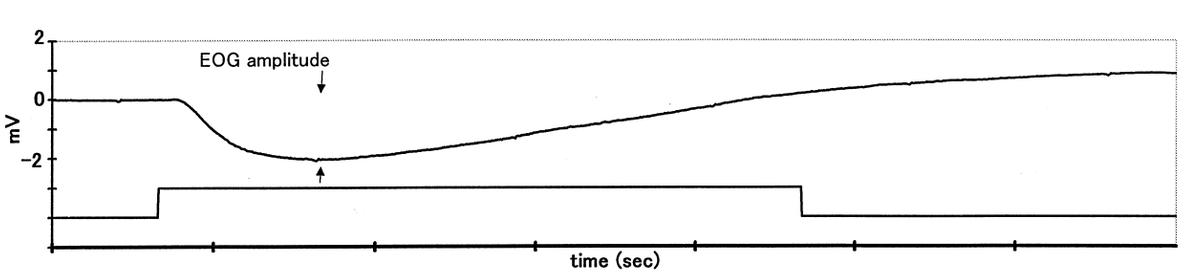

Fig. 1. An example of EOG induced by stimulation with air puff on the surface of the olfactory epithelium. The upper trace is electric potential change or EOG recorded in May, the non-breeding season. Lower trace indicates the application of the stimulus. Duration of the stimulus is 4 seconds. Ordinate: EOG amplitude (mV). Abscissa: time (sec).

