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Captive Breeding of the Four-eyed Turtle (*Sacalia quadriocellata*)

HE Bin[1], LIU Yuxiang[1], SHI Haitao[1,2 *], ZHANG Jie[1], HU Maogui[1], MA Yonggang[1], FU Lirong[1], HONG Meiling[1], WANG Jichao[1], Fong J. Jonathan[3] and Parham F. James[4]

[1] *College of Life Sciences*, *Hainan Normal University*, *Haikou* 571158, *Hainan*, *China*
[2] *Chengdu Institute of Biology*, *Chinese Academy of Sciences*, *Chengdu* 610041, *Sichuan*, *China*
[3] *Museum of Vertebrate Zoology*, *Department of Integrative Biology*, *University of California at Berkeley*, *Berkeley*, *CA* 94720-3140, *USA*
[4] *Alabama Museum of Natural History*, *University of Alabama*, *Box* 870340, *Tuscaloosa*, *AL* 35487-0340, *USA*

Abstract In 1998, a study on forty-five four-eyed turtles (*Sacalia quadriocellata*) was initiated to gather preliminary biological data of this species and to investigate the feasibility of its captive reproduction. In the following six years, no courtship behavior was found occurring in males and no oviposition in females. From 2004 to 2007, two successful techniques were applied to initiate reproductive behavior: 1) injecting exogenous reproductive hormones; and 2) reducing the stress of living in captivity. As a result of the hormone treatments, courtship behavior and copulation were observed during September and October, 2005. However, no courtship displays were seen from the CK males, which were not treated with hormones. Ovulation occurred between December and March, and the correlation was not significant between behavior of ovulation and food intake. Females laid only one clutch of eggs each year, with 2.47 eggs (n=34, range=1–4) at average, and 84 eggs were totally obtained, of which 13 were damaged, 52 were infertile and 19 fertile. Of the fertile eggs, nine were hatched with mean incubation period of 105.9 days (n=9, range=89–122 days) at temperature ranging from 24 to 27°C.

Keywords four-eyed turtle, *Sacalia quadriocellata*, captive breeding, hormone, stress, conservation

1. Introduction

The Four-eyed turtle [*Sacalia quadriocellata* (Siebenrock, 1903)] occurs in ponds and streams in woodland habitats of southern China, northern Laos, and northern Viet Nam (Zhao, 1998; Stuart *et al.*, 2001) and is found at the elevation from approximately 170 to 640 m (Gong *et al*., 2007). Recent research has shown that *S. quadriocellata* is genetically diverse and may comprise multiple species (Shi *et al*., 2008). Wild populations of this species are decreasing rapidly due to hunting and habitat destruction (DeBruin and Artner, 1987; Lau and Shi, 2000; Gong *et al.,* 2003). Consequently, it has been designated as endangered in the *China Red Data Book of Endangered Animals* (Zhao, 1998). Different populations of *S. quadriocellata* in China are genetically distinct and the IUCN (2006) listed the species as being threatened (Shi *et al*., 2008). With the survival of this species in the wild in jeopardy due to increasing habitat destruction and poaching, it is imperative to gather its biological and related data for conservation programs and create *ex situ* assurance colonies of this species.

In recent years, the utilization of captive turtles has been increasing, and that of wild endangered ones decreasing (Snyder *et al*., 1996; Turtle Conservation Fund, 2002). In some circumstances, successful breeding can help augment or preserve wild populations (CBSG, 2001), although several concerns have been raised with such an approach (Dodd and Seigel, 1991; Snyder *et al*., 1996; IUCN, 1998; Fong *et al*., 2007). Nevertheless, several turtle species have been successfully conserved with the help of captive breeding (Schleicher and Loehr, 2001; Schleicher, 2004; Symanski, 2004; Beil, 2005; Detlef and Marlies, 2005).

