Chelonian Conservation and Biology, 2021, 20(1): 103–108
doi:10.2744/CCB-1383.1

Reproductive Ecology of the Hainan Four Eye-Spotted Turtle (*Sacalia insulensis*) on Hainan Island, China

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Abstract. – **From 2000 to 2002, the reproductive ecology of the endangered Hainan four eye-spotted turtle (*Sacalia insulensis*) was studied on Hainan Island, China. A total of 147 adult females were captured, and their reproductive status was evaluated by palpation, X-ray imaging, and ultrasound. Twenty-two gravid females were under observation when they laid their eggs. We observed gravid females and nesting behavior along stream banks between December and April. Nest dimensions ranged from 5 to 6 cm in diameter and from 2 to 3 cm in depth. No females in our study produced more than 1 clutch per season. Clutch size average was 1.9 eggs (range = 1–3 eggs, SD = 0.56, *n* = 22) with an average egg mass of 16.05 g (range = 11.8–21.0 g, SD = 2.417, *n* = 21), an average egg width of 2.36 cm (range = 2.1–2.7 cm, SD = 0.135, *n* = 21), and an average length of 4.65 cm (range = 3.9–5.3 cm, SD = 0.400, *n* = 21). Hatching success rate was 53%, and hatchlings emerged after an average of 120.9 d (range = 98–148 d, SD = 14.94, *n* = 21) in late June to early July. The mean nesting site ground temperature was 24.43°C (range = 15.0°C–30.2°C, SD = 2.712, *n* = 7).**

Key Words. – ***Sacalia insulensis*; radio tracking; reproduction; nesting behavior; egg; Hainan; *Sacalia quadriocellata***

Turtles are one of the world's most endangered vertebrate groups. Based on the International Union for Conservation of Nature (IUCN) Species Survival Commission Tortoise and Freshwater Specialist Group assessments, 56% of all recognized turtles and tortoise species with sufficient data are categorized as threatened (critically endangered, endangered, or vulnerable; Rhodin et al. 2018). Asia, especially China, is receiving the most attention regarding the conservation of wild turtles because of the high number of threatened species found there (IUCN 2017). The freshwater turtle diversity of Hainan Island is especially rich (8 species) and distinctive for China (Gong et al. 2003). One genus of interest is *Sacalia* Gray 1870. Known for their unique eyespots on the back of the head, species of *Sacalia* are geographically restricted to southern China, Laos, and Vietnam. A genetic study of *Sacalia* using mitochondrial DNA revealed 4 mitochondrial clades within the 2 currently recognized species (Shi et al. 2008). One of these mitochondrial clades corresponds to samples of *Sacalia bealei* (Gray 1831), while the other 3 clades correspond to *Sacalia quadriocellata* (Siebenrock 1903). The population of *S. quadriocellata* from Hainan Province was found to be genetically distinct from those of other regions. Based on morphological and genetic analyses of *S. quadriocellata*, Lin et al. (2018b) elevated the Hainan Island population to species level, a result also supported by analysis of mitochondrial genomes (Lin et al. 2020). The available scientific name is *Sacalia insulensis* (Adler 1962), and the common name is the Hainan four eye-spotted turtle.

Sacalia insulensis remains a common wildlife species in pet markets because of its beautiful cranial ocelli (Gong et al. 2005). We undertook a long-term ecological study of *S. insulensis* on Hainan Island, China, collecting data on its population density, spatial distribution, habitat selection, behavior, and captive breeding (Shi et al. 2002; Gong et al. 2005, 2006, 2007; Liu et al. 2008, 2009a, 2009b; He et al. 2010). The present study presents baseline data on the natural reproductive ecology of *S. insulensis*, including reproductive season, nesting behavior, clutch size and mass, incubation period, and hatchlings. This work provides crucial data for conservation management plans and support for the passing of stricter legislation to protect this species.