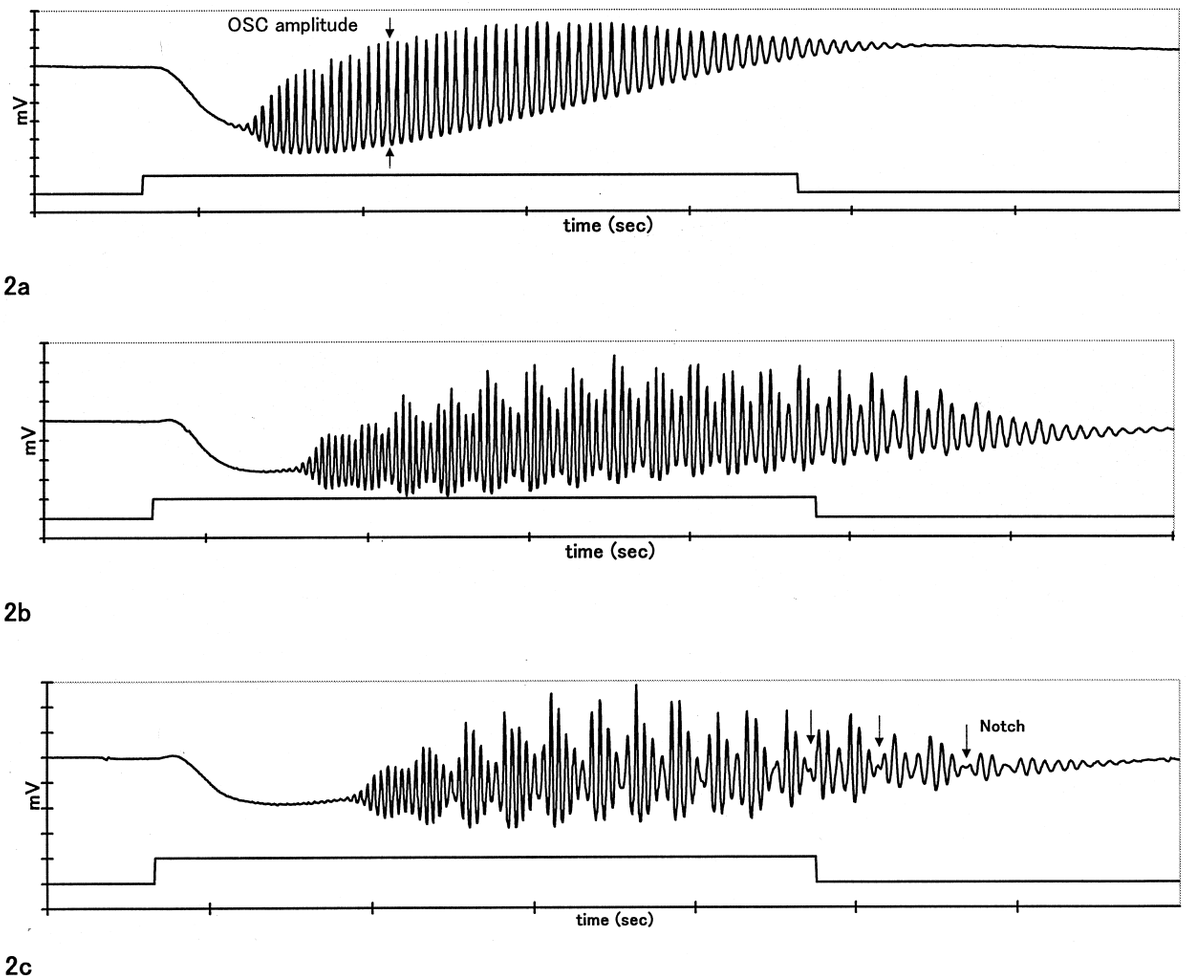

Fig. 2a, b, c. Three different types of OSC recorded in 56 animals in March, the breeding season. (a) An example of the simple OSC. (b) An example of the composite type OSC without notch. (c) An example of the composite type OSC with notch (shown by arrows) Upper traces are the OSC superimposed on the EOG. Lower traces are stimulation by air puff for 4 seconds.

Table 1. Incidences of three types of OSC observed in 56 individuals of *Bufo japonicus*.

Type name	Number of observed cases	(%)
Simple	34/56	61
Composite without notch	18/56	32
Composite with notch	4/56	7

that OSC originates in the central nervous system in response to the stimulus and is propagated back through the olfactory nerve to the olfactory epithelium. The other is that OSC originates in the peripheral receptor organ itself. To test these two possibilities, we cut the olfactory nerve and then observed the response of the olfactory epithelium to the stimulation. If the former assumption is correct, no OSC can be detected in the olfactory epithelium after cutting the nerve. If the latter assumption is correct, we still should be able to record OSC in the olfactory epithelium after cutting the olfactory nerve.

Before the cutting experiment, electric activity of both the olfactory epithelium and the olfactory nerve were recorded simultaneously. Olfactory stimulation induced electric potential changes in the olfactory nerve similar to the OSC in the olfactory epithelium (Fig. 3). Frequencies of the responses in the olfactory epithelium and nerve were 12.5 and 12.7 Hz, respectively, being practically identical. This result shows that the olfactory stimulation induced oscillatory responses in both the olfactory epithelium and nerve.

When the nerve was cut, OSC was still inducible in the olfactory epithelium, although there was a decrease in the amplitude after the deafferentation (Fig. 4). We also recorded electric potential changes from the peripheral cut-end of the olfactory nerve. The pattern of the recorded electric potential changes at the cut end was similar to that of the epithelium, but the polarity was reversed after the deafferentation. These experiments show the OSC in the olfactory epithelium was not propagated through the nerve, but generated in the epithelium itself. On the other hand, the OSC in the nerve was of electrotonic potentials or current spread from the epithelium.

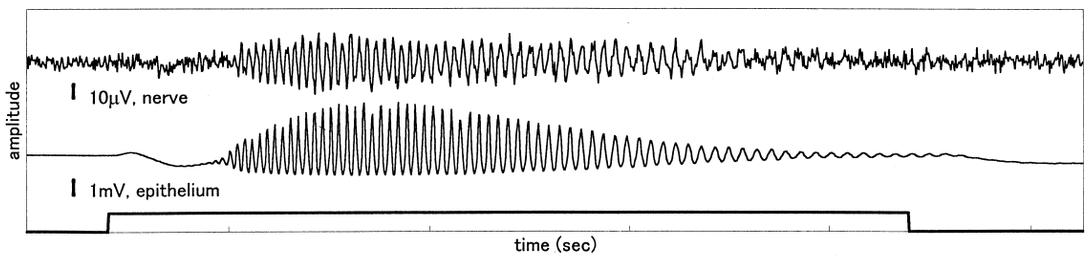

Fig. 3. An example of simultaneous recordings of OSC in the olfactory epithelium and oscillatory potentials in the olfactory nerve. The trace on the bottom indicates stimulation by odorized air for 4 seconds. Potentials were band-pass filtered between 0.5 Hz and 100 Hz in the recordings from the olfactory epithelium, and between 5 Hz and 1,000 Hz in the recordings from the olfactory nerve.
Frequencies of oscillation in the olfactory nerve and OSC in the olfactory epithelium were 12.7 and 12.5 Hz, respectively.

