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Annual Patterns of Nuptial Pad and Vocal Sac Development in the Male Chinese Bullfrog (*Rana rugulosa* Wiegmann)[1]

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Yung-Hsi Kao, Paul S. Alexander, Vivian V. Cheng Yang and John Yuh-Lin Yu (1994) Annual patterns of nuptial pad and vocal sac development in the male Chinese bullfrog (*Rana rugulosa* Wiegmann). *Zoological Studies* **33**(2): 153-159. The patterns of the changes in nuptial pad and vocal sac developments during an annual reproductive cycle of the Chinese bullfrog (*Rana rugulosa* Wiegmann) are reported for the first time. The frogs, hatched in April 1986, were collected from November, 1986 through November, 1987, at monthly intervals or more frequently during the breeding season, from a frog culture farm located in Taiwan. Nuptial pad development was histologically determined and vocal sac development was recorded by observing subgular wrinkling and pigmentation. In November and December, the nuptial pad epidermis was smooth and thin; the mucous gland diameter was small with a low cellular height. Nuptial pad epidermis and mucous glands developed rapidly in January and February. During the breeding season (March-July), the well-developed nuptial pad was rough, papillate and thick; mucous glands were hypertrophied and lumen secretions were abundant. Nuptial pads regressed during the postbreeding period (August-October). Vocal sac development coincided with both the breeding season (March-July) and the male calling season (March-September). Thus, both nuptial pad and vocal sac developments of *Rana rugulosa* exhibit a unimodal pattern during the annual reproductive cycle. The correlations between developments of these two secondary sexual characteristics and the plasma androgen levels are presented.

Key words: Seasonal changes of nuptial (thumb) pad and vocal sac, Anuran, Plasma androgen levels.

In adult male anurans the major secondary sexual characteristics, nuptial (thumb) pads and vocal sacs, are indicative of testicular androgen formation activity and reproductive activity. Functionally, nuptial pads can help the male cling to the female during amplexus at mating, and the vocal sacs can act acoustically as radiators and resonators attracting mating partners during the breeding period (Noble 1931, Duellman and Trueb 1986). Seasonal development patterns of male secondary sexual characteristics have been established in several anurans include *Bufo bufo* and *Rana nigromaculata* (Ting and Boring 1939), *R. erythraea* (Inger and Greenberg 1963), *R. esculenta* (Lofts 1964), *R. temporaria* (Lofts et al. 1972), *Hyla arborea* (Schneider 1977), *Bombina variegata* (Obert 1977), *R. limnocharis* (Alexander et al. 1979), *R. cyanophlytics* and *B. melanostictus* and *B. marinus* (Saidapur 1983), *R. catesbeiana* (Yoneyama and Iwasawa 1985), and *Pachymedusa dacnicolor* (Rastogi et al. 1986).

Rana rugulosa was first described by Wiegmann in 1853 , and is a comonly identified frog of southern and central China including Taiwan (Pope 1940, Liu 1950, Yuan 1952, Ting and Chu 1981). The Chinese bullfrog, *R. rugulosa*, is phylogenically closely related to the Indian bullfrog, *R. tigerina* (Pope 1940, Liu 1950). Frost (1985) separated these two subspecies into different species, *R. rugulosa* Wiegmann and *R. tigerina* Daudin.

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The Indian bullfrog (*R. tigerina* Daudin), inhabiting the tropical zone, is reported as exhibiting seasonal thumb pad changes with maximum development in the March-July breeding season (Saidapur and Nadkarni 1975, Saidapur 1983). We have undertaken a study investigating the annual patterns of testicular activity and secondary sexual characteristics of *R. rugulosa*. We have observed that this species exhibits a unimodal annual changes pattern in the testicular activity (Kao et al. 1993).

We report here the annual patterns of the changes in the nuptial pad and vocal sac development of the male *R. rugulosa* and their correlations with the changes in circulating androgen levels.

