Enhancement of Spermatogenesis from Spring Male Chorus for the Spectacled Toad *Duttaphrynus melanostictus* (Schneider, 1799)

雄性黑眶蟾蜍春季的合唱鳴聲對於生精作用的強化現象

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Abstract

The conspecific male chorus stimulation experiment was conducted for 33 days in the early breeding season in spring for the spectacled toad *Duttaphrynus melanostictus*, using 20 wild males captured shortly after their emergence from winter hibernation. The average number of sperm bundles of the test group (n=10) that received daily stimulation of male chorus was 56% higher than that of the control group (n=10) that did not have the chorus stimulation. Otherwise, there was no significant difference in food intakes, body condition (body lengths, and body, liver and fat body weights), and testicular size between the two groups after the experiment. The male chorus is not merely a vocal signal for attracting males and females for congregation, or a vocal warning in male-male competition and for territorial protection, but is also an acoustic stimulus that enhances spermatogenic activities to accelerate the sperm production for the reproductive success.

摘　要

於春季初期，我們使用剛出現的20隻黑眶蟾蜍進行爲期33天的雄蟾蜍鳴聲刺激實驗。雄蟾蜍在每天接受鳴聲刺激的實驗組(10隻)中，其平均的精子束數目比沒有接受鳴聲刺激的實驗組(10隻)，其精子束數目多出了 56%。除外，在鳴聲刺激與否的實驗後，於食物的取食量及身體條件狀況(如體長、體重、肝臟重及脂肪體重等)方面，實驗組與控制組間則無統計上的差異。兩棲類雄性的合唱鳴聲不只是吸引雄性與雌性聚集的訊號，或做爲雄性間彼此競爭領域的警告之用，春季雄蟾蜍合唱鳴聲對於同種雄蟾蜍的聽覺刺激，也有強化其生精作用並加速精子的生成，以利後續生殖成功的作用。

Key words: spermatogenesis, advertisement call, male chorus, spectacled toad *Duttaphrynus melanostictus*

關鍵詞：生精作用、宣告叫聲、雄性合唱、黑眶蟾蜍

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Introduction

Many of the anuran calls have been defined and classified according to acoustic characters and related behaviors (Duellman and Trueb 1994). It has been generally considered that male calls are used as vocal signals for attracting females and intraspecific recognition, and as vocal warning or threatening in male-male competition (Duellman and Trueb 1994; Tobias *et al*. 2004). The male calls are also known as acoustic stimuli that maintain the reproductive state of ovaries for *Alytes muletensis* (Lea *et al*. 2001) and enhance secretion of androgen for *Rana sphenocephala* (Chu and Wilczynski 2001) and *Hyla cinerea* (Burmeister and Wilczynski 2001). However, it still remained unclear whether male calls have any effects on spermatogenic activities of conspecific males, though there is evidence that the calls enable males to maintain the reproductive status of the testes in *Rana temporaria* (Brzoska and Obert 1980).

Spectacled toad *Duttaphrynus melanostictus* (Schneider, 1799) is a common tropical anuran distributed widely in Taiwan, southeastern China, Southeast Asia, and South Asia (Shieh 1993). In the central region of Taiwan, its spermatogenic cycle has been found to fluctuate seasonally in testicular sizes, spermatogenic activities, and plasma androgen levels. They rapidly increase in February, have a peak in March, and decline gradually in the rest of the year. The breeding season is suggested to be from middle February to May with a peak in March (Huang *et al*. 1997).

The courtship behavior of the spectacled toad is highly social; males congregate to emit advertisement calls and form a chorus along the edges of quiet water ponds. An advertisement call usually lasts for 15-25 seconds. It has been regarded as a vocal warning to approaching males to protect its breeding territory, and at the same time it attracts both males and females for congregation at the breeding ground to increase the breeding success (Shieh 1993). Since our previous observations in the wild showed that the male chorus continued nearly for weeks prior to having an amplexus behavior in March. We tested whether the spring male chorus affects testicular sizes and spermatogenic activities of male spectacled toads.