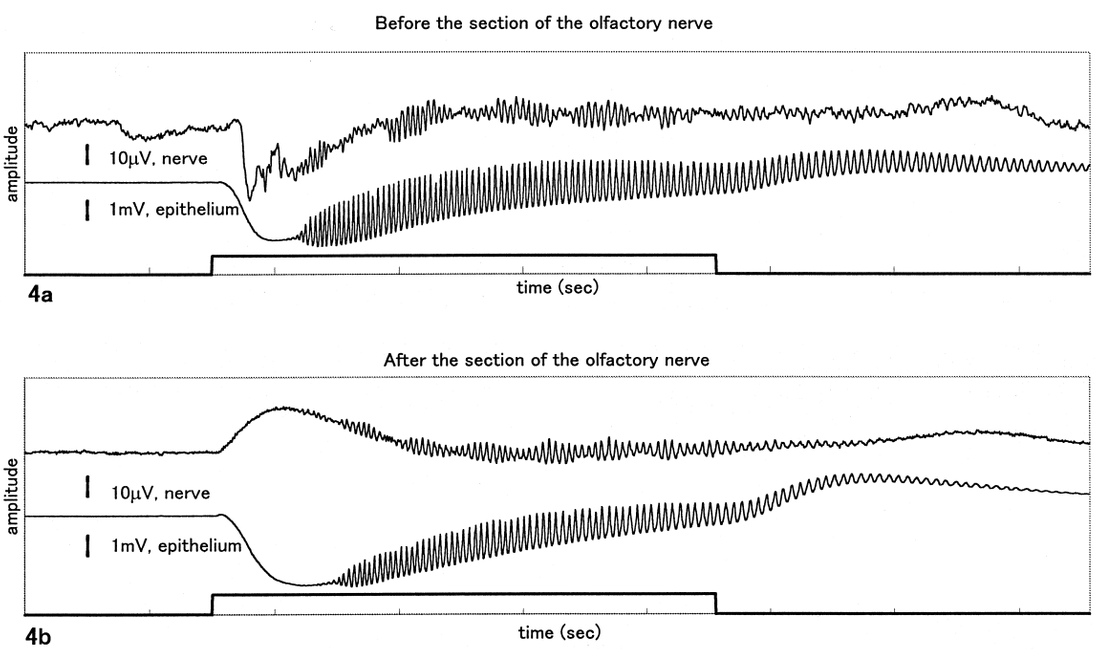

Fig. 4a, b. Potential changes in the olfactory nerve (top trace) and OSC in the olfactory epithelium (middle trace) were recorded simultaneously before (a) and after (b) sectioning the olfactory nerve. The trace in the bottom indicates stimulation by odorized air for 4 seconds. Potentials were band-passed between 0.5 and 100 Hz in both recordings from the olfactory epithelium and nerve.

Effect of Surgical Separation of the Olfactory Epithelium into Two Parts

The composite OSC may have derived from two independent OSCs with slightly different oscillatory frequency generated simultaneously at different sites of the olfactory epithelium. To test this possibility, we surgically separated the olfactory epithelium into two different portions and compared OSCs recorded from the two portions.

Before surgery, we placed recording electrodes in the anterior and posterior portions of the olfactory epithelium, applied the olfactory stimulation and recorded OSC simultaneously from the two portions (Fig. 5a). Then, the anterior and posterior epithelia were transected with a surgical knife and the gap was filled with liquid paraffin for insulation. Potential changes following stimulation were recorded from the two portions simultaneously. Nine animals were used for this transection study. Before the transection, frequencies of OSC in the two portions were the same in 6 out of the 9 animals. In the remaining 3, differences in oscillatory frequency were small if any (Table 2). However, after transection of the epithelium, oscillatory frequencies were significantly different between the anterior and posterior portions (Fig. 5b and Table 2). The mean differences of oscillatory frequencies between anterior and posterior parts after the transection was 1.97±0.45 Hz, although the mean difference before the transection was small and not significantly different from 0 (0.12±0.06 Hz).

Annual change in EOG

The mean EOG amplitude varied from month to month. The largest mean EOG amplitude (mean±SEM, standard error of the mean; 2.10 (0.84 mV, n=5) was observed in May and the smallest (0.22±0.06 mV, n=6) in September (Fig. 6). Monthly variation in the EOG amplitude was statistically significant (p<0.01 by the Kruskal-Wallis test).