MATERIALS AND METHODS

Animals

A total of 347 sexually mature male *Rana rugulosa* were collected from a frog culture farm located in Tungshih (23°40'N, 120°15'E), Yunlin County, Central Taiwan from November of 1986 through November, 1987, for testicular activity investigation (Kao et al. 1993). One hundred and eighty of these animals were used for studying nuptial pad and vocal sac development.

Sampling and histology of nuptial pads and vocal sacs

Frogs were placed into a wet fishing net and anesthetized with diethyl ether (Kao et al. 1993). The two nuptial pads were fixed in Bouin's fluid and examined histologically. Vocal sac development was recorded by observing subgular wrinkling and pigmentation. Bouin's-fixed nuptial pads were wax-embedded, sectioned at 7μm, and stained with Harris' hematoxylin and eosin. The state of nuptial pad development was determined by epidermal thickness, and glandular cell height mearsurment with an ocular micrometer using Lofts' method (Lofts 1964). The diameter of cross-sectioned nuptial pad glandular tubules was also measured with an ocular micrometer. The average of 20 measurements selected from four representative cross-sections was taken as an organ index for each individual.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range tests were used to examine differences among the numerous means of epidermal thickness, mucous gland tubule diameter, and mucous gland height of the nuptial pad development of *R. rugulosa*. Linear correlations between plasma androgen levels and the nuptial pad developments were performed. Androgen concentrations in blood plasma were obtained from the previous study (Kao et al. 1993). Probability levels of 0.05 and 0.01 were used to indicate significance in means and correlations comparisons (Steel and Torrie 1980).

RESULTS

Sexual dimorphism of secondary sexual characteristics in the Chinese bullfrog is illustrated in Fig. 1. As indicated, the male frogs possess nup-

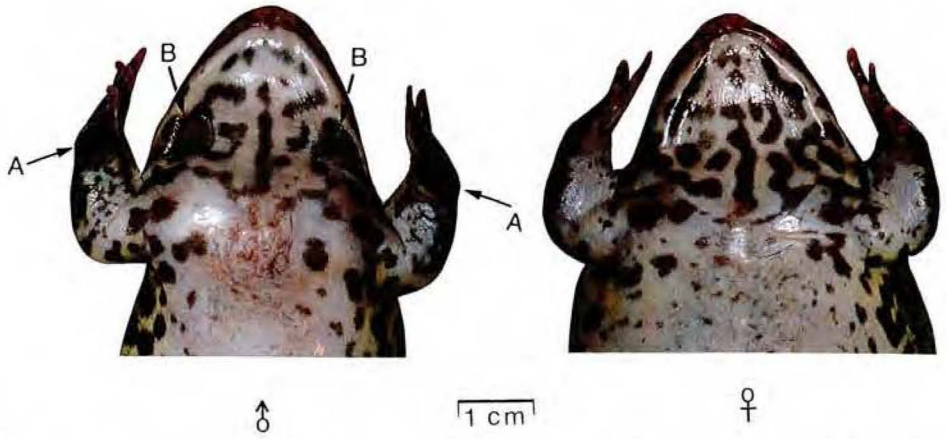

Fig. 1. Photograph showing sexual dimorphism of nuptial pads (A) and vocal sacs (B) in *Rana rugulosa*.

tial pads on the inner surface of the first finger and external vocal sacs in the subgular. These two secondary sexual characteristics are absent in the females.

Histological observations of seasonal changes in nuptal pad epidermis and mucous glands are typically shown in Fig. 2. During the early hibernation period from November through December, the epidermal layer had a thin, smooth surface. Mucous gland diameter was small and cellular height was low (Fig. 2A). Both the epidermis and mucous glands developed rapidly during the late hibernation period from January through February. During the breeding season from March through July, the epidermal layer was thick, rough and papillate with keratinized spikes. The mucous glands were large in diameter and hypertrophied. Additionally, the mucous glandular cells were fully developed, and mucus secretions were found in glandular lumen (Fig. 2B). During the postbreeding period from August through October, the epidermis and mucous gland regressed (Fig. 2C).