* Corresponding author: Prof. SHI Haitao, from the College of Life Sciences, Hainan Normal University, with his research focusing on ecology, taxonomy, conservation biology and exotic species invasion of turtles.
E-mail: haitao-shi@263.net
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However, all documented captive breeding work in the past on *S. quadriocellata* was unsuccessful (Xia, 1983; Rodel, 1985; Zhou, 1997; Song and Yu, 2002), although it is possible that successful attempts have gone unreported. In 1998, we started a study to gather the data of reproductive biology of *S. quadriocellata*. In the following six years, no courtship behavior was found in males and no oviposition in females. As for this, two possible factors might be: 1) an unsuitable captive environment causing reduced gonadal development and the lack of reproductive hormones; and 2) insufficient nutrition to promote follicle maturation. Several techniques were applied to initiate reproductive behavior: injecting exogenous reproductive hormones, reducing the stress of living in captivity, and comparing the food intake of individuals that did and did not ovulate. This paper reports these techniques that have successfully promoted the captive breeding of *S. quadriocellata*.

2. Materials and Methods

2.1 Animals In 1998, 45 individuals of *S. quadriocellata* (17 males and 28 females) were captured from Qiongzhong County in Hainan Province, China. All the husbandry methods including captive environment, diet, and water quality followed those in Wang *et al.* (2005) and Liu *et al.* (2009). Males and females were kept in separate 60 × 80 cm indoor pools. During our experiments, cinematographic analysis was used to document and describe mating behaviors, nesting and oviposition, and is described in detail by Liu *et al.* (2008). Vitellogenic follicles and eggs were examined monthly starting from August 2006 to May 2008 using an ultrasonography protocol similar to that described by Rostal *et al.* (1990).

2.2 Hormone injecting From August 2004 to 2007, two kinds of hormones were used to induce reproduction in 30 individuals of *S. quadriocellata* (12 males and 18 females), that is, Luteinizing hormone-releasing hormone analogue (LHRH-A3) and human chorionic gonadotropin (HCG) (Ningbo Second Hormone Factory in Ningbo, Zhejiang Province, China). The hormones were injected into the hind leg muscles of both males and females to stimulate courtship behavior as follows: LHRH-A3, males 4 μg/kg and females 8 μg/kg; and HCG, males 800 IU/kg and females 1600 IU/kg. During the period of 2004–2007 starting in August, the injections were made every 10 days for a total of 10 times every year, while no injection was made in 2008 and 2009. After the hormonal injections, all behaviors were observed monthly from August to December. Other individuals (10 females and 5 males) were only injected with saline for control.

2.3 Reducing stress To reduce the stress of living in captivity (e.g., feeding, washing, handling, chasing, and noise), five females and five males were chosen and kept in an outdoor pond (10 m^2 with a shelter) from August 2007 to June 2008. Other individuals (23 females and 12 males) were kept in indoor pools, one individual per pool. The food ingested by every female in indoor pools was recorded and compared by using a *t*-test. From August 2008 to June 2009, 18 females and 12 males were kept in outdoor pond and other individuals (10 females and 5 males) kept in indoor pools.

2.4 Nesting and incubation Gravid turtles (determined by ultrasonography) were kept in indoor pools included a nesting box (30 cm × 20 cm × 10 cm) each filled with substrate (soil from wild nesting area). After oviposition for 48 hours, eggs were labeled, weighed on an electro-balance and measured with calipers, and then transferred into an incubator (cedar box with soil for substrate). The eggs were placed separately and covered with 1 cm soil to optimize gas exchange and water retention. The incubation conditions were controlled by a climate incubator, with temperature ranging from 24 to 27°C, and relative air humidity kept at 90%. Fertility of eggs was checked by candling. In most fertile eggs, a chalky-white band appeared darker than the translucent parts of the eggshell when candled 24–96 hours after oviposition. The size of the band became larger during the first 1–2 weeks of development and occupied almost the entire interior of the eggs. In contrast, the infertile eggs showed no changes in morphology throughout the duration of incubation. Incubation period, which is defined as the period of time between oviposition and emergence of the hatchlings, was recorded for each fertile egg.

3. Results

3.1 Mating After the treatment with exogenous reproductive hormones, the first courtship behavior was observed in October 2004. Throughout the hormone treatment from 2004 to 2007, males engaged in a period of intense courtship activity from August to October, 2005. After males and females were placed together, courtship behavior immediately occurred and lasted nearly continuously from September to October, al-

though copulation could rarely be observed. The quantitative study on the courtship and copulation display of *S. quadriocellata* in captivity was reported by Liu *et al.* (2008). In October 2005, complete courtship behavior was observed in the males and one individual ejaculated in the water after copulation. The successfully copulated rate of males was 4.17% (Liu *et al.*, 2008), and no courtship was observed for the males not treated with hormones.