METHODS

The present study was conducted from 2000 to 2002 on Hainan Island, China. We captured turtles using aquatic traps baited with salted fish. For each captured female, we measured its mass, midline carapace length, maximum carapace width, and midline plastron length and palpated its body cavity to check for the presence of shelled eggs. Because palpation has been shown to underestimate the frequency of gravid females (Keller 1998), we needed a more accurate method to identify gravid females, as our access to radiography was limited. Females suspected of being gravid were taken to a nearby hospital and checked with X-ray imaging (Kuchling 1999) and ultrasound scanning (Rostal et al. 1990). For the subset of females with hard-shelled eggs, we attached a radio transmitter (AVM, 216,000–216,999 MHz) and irradiance diode to the carapace before releasing individuals at the points of capture. During the radio-tracking period of the study, all females were located once per hour (24 times/d). When the gravid female climbed onto the bank to nest, we began continuous observation until oviposition was completed. We minimized disturbance by observing turtles using binoculars during the day and night-vision glasses during the night. Due to personnel limitations, we were not able to track all individuals until nesting. For the subset of nests that were successfully located, the eggs were excavated and measured (mass, measured to the nearest 0.1 g using an electronic balance; length and width, measured to the nearest 0.1 cm using calipers) at the nesting site, and then reburied in their original positions. We continued to monitor turtles with radio tracking once a week throughout the entire nesting season. For each subset of nesting sites that we successfully located via radio tracking, 6 microhabitat characteristics were recorded (distance from stream, height above water surface, slope, percent vegetation coverage, soil humidity, and air temperature). Relative clutch mass (RCM) was calculated as RCM = clutch wet mass/female body mass before oviposition. We analyzed the correlation of gravid female mass and carapace length with clutch size and clutch mass and egg mass with egg width, egg length, hatchling weight, and hatchling carapace length. All data were $\log_{10}$ transformed to linearize allometric data for linear regression analysis. We also calculated slope and 95% confidence intervals (CI) for comparisons that were significant in regression analysis (King 2000; Iverson et al. 2019). We covered each nest with wire mesh (approximately 10 cm tall) to prevent predation and facilitate data collection. We checked nests and recorded ground temperatures at nesting sites with a standard thermometer 3 times daily (at 0800, 1400, and 1800 hrs) until the emergence of hatchlings. Incubation time for each nest was defined as the number of days between oviposition and the emergence of the first hatchling.

RESULTS

General Information. — A total of 147 adult female *S. insulensis* were examined for eggs via palpation. Of these, 36 individuals were confirmed gravid by ultrasound scanning and X-ray photography (examples in Fig. 1). A total of 22 females had hard-shelled eggs and were tracked via radio telemetry. For these 22 females, mean mass was 262.34 g (range = 216.0–319.5 g, SD = 31.501), mean carapace length was 12.77 cm (range = 11.3–14.0 cm, SD = 0.672), mean carapace width was 9.17 cm (range = 8.0–10.8 cm, SD = 0.553), and mean plastron length was 11.10 cm (range = 10.3–12.2 cm, SD = 0.509). Due to personnel limitations, we were able to observe the nesting behavior of only 13 of the 22 females with hard-shelled eggs. The remaining 9 female turtles were found at their nesting sites by radio tracking. In order to keep the nest intact, we excavated portions of 13 nests and measured 21 eggs. Nest-site characteristics were measured for 10 nests, while soil surface temperatures were recorded for 7 nest sites.

Reproductive Season. — The reproductive season lasted from December to April. The earliest hard-shelled eggs were detected on 29 December 2000, and the earliest deposition of eggs was 38 d later on 5 February 2001. Most females nested during February and March, with the latest nesting occurring on 21 April 2002.

Nesting Behavior. — We were able to observe details of nesting behavior of 13 females. We divided nesting behavior of *S. insulensis* into 4 stages: 1) nest-site selection, 2) nest excavation, 3) egg laying, and 4) egg covering and camouflaging. Tracked gravid females were typically found on stream bottoms among stone crevices and underwater roots. Nesting was completed in a single emergence in 62% of cases (8 of 13) and in 2 trips for 23% of cases (3 of 13). In the most extreme case, 1 female made 5 trips to complete nesting. In the final case, the female laid 3 eggs in the stream rather than on land.

After nest-site selection, the female dug the nest using her hind limbs 5–10 times, rotated 180° clockwise, and continued digging the nest with her forelimbs. The female would repeat this behavior, alternating between using the hind limbs and forelimbs to push the soil away from her body. The observed females all exhibited this nest digging behavior, with the exception of 1 individual that laid eggs in brush without digging a nest or covering the eggs. The average time for nest digging was 115.5 min (range = 40–150 min, SD = 102.23). The shape of nests was irregular, to a depth of 2–3 cm and width of 5–6 cm.