Significant annual changes occured in both the epidermis and the mucous gland ($p < 0.01$, ANOVA test). Epidermal thickness, mucous gland cellular height, and nuptial pad tubular diameter changes are quantitatively summarized in Table 1. In February, epidermal thickness, and glandular tubule diameter, and nuptial pad cell height increased rapidly ($p < 0.05$, Duncan test) from the hibernation period (December-January). These nuptial pad parameters peaked with some fluctuations from March through July and then declined from August to November. During an annual cycle of the male *R. rugulosa*, the difference between maximal and minimal mean values in the diameter of the mucous gland, in the height of the mucous gland cells, and in the thickness of the epidermis were 4, 3, and 1.5 fold, respectively. Results also revealed that maximal nuptial pad development occured when the androgen levels were highest during the breeding season. The correlation coefficients among the three parameters of the nuptial pads , and the correlation coefficients between these three parameters and the circulating androgen levels are shown in Table 2.

Morphologically, the well-developed vocal sacs in the subgular had a loose and obviously wrinkled surface with dark blackish pigments. As indicated in Fig. 3, fully developed vocal sacs existed in most frogs studied during the calling season from March through September, with maximal frequency of occurrence during the breeding season from March through July. We also observed vocal sacs

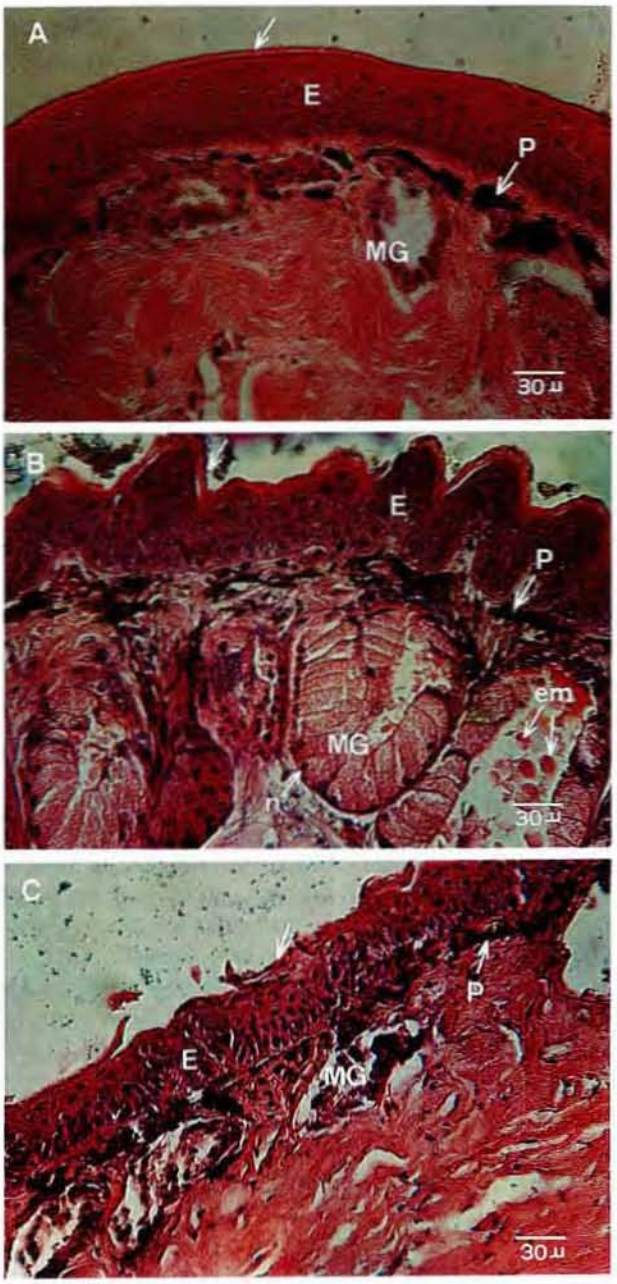

Fig. 2. Microscopic photographs showing seasonal changes in the epidermis and mucous glands of the nuptial pads of male *R. rugulosa*. The pigment cells (P) were located between the epidermis and mucous glands.

A. Hibernation (December). The epidermal layer (E) had a smooth (arrow) and relatively thin surface. The mucous glands (MG) were small in diameter, and cuboidal in the cellular shape.