3.2 Follicle development and ovulation Ovarian follicles, atretic follicles, and oviductal eggs were identified using ultrasound. Follicles developed either under normal conditions or with induction by exogenous hormone, with an ovulation period from December to March. 2 females in 2008 and 5 females in 2009 of which follicle development never occurred, began to ovulate when placed in the outdoor pond that reduced captive stress. 10 females in the indoor pools (with serious captive stress) and 2 females in the outdoor pond never exhibited follicular development. We compared the food ingested before and during ovulation (from November 2007 to April 2008), and there was no significant difference between the turtles that did and did not ovulate (t=0.150, df=21, p=0.882).

3.3 Nesting Totally, 84 eggs in 34 clutches were produced. Of them, 13 were damaged, 52 were infertile, and 19 fertile. Of all the eggs, 22.6% (19/84) were fertile, the percentage of the fertile eggs was 36.4% from 2005 to 2008 (with hormone injection from 2004 to 2008) and 7.5% in 2009 (with no hormone injection in 2008). Of all the 34 clutches, 8 were hormonally induced, 18 were a result of reduced captive stress, and eight resulted with no treatment. The data on treatment groups and egg production are shown in Table 1. Oviposition behavior was displayed from January to June, peaking in March and April. Each female was found to lay a single clutch each year, with an average clutch size of 2.47 eggs (n=34, range=1–4). Clutch sizes of two and three eggs were the most common (76.5% of all clutches). The average weight of an egg was 12.8±1.7 g (n=71, range=8.9–16.9 g), with a mean long diameter of 42.9±3.0 mm (n=71, range=35.4–49.4 mm), and a mean short diameter of 22.1±1.0 mm (n=71, range=20.2–24.1 mm). 33 eggs were laid in water (11 eggs were broken, 2 eggs were fertile) and 51 in soil (2 eggs were broken, 17 eggs were fertile).

Table 1 Data on the captive breeding of *S. quadriocellata* from 1998 to 2009

Clutch number	ID of turtle	Clutch size	Spawning date	Nesting site	Mass (g) and size (mm) of egg				Fertility of clutches (%)	Treated techniques
1	200	1	15 Jan. 2005	Water	*				0	HI
2	245	4	16, 30 May; 3, 6 Jun, 2005	Water	12.4 42.3×22.5	12.7 44.4×20.8	10.4 39.1×21.0	10.5 38.9×20.8	0	HI
3	285	4	8,9,11,13 May, 2006	Water	*	*	*	*	0	NT
4	200	2	8 May, 2007	Water	*	*			0	HI
5	035	3	22 Jan, 2007	Soil	10.0 41.1×21.0	10.5 38.9×20.8	10.1 39.8×20.8		0	HI
6	285	3	21 Mar, 2007	Soil	14.0 43.6×23.0	13.0 44.1×23.0	13.0 40.0×23.5		100	NT
7	242	3	1 Apr, 2007	Soil	10.4 37.8×22.0	11.1 40.7×21.7	8.9 41.1×22.2		100	HI
8	024	1	23 Apr, 2007	Soil	*				0	NT
9	285	3	30 Jan, 6 May, 8 May, 2008	Water	11.9 42.8×21.6	13.5 44.5×21.9	12.5 46.3×21.4		0	NT
10	299	1	26 Mar, 2008	Water	9.5 38.2×20.3				0	RCS
11	035	2	3 Apr, 2008	Water	14.6 44.3×23.2	12.5 40.3×22.9			100	HI
12	242	3	7 Apr, 2008	Soil	13.9 41.0×23.9	14.2 44.3×23.2	14.9 45.2×22.8		66.7	NT
13	291	2	10 Apr, 2008	Soil	14.2 47.3×22.0	13.6 45.8×22.1			100	NT

(Continued Table 1)