After digging the nest, females rested for an average of 3.9 min (range = 5–12 min, SD = 2.71), after which egg laying began. Females deposited eggs relatively quickly, with an average oviposition time of 5.9 min (range = 1–8 min, SD = 5.14). Once oviposition was completed, females took on average 64.0 min (range = 20–110 min, SD = 35.87) to cover and camouflage the eggs with the previously excavated soil and fallen leaves. While measuring eggs, we happened to move a female who was in the process of covering her eggs. Surprisingly, she continued to exhibit the egg-covering behavior even though she was not at her nesting site and there were no eggs below her. At 11 of the 13 nesting sites, turtles left their nest sites immediately after egg covering was completed, while 1 individual did not dig a nest and 1

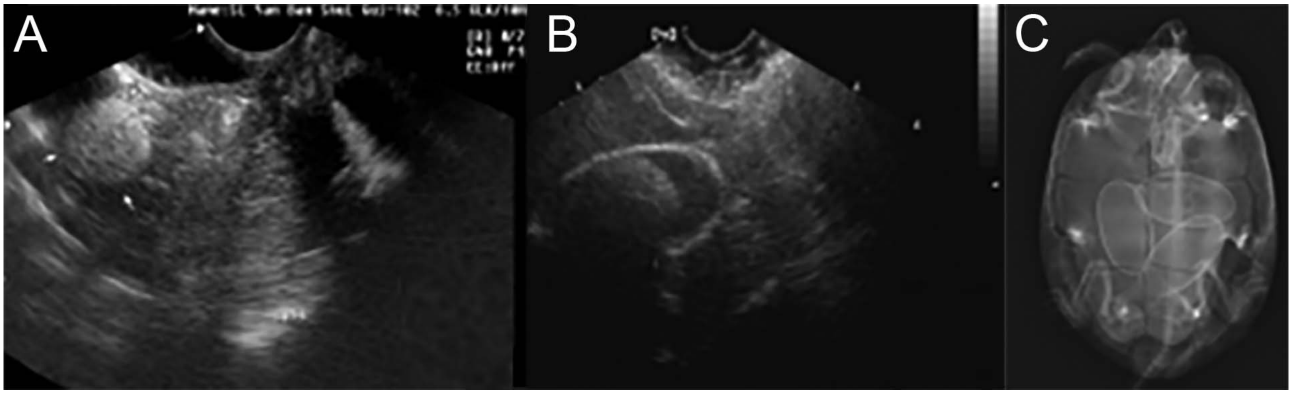

Figure 1. Examples of imagery used to verify that female *Sacalia insulensis* were gravid. (A) Ultrasound scan of a follicle. (B) Ultrasound scan of a hard-shelled egg. (C) X-ray photograph of 3 eggs.

individual stayed at the nesting site for more than 2 hrs before leaving.

Nest-Site Characteristics. — Based on the observation of 10 nest sites, we concluded that *S. insulensis* lays eggs along stream banks where the substrate consists of loose, black soil. The nest sites were on average 7.32 m away from the stream (range = 0.8–14 m, SD = 6.61), 4.82 m higher than the water surface (range = 0.5–14 m, SD = 1.341) and with an average slope of 28.5° (range = 5°–60°, SD = 15.32). Mean vegetation coverage was 85.3% (range = 50%–95%, SD = 9.84) and was composed primarily of shrubs and trees. The mean soil humidity of nest sites at egg-laying time was 29.84% (range = 26.3%–37.8%, SD = 4.342), and the mean air temperature was 20.63°C (range = 17.6°C–24.7°C, SD = 3.523).