B. Breeding season (April). The epidermis (E) showed a papillated surface (arrow) with keratinized spikes. The mucous glands (MG) were considerably hypertrophied and epithelium became elongated with the nucleus (n) closed to the basement membrane. The lumen of the mucous gland was observed with eosinophilic materials (em) secreted by the glandular epithelium.

C. Post-breeding season (September). The epidermal layer (E) became smooth and nonpapillated (arrow). The mucous glands (MG) became regressed.

Table 1. Changes in the nuptial pad development and plasma androgen levels during an annual reproductive cycle of male *R. rugulosa*

month[1]	N[2]	Nuptial pad development[3]			Plasma Androgen[3,4] (ng/ml)
		Epidermal thickness[3] (μm)	Moucous gland tubule diameter[3] (μm)	Mucous gland cell height[3] (μm)	
1986					
November 24	10	5.5 ± 1.1	61.6 ± 1.9	8.4 ± 0.9	0.72 ± 0.22
December 2	10	64.9 ± 2.9	64.5 ± 1.5	10.3 ± 0.6	0.30 ± 0.02
1987					
January 2	9	73.1 ± 1.1	78.4 ± 1.7	15.8 ± 1.0	0.51 ± 0.07
February 26	10	87.9 ± 2.4	111.1 ± 8.2	34.6 ± 2.6	0.48 ± 0.12
March 12	8	89.7 ± 2.5	110.0 ± 14.5	37.3 ± 2.7	0.55 ± 0.10
26	8	83.7 ± 4.3	111.7 ± 9.8	32.8 ± 3.0	1.18 ± 0.15
April 2	9	82.6 ± 2.4	138.6 ± 2.0	32.3 ± 1.6	3.75 ± 0.85
9	9	87.2 ± 2.4	114.4 ± 6.2	35.4 ± 1.8	5.20 ± 1.20
16	9	88.9 ± 5.5	153.9 ± 9.9	38.5 ± 2.1	4.15 ± 0.91
23	9	91.0 ± 2.0	140.1 ± 11.3	35.0 ± 2.3	11.10 ± 2.10
30	10	91.9 ± 3.1	152.5 ± 10.6	38.2 ± 2.1	12.00 ± 2.35
May 7	9	85.5 ± 6.0	125.7 ± 8.0	36.3 ± 1.8	14.35 ± 1.02
21	9	83.3 ± 5.1	116.9 ± 4.8	31.5 ± 2.6	7.95 ± 1.10
June 4	9	90.3 ± 4.9	122.3 ± 7.8	32.1 ± 0.7	8.60 ± 1.20
28	9	85.4 ± 1.5	132.9 ± 8.3	35.8 ± 0.9	3.05 ± 0.50
July 29	9	81.9 ± 2.9	128.6 ± 2.7	33.9 ± 1.3	2.30 ± 0.36
August 29	9	73.0 ± 3.8	107.5 ± 2.2	22.0 ± 0.8	1.20 ± 0.45
September 24	8	66.3 ± 3.7	71.5 ± 1.8	15.3 ± 1.2	0.91 ± 0.26
October 30	8	64.8 ± 3.6	79.8 ± 2.5	18.3 ± 1.2	0.60 ± 0.16
November 23	9	60.1 ± 3.2	71.9 ± 1.8	18.6 ± 0.8	1.35 ± 0.91

[1]The frogs were collected monthly or more frequently during the breeding season from March through July.
[2]Number of animals.
[3]The data are expressed as mean ± SE.
[4]Data from Kao et al. (1993).