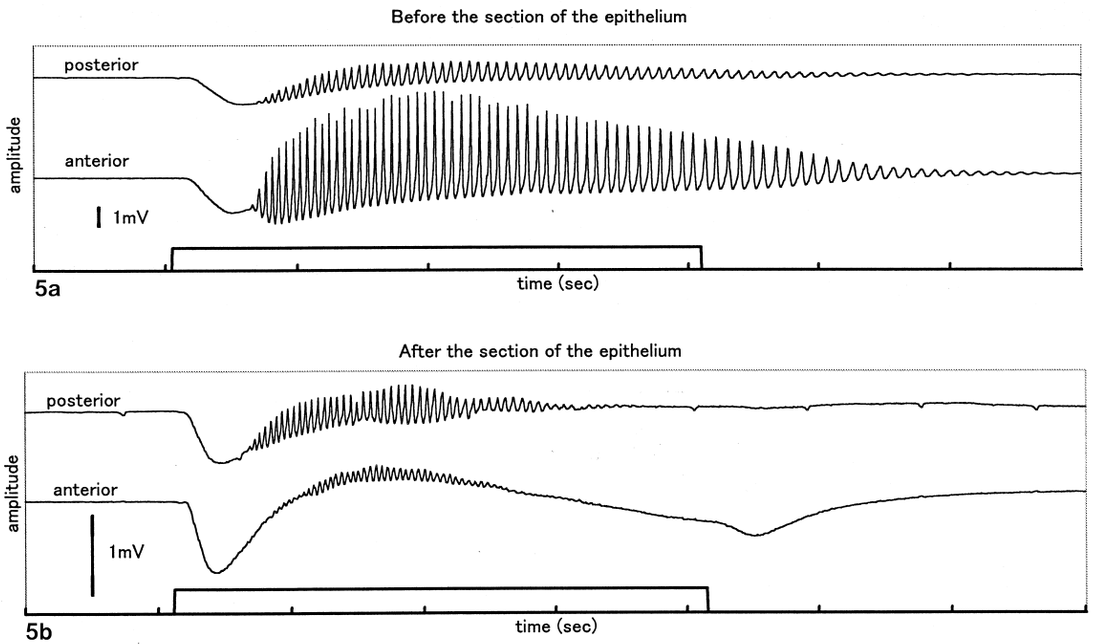

Fig. 5a, b. OSC were recorded in the anterior portion (middle trace) and the posterior portion (upper trace) of the olfactory epithelium simultaneously before (a) and after (b) separation of the olfactory epithelium into the two portions by sectioning. The trace in the bottom indicates stimulation by odorized air for 4 seconds. Frequencies of the OSC in the two portions before the separation (a) were identical (15.87 Hz). After the transection (b), frequencies were 22.73 Hz in anterior portion and 19.23 Hz in posterior portion. These recordings were obtained in animal number 3 from Table 2.

Table 2. Frequencies of OSC in the anterior and posterior portions of the olfactory epithelium and absolute frequency differences between the two portions before and after separation of the two portions by sectioning in the toad, *Bufo japonicus*.
The mean of the frequency differences before the separation did not differ significantly from zero by Student's *t*-test ($p > 0.05$), but the mean of the frequency differences after the separation differed significantly from zero by Student's t-test ($p < 0.05$).

	Before the section			After the section		
Individual	anterior	posterior	difference	anterior	posterior	difference
1	20.00	20.00	0.00	20.41	22.22	1.81
2	17.54	17.54	0.00	19.61	15.87	3.74
3	15.87	15.87	0.00	22.73	19.23	3.50
4	17.24	17.54	0.30	21.28	19.61	1.67
5	18.87	18.87	0.00	20.41	18.87	1.54
6	18.87	18.52	0.35	22.22	18.52	3.70
7	16.95	16.95	0.00	17.86	18.18	0.32
8	20.00	19.61	0.39	19.23	20.00	0.77
9	14.29	14.29	0.00	15.38	14.71	0.67
average	17.74	17.69	0.12	19.90	18.58	1.97
SEM	0.64	0.61	0.06	0.75	0.74	0.45

When we divide data of the monthly EOG amplitudes into two groups of the earlier half (January to June) and the later half (July to December) of the year, and compare the amplitude between these two periods, the difference is highly significant ($p < 0.001$ by Dunn's test). However, when we divide the data into two groups of the long day period (April to September) and the short day period (October to March), the difference in the EOG amplitude between these two periods is not significant (p being close to 1 by Dunn's test).