Materials and Methods

Experimental toads

Spectacled toads were rarely found in the cold month of January, 2006. On 29 January to 2 February when the weather warmed up for a time, the toads suddenly emerged from their winter hibernation and started to call intermittently. We captured 75 male toads during this 4-day period at the Endemic Species Research Institute campus and its vicinity in the central Taiwan. They were immediately brought to the laboratory, and their snout-vent lengths were measured and body weights were taken. Twenty adult males with similar sizes were selected from the captures for the experiment. They were divided into two groups, the test group and the control group, each with 10 males. In order to avoid the body size difference between the two groups, ten pairs of almost equal sizes were selected. There were no significant differences in both body lengths and weights between the two groups (Table 1). Each male was kept in a terrarium (60×30×20 cm) with soil and gravel covered with dry leaves and a dish of water.

The experimental males of both groups were acclimated in the laboratory for three days. Each day the two groups were placed under ambient environments outside the laboratory for the day time, and transferred into two separate sound-proof rooms, one for the control group and the other for the test group, for the night. The rooms were mostly free from disturbance and were exposed to a natural photoperiod. Ten live mealworms (larvae of *Tenebrio molitor*) were provided in each terrarium as a daily ration. The number of the worms decreased in the following morning was recorded as the daily food intake for the day.

Acoustic stimulus

In February of the previous year (2005), we recorded male chorus of spectacled toads with a Sony TC-D5M cassette tape recorder in the field where the experimental toads of this study were collected. The chorus had the fundamental frequency about 1.73 kHz, belonging to the typical spectral character of advertisement calls during the breeding season (Shieh 1993). The analog sound was transferred into the digital file with sampling frequency of 48 KHz and the sample size of 16 bits. It was used as the source of acoustic stimulus for this study.

Acoustic experiment

Immediately after the acclimation, the acoustic stimulation experiment was conducted for 30 days, started on 5 February and ended on 8 March, 2006. The test group and the control group were placed under ambient environments outside the laboratory for the day time and in the separate sound-proof rooms for the night, and a daily ration of 10 mealworms, similar to the conditions used for the acclimation.

During the experimental period, the digital file of male chorus was played in the room of the test group every night from 20:00 to 24:00 through a PC with the Windows Media Player software and two small speakers in front of the terraria at a sound pressure level of 75 dB at the distance of 1m to mimic the field condition.

A sound recorder (SONY IC Recorder) was set in the control group room to record if there were any calls or sounds made by the toads during both acclimation and experimental periods. Also, a same recorder was set in the room of the test group to record calls or sounds from the toads and the chorus from the speakers. The calls and sounds recorded were compared to the male chorus from the digital files to identify whether the test toads made chorus in response to the acoustic stimulus, or made any other particular calls or sounds.

Data collection and analysis

At the end of the experiment period, for each of the toads, the snout-vent length was measured and the weight was taken. They were then anesthetized and dissected, and their livers and fat bodies were removed and weighed. Both testes were removed and were weighed individually, and the lengths and diameters measured. The averages of both testes were used in data analysis. Both testes were fixed in the Bouin's solution. Histological slides were made from the horizontal middle section of each of the testes at a thickness of 5 μm. It was stained with hematoxylin and eosin solutions (H&E), following the standard histological slide preparation procedure (Ross *et al*. 1989). The slides were examined under a light microscope at a 100X magnification. Sperm bundles tubules was usually clustered, and the number of sperm bundles in the seminiferous were counted. The bundles in both testes were averaged as the intensity of spermatogenic activity (Huang *et al*. 1997). The total number of mealworms consumed during the experimental period was computed by adding up the daily consumptions. Paired t-test was used to compare between the test group and the control group for each of the variables studied.

Results and Discussion

It was observed that male toads of the control group were fairly quiet during the acclimation and experimental periods. They did not make chorus (advertisement calls) in the nights but occasionally made low and short sounds. These sounds might be regarded as vocal relieves to stress or fright caused by the daily handlings and unfamiliar terrarium environments. For the test group the male toads behaved fairly similar to that of the control group; they did not make chorus but only a few stress or fright sounds. They did not make vocal responses to the chorus stimulus.