Clutch number	ID of turtle	Clutch size	Spawning date	Nesting site	Mass (g) and size (mm) of egg				Fertility of clutches (%)	Treated techniques
14	024	3	19 Apr, 2008	Soil	12.2 44.4×22.0	12.3 41.9×21.9	12.3 42.0×22.2		66.7	NT
15	197	2	22 Apr, 2008	Soil	14.5 49.4×21.9	13.4 48.0×21.7			100	NT
16	253	2	24 Apr, 2008	Soil	15.0 46.4×23.0	14.4 45.3×22.7			0	HI
17	245	3	18 May, 2008	Soil	12.2 44.4×21.0	12.3 41.9×21.9	12.3 42.0×22.2		0	HI
18	041	2	18 May, 2008	Soil	12.1 44.6×21.3	11.6 43.4×21.0			0	RCS
19	253	2	25 Mar; 28 Mar, 2009	Soil	14.5 44.3×22.6	14.2 44.6×22.7			100	RCS
20	035	3	25 Mar, 2009	Soil	11.3 42.0×21.5	10.6 40.7×21.6	15.6 47.6×23.0		0	RCS
21	037	4	25 Mar, 2009	Water	*	10.8 40.0×21.2	10.3 38.2×21.0	10.3 36.1×21.7	0	RCS
22	242	3	25 Mar; 31 Mar, 2009	Water	*	13.2 41.1×23.3	14.7 45.7×22.9		0	RCS
23	245	3	31 Mar, 2009	Water	10.2 39.9×20.2	12.6 45.0×21.7	*		0	RCS
24	176	3	1 Apr, 2009	Soil	12.6 42.5×22.0	13.0 43.2×22.6	11.4 41.7×21.0		0	RCS
25	200	1	7 Apr; 12 Apr, 2009	Water	*				0	RCS
26	024	3	10 Apr, 2009	Soil	13.8 38.2×21.1	11.6 35.4×23.3	13.7 43.3×23.1		0	RCS
27	291	3	11 Apr; 14 Apr, 2009	Soil	14.3 44.4×23.2	12.2 38.2×23.1	14.2 45.0×23.0		0	RCS
28	285	3	12 Apr; 27Apr, 2009	Soil	15.3 44.2×23.7	15.2 48.1×22.5	13.7 39.3×23.9		0	RCS
29	197	2	14 Apr, 2009	Water	16.9 45.9×24.0	14.8 46.9×22.4			0	RCS
30	299	2	14 Apr, 2009	Soil	16.6 46.5×24.1	14.0 46.9×21.9			50	RCS
31	041	1	15 Apr, 2009	Water	12.9 42.4×21.9				0	RCS
32	256	3	16 Apr; 10 May, 2009	Soil	11.3 41.1×20.9	15.5 45.1×23.3	*		0	RCS
33	022	2	19 Apr, 2009	Soil	13.1 45.7×21.0	12.9 44.9×21.4			0	RCS
34	031	2	11 May, 2009	Water	12.8 42.4×22.0	13.0 43.6×21.9			0	RCS

Note: HI: Hormone injecting; RCS: Reduced captive stress; NT: No treatment; * = Eggs broken during oviposition

3.4 Incubation From 1998 to 2009, 9 of the 19 fertile eggs were successfully hatched, with a rate of 47.4%. The average of incubation period was 105.9 days (n=9, range=89–122 days) at temperatures ranging from 24 to 27°C (Table 2). The hatchlings had soft carapaces, with curled margins that gradually became flattened after several hours with no yolk sac. The color of the plastron was a darkly bright orange and the carapace was olive. Present on the top of the head of each hatchling were four yellow eye-spots, each with a black dot in the center. Unlike adult *S. quadriocellata*, hatchlings were monomorphic in terms of eye-spots, so it was impossible to determine the gender of hatchlings. After hatching, all individuals were raised in our laboratory.