Annual change in the OSC

Incidence of OSC Unlike EOG, incidence of detectable OSC in response to the stimulation with air alone varied among animals. The proportion of the number of animals with detectable OSC to the total number of animals examined was calculated and used as the incidence of the OSC in each month. Data of males and females were pooled in this analysis, because there were no sexual differences in the amplitudes of EOG and OSC, and incidence of OSC during the breeding season (Table. 3). OSC occurred throughout the period from

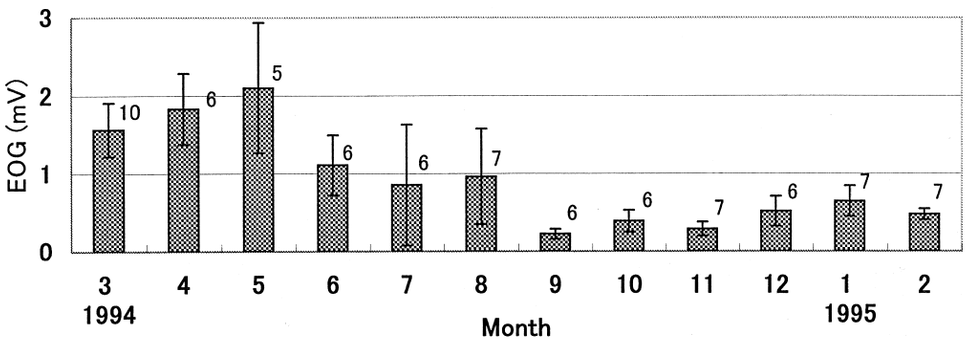

Fig. 6. Monthly changes in the mean EOG amplitude with SEM in wild toads during a 12 months period from March 1994 to February 1995. The number of animals used for calculation is indicated above each column. The difference in the mean between two periods of January to June and July to December was highly significant ($p<0.001$) by Dunn's test.

Table 3. Sexual difference in the incidence and amplitude of the EOG and OSC during the breeding season.
Data are expressed as mean±SEM. The number of animals is given in parentheses.
The OSC incidence is expressed as the ratio of the number of individuals with a detectable OSC to the number of individuals examined. There were no significant differences between males and females in both EOG and OSC.

	EOG amplitude (mV)	OSC amplitude (mV)	Rate of OSC incidence
Male	1.44±0.12 (63)	2.23±0.47 (32)	0.508 (32/63)
Female	1.47±0.17 (49)	1.94±0.41 (24)	0.490 (24/49)

January to June and not from July to December. The incidence of OSC was highest (rate=0.6, n=10) in March, the breeding season, and then decreased gradually month by month until July when it reached 0 (Fig. 7). The OSC appeared in January again just before the next breeding season.

Amplitude of OSC Monthly means of OSC were calculated for those individuals that showed OSC and also for all individuals including both responding and non-responding individuals. Since both means showed similar annual changes, we presented annual changes of monthly means for all individuals (Fig. 8). The mean amplitude was highest (0.99±0.55

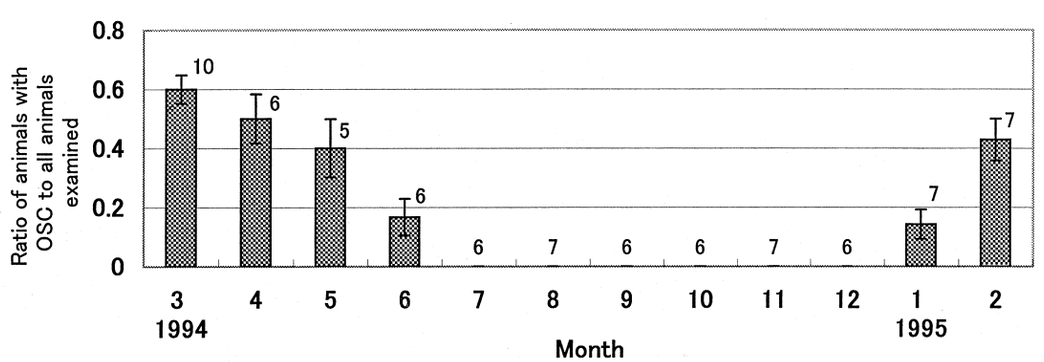

Fig. 7. Monthly changes in the incidence of the OSC in wild toads during the period from March 1994 to February 1995. Incidence of OSC is expressed as the ratio of the number of animals that showed OSC to the total number of the animals examined. Vertical bars indicate the standard error of the binomial ratio.