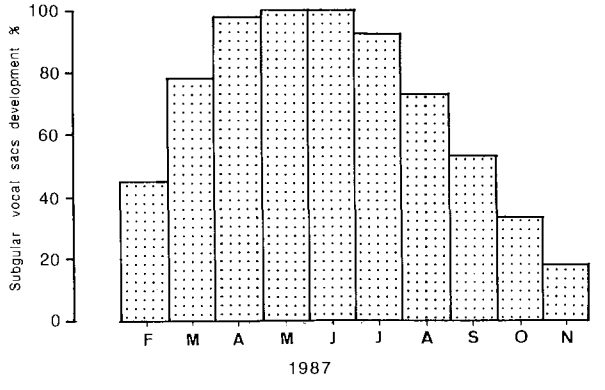

Fig. 3. Monthly changes in the frequency of well-developed subgular vocal sacs of *R. rugulosa* from February through Novermber of 1987.

to attain a maximum size during the active breeding season.

DISCUSSION

The present study has demonstrated that pronounced annual changes occur in nuptial pad and vocal sac development of *R. rugulosa*. The synchronized development of nuptial pads and vocal sacs generally coincides with circulating androgen levels and male reproductive activity.

Studies on annual pattern changes in nuptial (thumb) pad development have focused on male anurans located in temperate and tropical zones, and observed patterns have been considered as either cyclic (seasonal) or noncyclic (nonseasonal) (Inger and Greenberg 1963, Saidapur 1983). The

cyclic annual nuptial pad development pattern commonly found in temperate zone species, such as *R. esculenta* (Lofts 1964) and *R. temporaria* (Lofts et al. 1972), exhibits marked seasonal variation throughout the year. The noncyclic pattern is characteristic of most anurans inhabiting the tropical zone, like *R. erythraea* (Inger and Greenberg 1963) and *R. cyanophlyctis* (Saidapur 1983), which display little seasonal variation in nuptial pad development; however, *R. tigerina* and *B. marinus*, also located in tropical India, are known to show significant seasonal variation in nuptial pad development. Consequently, diverse species specificity exists in annual nuptial pad development pattern of the anurans inhabiting temperate and tropical zones. As evidenced by changes in epidermal thickeness and in mucous gland tubular diameter and cellular height (Fig. 2), male *R. rugulosa* inhabiting subtropical Taiwan displays pronounced seasonal changes in nuptial pad development. We have observed sharp increases in nuptial pad epidermal thickness, glandular height and tubular diameter in *R. rugulosa* in February although the values of plasma androgen remained low. Such skewed changes between circulating androgen levels and nuptial pad development could be possibly explained by the great increase of nuptial pad androgen receptors at this time; or other androgens such as androstenedione and androstenediol, which were not measured by the radioimmunoassay as reported previously (Kao et al. 1993), increase sharply. We also found that a refractory nuptial pad regression follows the decrease in plasma androgen at the end of the breeding season. This pattern in *R. rugulosa* is different from that of other anurans, such as *R. esculenta* (Lofts 1964) and *R. temporaria* (Lofts et al. 1972), and *R. tigerina* (Saidapur and Nadkarni 1975), which show a drastic regression in the nuptial pad development during the middle or late breeding season. Studies on male *R. rugulosa* nuptial pad androgen receptor dynamics, like those of *R. esculenta* established by Delrio et al. (1980), and d'Istria and Delrio (1989), may help to shed light on such phenomena.

Nuptial pad development regulation in anurans has been studied in several species at various laboratories. Izzo et al. (1982) reported that nuptial (thumb) pad development in *R. esculenta* is regulated by the interaction of temperature and androgen hormone. Rastogi et al. (1978) showed that high temperature (28°C) retarded thumb pad development and low temperature (4°C) seemed to maintain. Lofts (1974) and Saidapur (1983) both reported that nuptial pad development closely correlates with the changes in the size, nucleus, and number of Leydig cells (the major androgen-producing cells) and with activity of 3β-hydroxysteroid dehydrogenase (the main enzyme of androgen synthesis) within the same cells and concluded that nuptial pad is androgen-dependent. Rastogi et al. (1986) also reported that nuptial pad developments in *Pachymedusa dacnicolor* were highly correlated with plasma androgen levels and might be primarily induced and stimulated by androstenedione. We have observed high correlations between plasma androgen levels and epidermal thickness, as well as mucous gland tubular diameter of the nuptial pad development of *R. rugulosa* during an annual cycle (Table 2). As indicated previously in Kao et al. 1993 only testosterone and dihydrotestosterone in the plasma were quantified; other androgens such as androstenediol and androstenedione (which may be present in *R. rugulosa*) were not