Despite of the experimental conditions that might cause the stress to the experimental toads, the daily mealworm consumption averaged about 3 worms without significant difference between the test group and the control group (t=0.17, df=9, p=0.87). Also, the snout-vent lengths (t=-1.65, df=9, p=0.13) and body weights (t=-0.24, df=9, p=0.82) of both groups at the end of the experiment were not significantly different (Table 1). Evidently, the food intakes of the toads were sufficient to maintain the body condition, and the acoustic stimulus did not affect both food consumption and body condition. Liver weights (t=-0.37, df=9, p=0.72) and fat body weights (t=-0.21, df=9, *p*=0.84) were also not affected by acoustic stimulus (Table 1). On the other hand, the acoustic stimulus affected significantly the spermatogenic activities (*t*=-4.34, df=8, *p*=0.002) but not the testis sizes (weight, *t*=-1.15, df=8, *p*=0.28; length, *t*=0.51, df=8, *p*=0.63; diameter, *t*=-1.39, df=8, *p*=0.20) (Table 1). The sperm bundles produced by the test group were 56% higher than those of the control group.

Table 1. A comparison of body lengths, body weights, liver weights, fat body weights, testes sizes, sperm bundles, and daily mealworms consumptions (mean ± standard deviations; sample sizes in parentheses) of the experimental male spectacled toads between the control group and the test group

Variables	Control group	Test group
At the time of capture		
Snout-vent length (mm)	58.5 ± 7.8 (10)	58.8 ± 8.7 (10)
Body weight (g)	19.7 ± 6.8 (10)	19.5 ± 6.7 (10)
End of acoustic experiment		
Snout-vent length (mm)	58.9 ± 8.1 (10)	60.6 ± 8.6 (10)
Body weight (g)	22.5 ± 9.2 (10)	22.9 ± 7.0 (10)
Liver weight (mg)	787.8 ± 406.5 (10)	819.6 ± 239.0 (10)
Fat body weight (mg)	381.0 ± 249.7 (10)	358.0 ± 227.6 (10)
Mealworms consumption (30 days)	87.0 ± 34.2 (10)	84.5 ± 35.8 (10)
Testes weight[1/] (mg)	38.6 ± 16.9 (9)	42.8 ± 18.5 (9)
Testes length[1/] (mm)	11.3 ± 4.8 (9)	10.5 ± 2.0 (9)
Testes diameter[1/] (mm)	2.4 ± 0.8 (9)	2.7 ± 0.4 (9)
Number of sperm bundles[2/]	153.3 ± 79.7 (9)	239.6 ± 109.2 (9)

[1/] Average values of both testes of individuals.
[2/] Significant difference between the two groups at 1% level (paired t-test, *p*<0.01).

The experimental period of this study corresponded to the early breeding season when male spectacled toads started gathering at water edges and emitting the advertisement calls. The sex ratio during this period was biased extremely toward males (Shieh 1993). The male chorus continued nearly for a month prior to having an amplexus behavior in March. Such a long period of the premating male chorus can not be simply explained for attracting males and females for gathering or protecting their territories per se (Duellman and Trueb 1994; Tobias *et al*. 2004). The male calls are known to be one of the energetically most expensive activities of ectothermic vertebrates (Taigen and Wells 1985; Pough *et al*. 1992; Emerson and Hess 2001). Continuous callings also cause frogs to expose themselves in dangers. Therefore, besides attraction, recognition or competition, the long period of male chorus before the arrival of females might have other biological significances. The results of this study suggest that the male chorus plays an important role in male reproductive cycle as an acoustic stimulus that enhances spermatogenic activities to accelerate the sperm production.

The acoustic stimulus caused the enhancement of spermatogenic activities without affecting the testicular sizes for spectacled toads in spring (Table 1). Similar result was found in *Rana shenocephalia* (Wilczynski and Chu 2001). However, the grass frog *Rana temporaria* exposed to conspecific calls in fall resulted in larger testes (Brzoska and Obert 1980). The above-mentioned difference in testicular growth to male chorus was explainable by the fact that the testicular development with different degrees of seasonal gonadal maturation (Wilczynski and Chu 2001). Male chorus increases testicular sizes in fall when the growth in size is in progress, and enhances spermatogenic activities in spring when the spermatogenesis is just in progress.

A pathway of auditory stimulus to testicular growth in anuran has been suggested to be through the tympanic membrane to the basal forebrain and then to the endocrine control center for GnRH secretion (Neary 1988; Wilczynski *et al*. 1993). Brzoska and Obert (1980) reported that acoustic signals influence the hormone production of testes in *Rana temporaria* and suggested that conspecific chorus could modulate sex steroid levels and thereby mediate the reproductive state of male frog by positive feedback. In this study, we speculated that the conspecific chorus stimulation might also cause the secretion of the male hormone that promotes the testicular maturity, and then lead the spermatogenesis to advance testes maturation.

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