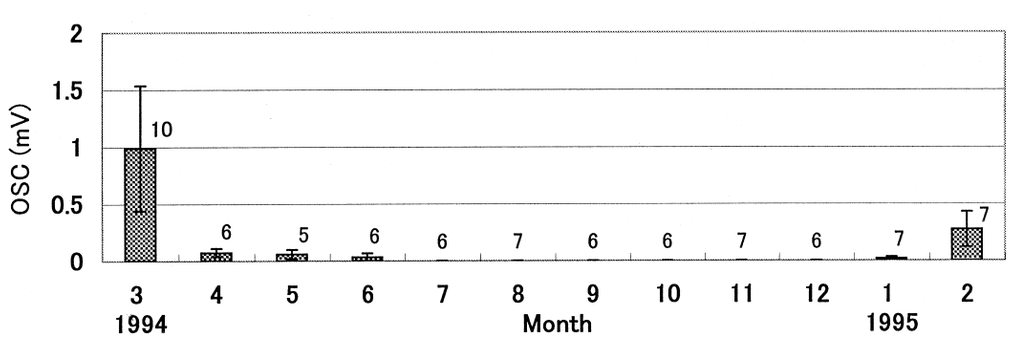

Fig. 8. Monthly changes in the mean OSC amplitude in wild toads during the period from March 1994 to February 1995. Data are shown in means±SEM for toads captured in each month.

mV) in the middle of the breeding season, March. It was low or nil in the other months, although the second highest mean level was observed in February.

DISCUSSION

In the present study, we described properties of the oscillatory electric potentials (OSC) that appear superimposed on the EOG in the toad. We also found that a series of electric changes corresponding to OSC was simultaneously induced in the olfactory nerve. Oscillations of EOG and electric potential in the olfactory bulb were described in the frog, *Rana temporaria*, by Ottoson (1959). In the rabbit, Adrian (1955, 1957) also made similar observations. These investigators assume two possible origins of OSC in the olfactory epithelium, one is the peripheral and the other is the central origin. We showed that the olfactory denervation had no effect on the induction of OSC in the olfactory epithelium. This supports the idea that OSC is produced by direct response of the olfactory epithelium to the air stimulus and not by activity of the olfactory nerve.

Our data from the transection of the olfactory epithelium indicate that OSC can be generated in at least two different sites of the olfactory epithelium. The OSC may be generated in a group of cells at a single site of the olfactory epithelium and propagate all over the epithelium. Most often observed simple type OSC may have been induced in such a way (Fig. 2a). When other sites of the epithelium are involved in initiating OSC, a composite type of OSC may be formed and a slight difference in the frequency of oscillation between the two OSCs caused a beat wave. The notch may be caused by a phase lag between the two OSCs.

OSC, we observed in the toad, may be formed with synchronous discharge of a number of sensory cells in the epithelium as suggested by Ottoson (1959). Kurahashi and Shibuya (1989) reported that repetitive spike discharges were recorded from isolated olfactory receptor cells in the newt of *Triturus pyrrhogaster*. It is probable that OSC can be detected in other amphibian species if we carefully observe.

We originally started the present study from behavioral studies of the toad. We expected the presence of a specific electric activity during the breeding migration. The OSC can be a strong candidate for it, because the incidence of OSC and also the amplitude of OSC increase gradually at the beginning of the breeding season and reach the maximal levels in the breeding month. Increase in odor acuity during the breeding season is known in the European Starling, *Sturnus vulgaris* (Clark and Smeraski, 1990). We may consider a relation of OSC and pheromone, since a peptide pheromone secreted by the male newt is known to trigger the courtship behavior in the female newt (Kikuyama *et al*., 1997). However, in the toad, the relation between OSC to sex attractant or pheromone may be negated, because the courtship behavior is triggered by visual and tactile senses (Ishii and Itoh, 1992) and OSC is induced in both sexes in the toad. The OSC is more likely to be involved in migrating behavior than the courtship behavior.

The close correlation of OSC with the breeding season that we found in the toad suggests the presence of an endocrine mechanism inducing OSC. Studies to determine correlation of endocrine activity and OSC parameters are now being conducted.

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