Clutch Size, Incubation, and Hatchlings. — All females produced only 1 clutch per breeding season. Clutch size ranged from 1 to 3 eggs; 23% of clutches had 1 egg (5 of 22), 68% of clutches had 2 eggs (15 of 22), and 9% of clutches had 3 eggs (2 of 22). Mean egg mass was 16.05 g (range = 11.8–21.0 g, SD = 2.417, $n = 21$). Eggs were elliptical, with a mean width of 2.36 cm (range = 2.1–2.7 cm, SD = 0.135, $n = 21$) and mean length of 4.65 cm (range = 3.9–5.3 cm, SD = 0.400, $n = 21$). The eggs were smooth and ivory white in color, with the yolk visible through the shell (Fig. 2).

Clutch size was not correlated with gravid female mass or carapace length ($r = 0.50$, $F_{1,10} = 3.30$, $p = 0.099$, and $r = 0.25$, $F_{1,10} = 0.69$, $p = 0.43$, respectively). Similarly, clutch mass was not correlated with female mass or carapace length ($r = 0.44$, $F_{1,10} = 2.44$, $p = 0.15$, and $r = 0.19$, $F_{1,10} = 0.38$, $p = 0.55$, respectively). Additionally, there was a significant negative correlation of clutch size with egg mass ($r = -0.59$, $F_{1,10} = 5.26$, $p = 0.045$; slope = –0.28, 95% CI = –0.553–0.008), and there was no correlation of clutch size with egg width ($r = -0.44$, $F_{1,10} = 2.41$, $p = 0.15$) or egg length ($r = -0.55$, $F_{1,10} = 4.38$, $p = 0.063$). *Sacalia insulensis* had a mean RCM of 11.5% (range = 7.8%–16.7%, $n = 10$). Mean egg mass and mean egg width within a clutch were not correlated with female carapace length ($r = -0.21$, $F_{1,10} = 0.44$, $p = 0.52$, and $r = -0.09$, $F_{1,10} = 0.075$, $p = 0.79$, respectively). Egg mass was significantly correlated with egg width ($r = 0.86$, $F_{1,10} = 28.47$, $p < 0.001$; slope = 0.338, 95% CI = 0.197–0.479), egg length ($r = 0.89$, $F_{1,10} = 38.89$, $p < 0.001$; slope = 0.472, 95% CI = 0.303–0.640), hatchling mass ($r = 0.95$, $F_{1,10} = 94.88$, $p < 0.001$; slope = 1.065, 95% CI = 0.822–1.309), and hatchling carapace length ($r = 0.90$, $F_{1,10} = 40.44$, $p < 0.001$; slope = 0.364, 95% CI = 0.237–0.492). The average incubation time was 120.9 d (range = 98–148 d, SD = 14.94, $n = 21$). Most hatchlings emerged between the end of June and early July. The average soil surface temperature of the nest site was 24.43°C (range = 15.0°C–30.2°C, SD = 2.712, $n = 7$). Hatching success rate was 53% (21 of 40 eggs). We observed 21 hatchlings over the study duration, with an average mass of 10.34 g (range = 7.3–13.2 g, SD = 1.773), average carapace width of 3.53 cm (range = 2.7–4.2 cm, SD = 0.423), and average carapace length of 4.12 cm (range = 3.6–4.6 cm, SD = 0.271).

DISCUSSION

Data on the reproductive biology of turtles are critical for understanding their ecology and conservation. These data are key to documenting the limited reproductive output of many turtle species (Gibbons et al. 1982). The paucity of reproductive data has hampered the evaluation of the survival status of *Sacalia*, which includes 3 species (*S. bealei*, *S. insulensis*, and *S. quadriocellata*). Lin et al. (2018a) described the reproductive ecology of *S. bealei*, while He et al. (2010) described captive breeding in *S. insulensis*. We focus our comparisons of our data on wild *S. insulensis* reproduction with these other *Sacalia* studies but also extend comparisons to other relevant turtle studies.