Table 2 Successful breeding results of *S. quadriocellata* in captivity from 2007 to 2009

Hatchling size	Clutch size	Date hatched	Incubation period (days)	Carapace length (mm)	Hatchling mass (g)
1	06	19 Jun, 2007	089	41.48	9.36
2	06	1 Jul, 2007	101	40.50	9.63
3	07	3 Jul, 2007	093	39.40	7.44
4	07	4 Jul, 2007	094	37.32	7.40
5	11	23 Jul, 2008	111	39.88	8.50
6	12	28 Jul, 2008	112	39.42	8.48
7	14	13 Aug, 2008	116	36.60	7.40
8	15	15 Aug, 2008	115	40.18	8.11
9	19	25 Jul, 2009	122	37.41	9.30

4. Discussion

While most females experienced the same reproductive cycle in wild, oviposition of some individuals in our study shifted from January-April to May-June (Shi *et al.*, 2002). The phenomenon of clutches being laid in water can be explained by the absence of suitable nesting sites in the indoor pond (Loehr, 1999). The delayed oviposition in 2008 was probably a result of unusually low temperatures in southern China from January to February (The mean temperature of water in our laboratory was 14.1±1.8°C in 2008 and 16.4±1.9°C from 2005 to 2007).

We found the incubation period in captivity (105.9 days) was different from the data from wild in Hainan Province, where the incubation period was more than 120 days (Shi *et al.*, 2002). The shorter period in captivity might be caused by the higher incubation temperature in captivity compared to the wild (lower than 25°C). It is unclear whether the shorter incubation period had any effect on the hatchlings. Since we were not able to distinguish the sex of the hatchlings, it was not possible to determine whether temperature sex determination was present in *S. quadriocellata*.

There is growing interest in many of the world's zoos and wildlife parks for breeding endangered species. We initiated this study on captive breeding of the *S. quadriocellata* in 1998, but there was no reproduction in the subsequent six years. Possible factors causing the lack of reproduction were: 1) insufficient nutrition to promote follicle maturation; and/or 2) an unsuitable captive environment or captive stress causing reduced gonadal development and the lack of reproductive hormone. Kuchling (1999) mentioned that an abundance of food before and during the ovulation period would be a key factor in reproduction for some turtle species since follicle maturation was energetically expensive. We tested this by comparing the food intake of individuals that did and did not ovulate and was found with no significant difference. Therefore, in our study, the lack of adequate food in winter and spring was not a factor for the lack of reproduction by captive female *S. quadriocellata*.

The transfer of wild turtles into captivity can seriously disturb or alter reproductive processes (Kuchling and Bradshaw, 1993). In captivity, plasma concentrations of gonadal steroids can decrease (Kuchling, 1999) and turtles respond with increased levels of corticosterone in the face of captive stress (Tyrrell and Cree, 1998). The *S. quadriocellata* in our study became hypertrophic and showed an increase in subcutaneous fat immediately after being brought to the laboratory. These symptoms are often the indication that corticosterone is being secreted in excess, since corticosterone can have anabolic effects of promoting lipogenesis if the energy demand is being met by adequate food availability (Berdanier, 1989; Boswell *et al.*, 1994; Holberton *et al.*, 1996). Studies on reptiles have shown that corticosterone directly inhibits mating behavior and testicular function in a variety of species, e. g., Italian wall lizard, *Podarcis sicula* (Manzo *et al.*, 1994) and red-sided garter snake, *Thamnophis sirtalis parietalis* (Moore and Mason, 2001). We believe that the stress of captivity contributed to the lack of reproductive hormones, directly leading to the stagnation of the gonadal development and suppression of mating behavior in *S. quadriocellata*. Two females in 2008 and five in 2009, in which follicle development never occurred, began to ovulate when placed in the pond that reduced captive stress. It seems that reduction of the captive stress for one year can stimulate reproduction. Also, after exogenous hormones were injected in 2004 to 2007, reproductive

behavior was displayed, compared to the individuals in the control group (with no exongenous hormone injection), who showed no signs of reproductive behavior. The idea of reproductive hormones stimulating reproduction was supported when comparing years when hormones were injected and not injected. From 2005 to 2008, when hormones were injected, there was a 36.4% fertility rate, while lack of the injections in 2008-2009 resulted in a low fertility rate of 7.5%. This also indicated the hormone treatment of males was beneficial to egg fertility because the only change in methodology was hormone injection.

These results show that a major factor in the lack of reproduction of *S. quadriocellata* in captivity is an unsuitable captive environment that causes the reduction of gonadal development and the lack of reproductive hormones. Two useful methods in resolving this phenomenon are to reduce the captive stress in captive environments and to inject exogenous reproductive hormones.

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