Table 2. Correlation analyses of nuptial pad developments and their relations to the plasma androgen levels during an annual cycle of male *R. rugulosa*

Variable 1	Variable 2	*r*
Androgen level	Epidermal thickness	0.603**
Androgen level	Mucous gland tubular diameter	0.595**
Androgen level	Mucous gland cellular height	0.439^{+}
Epidermal thickness	Mucous gland tubular diameter	0.732**
Epidermal thickness	Mucous gland cellular height	0.684**
Mucous gland tubular diameter	Mucous gland cellular height	0.841**

Data of plasma androgen levels was from Kao et al. (1993).
Correlation coefficients (*r*) were analyzed from the monthly means of the parameter.
** $p<0.01$; $^{+}$ $p<0.10$.

measured. Consequently, the correlations between plasma androgen and nuptial pad developments in *R. rugulosa* could be underestimated. Furthermore, plasma androgen levels of *R. rugulosa* are elevated during the breeding season when the temperature is high (Kao et al. 1993). The *R. rugulosa* pattern is different from that of *R. esculenta* (Rastogi et al. 1978), but is similar to that of *P. dacnicolor* (Rastogi et al. 1986).

In male *R. rugulosa*, vocal sacs are in pairs in the subgular. This observation is different from those of Ascaphids, Pipids and some Discoglossids of Anuran, which have no vocal sacs and consequently are considered primitive Anurans groups (Salthe and Mecham 1974). Vocal sac development patterns in *R. rugulosa* observed in this study are similar to those of *R. limnocharis* described by Alexander et al. (1979), who reported anuran exhibition of seasonal changes in subgular wrinkling and pigmentation. In male anurans, such as *Acris gryllus* and *Bufo fowleri* (Jones et al. 1972), vocal sac development is physiologically stimulated and maintained by androgen. We also observed that during an annual cycle of male *R. rugulosa* vocal sac development coincides with plasma androgen levels.

The functions of mucus secreted by the specialized nuptial pad mucous glands in anurans were credited with helping the amplexing behavior (Noble 1931). Full development of vocal sacs of this species is coincident with both male calling season from March through September and the breeding season from March through July. This observation supports the proposal that the vocal sacs of male *R. rugulosa* play an important role during the mating call, similar to those reported by Wells (1977).

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雄性虎皮蛙性墊及鳴囊發育之年周期型態

高永旭　歐保羅　鄭　葳　余玉林

本研究爲探討雄性虎皮蛙，在年生殖周期期間，性墊及鳴囊發育之變化型態。按月於台灣省雲林縣東勢鄉牛蛙養殖場(位於東經120度15分，北緯23度40分附近)採樣成蛙一次，或於生殖季節每月採樣多次。該蛙之蝌蚪孵出是民國75年 4 月。採樣期間自民國75年11月起至76年11月止。以組織學切片法，鏡檢性墊發育；以肉眼觀察法，依據鳴囊皺紋和顏色之出現及程度，記錄鳴囊發育。結果顯示：十一月至十二月，性墊表皮層表面平滑，表皮層厚度薄；皮下黏液腺管徑小及腺體細胞高度低。一月至二月，性墊表皮與皮下黏液腺急速發育。在生殖季節期間(三月至七月)，性墊表皮層呈不平滑及乳突狀，其厚度亦增加至最大；性墊皮下黏液腺甚爲發達，並發現在腺體之空腔有許多嗜酸性分泌物質。在生殖季節後(八月至十月)，性墊逐漸萎縮。鳴囊於生殖期間發育非常發達，並與鳴叫季節期(三月至九月)一致。因此，雄性虎皮蛙在年生殖周期中呈現出單一型態之性墊及鳴囊發育。本報告亦提出雄性虎皮蛙性墊及其鳴囊發育與其血漿雄性素之相關性。

關鍵詞：性墊及鳴囊之季節性變化，無尾兩棲類，血漿雄性素型態。