Reproductive Season. — Lin et al. (2018a) reported that the nesting season of wild *S. bealei* is in May, which differs from *S. insulensis*, in which the majority of nesting occurs in February and March. The pattern of reproduction

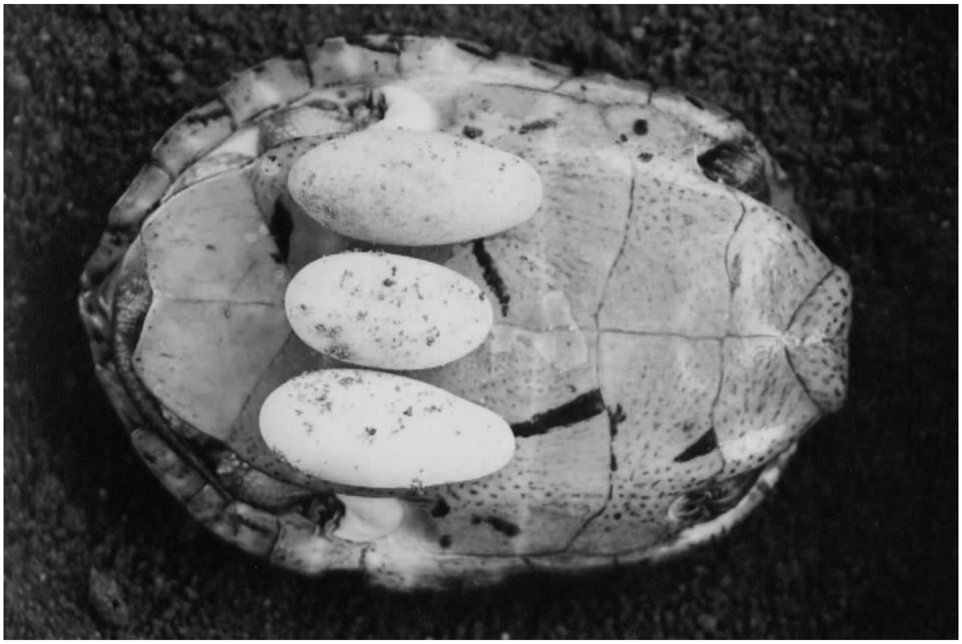

Figure 2. Female *Sacalia insulensis* with its eggs. Photo by H. Shi.

displayed by a species often correlates with the temperatures required for adult activity, embryonic development, and hatchling survival (Moll 1979). Rainfall seems to be the main factor dictating nesting in tropical species (Kuchling 1999). The reproductive season of *S. insulensis* is probably determined by the variability of rainfall in the region. After approximately 4 mo of incubation, most eggs hatch in late June and early July, just before the start of the rainy season (July–October) on Hainan Island. *Sacalia insulensis* cover their eggs with only 1–2 cm of soil, which is not enough to withstand the scour of heavy rains on the eggs.

The reproductive season of *S. insulensis* starts earlier than almost all of the other 7 turtle species living in the region (Table 1). We suspect the reason for the early start of reproduction in *S. insulensis* is its distinct microhabitat preferences. *Sacalia insulensis* inhabits ravine streams, where the high canopy density and humidity result in low microhabitat temperatures (24.2°C; *pers. obs.*). Therefore, the incubation period of *S. insulensis* requires more time (mean 120.9 ± 14.94 d SD). Lin et al. (2018a) reported a similar phenomenon in *S. bealei*, where the average incubation period was 94.7 d at a mean temperature of 25.1°C. Although the lower microhabitat temperatures result in a longer incubation period, the low incubation temperature may be an important factor for the development of embryos of *S. insulensis*. Goode and Russell (1968) found that the developing embryos of *C. expansa*, which inhabits the Murray River in New South Wales, must survive low temperatures for a long period before incubation is successful. Although further studies are needed to understand the relationship between temperature and incubation success in *S. insulensis*, our results suggest that the earlier nesting of *S. insulensis* might be shaped by both the low microhabitat temperature during incubation and the "deadline" of the dry season, during which there is lower risk of flooding than in the wet season.

Clutch Size, Egg Size, and Reproductive Strategy. — Clutch size, egg size, and number of clutches may be regarded as an adaptive compromise for survival achieved

Table 1. Comparison of nesting season and clutch size for wild *Sacalia insulensis* (present study) to studies of other wild Chinese geoemydids and captive *S. insulensis*. The relative clutch mass (RCM) for the captive *S. insulensis* and wild *Mauremys sinensis* and *Cuora galbinifrons* was estimated from the reported averages of the clutch size, egg mass, and female mass. In the case of captive *S. insulensis*, the average mass was taken from the current study. For all other studies, the RCM was measured per clutch.

Species	Nesting season	Average clutch size	Average egg mass (g)	Average female mass (g)	RCM (%)	Source
Sacalia insulensis	Captivity	2.5 (1–4)	12.8	265	12.1 (estimated)	He et al. (2010)
S. insulensis	Feb–Mar	1.9 (1–3)	16	265	11.5 (7.8–16.7)	Present study
Sacalia bealei	May	2.2 (1–3)	14.8	330	9.5 (5.1–12.2)	Lin et al. (2018a)
Cuora mouhotii	Jun–Jul	3.0 (1–5)	21.3	665	9.4 (5.3–14.6)	Wang et al. (2011)
Mauremys sinesis	Mar–Jun	12.6 (7–17)	8.0	1444	7.0 (estimated)	Chen and Lue (1998)
Cuora galbinifrons	May–Jul	1.2 (1–3)	31.4	746	5.5 (estimated)	Wang (2010)
Cuora flavomarginata	May–Jul	1.6 (1–3)	22	593	5.3 (2.3–9.2)	Chen and Lue (1999)

over millions of years of evolution (Moll 1979). Legler (1981, 1985) classified Australian chelids into 2 broad categories based on reproductive characters: 1) temperate pattern of spring ovulation and nesting, small eggs, and short incubation times and 2) tropical pattern of dry season ovulation and nesting, large eggs, and long incubation times. The 2 patterns are believed to reflect the regions in which the species first evolved (Kennett 1999). In our study area, *S. insulensis* appear to follow a tropical pattern. How parents divide their available reproductive energy between the size and number of offspring has a profound effect on parental reproductive success. Theory suggests that the relationship between offspring size and offspring fitness is of fundamental importance to the evolution of parental reproductive strategies (Rollinson and Hutchings 2013). For the smaller species of geoemydids, it is common that clutch size varies from 1 to 3 large eggs (Moll and Moll 1990), as supported by studies of wild geoemydids in China (Table 1). Female *S. insulensis* lay a single clutch each year, with an average clutch size of 1.9 eggs and a mean RCM of 11.5% (range = 7.8%–16.7%). When compared with S. *bealei* (average clutch size of 2.2 and mean RCM of 9.5%, range = 5.1%–12.2%; Lin et al. 2018a), it appears that *S. insulensis* might have a reproductive strategy (small clutch size and large egg size) similar to that of S. *bealei*. In the study of He et al. (2010) in captivity, each female *S. insulensis* laid a single clutch annually, with an average clutch size of 2.5 eggs (n = 34, range = 1–4), with clutch sizes of 2 or 3 eggs being the most common (76% of all clutches). Within a turtle species, clutch size usually increases with body size and age, but this trend may be absent in species laying small clutches (Moll 1979). We also found no significant correlation between female body size (carapace length) and clutch size, but this result might be related to our limited sample size as well as a potential bias due to our selection of gravid females by palpation (Keller 1998).

A side effect of small clutch sizes is that wild populations recover more slowly after any population decline, which is worrisome for conservation. Gong et al. (2005) reported that trade in wild turtles occurred in all cities and counties of Hainan Province and that 90% of turtles in the trade are *S. insulensis*. Hence, low reproductive output, combined with overharvesting and other human interference (e.g., habitat degradation), results in *S. insulensis* being in danger of extinction. To combat this threat, it is urgent to elevate the protection of this species and prevent poaching, especially during the reproductive season.

Acknowledgments

The present study was supported by the National Natural Science Foundation of China (No. 31772486 to H.S. and No. 31860608 to J.W.). The methods of this study were approved by the Animal Research Ethics Committee of Hainan Provincial Education Centre for Ecology and Environment, Hainan Normal University (HNECEE-2007-002), and were carried out in strict accordance with the institutional guidelines. We thank Hainan Medical University for assistance in X-ray imaging and ultrasound scanning. We thank Y.L. Fu, B.L. Fu, and many undergraduate students for their assistance during the fieldwork. We are grateful to J.R. Buskirk and K.H. Kikillus for providing constructive comments on a previous version of the manuscript. We thank 2 anonymous reviewers for providing constructive comments on this manuscript.

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Received: 19 March 2019

Revised and Accepted: 29 August 2020

Published Online: 24 May 2021

Handling Editor: Peter V. Lindeman