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Reproductive ecology of the Beal's-eyed turtle (*Sacalia bealei*)

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The Beal's-eyed turtle (*Sacalia bealei*) is endemic to southeastern China and endangered due to poaching and habitat loss. Knowledge of *S. bealei* ecology is lacking and this study provides baseline information of its reproduction in a natural environment. We studied the reproductive ecology of *S. bealei* using X-ray, spool-and-line tracking, and direct observation. Six nesting females were successfully tracked and their nesting behaviors were documented in detail. Females produced only one clutch per year, with a mean clutch size of 2.2 eggs (range 1–3). The white, hard-shelled eggs were ellipsoidal with a mean length of 45.50 mm, a mean width of 23.20 mm, and mean weight of 14.8 g. The relative clutch mass (RCM) was 9.47%, while the relative egg mass (REM) was 4.60%. The mean incubation period was 94.7 days with a mean nest temperature of 25.08°C. Hatchlings had a mean weight of 9.7 g, carapace length of 40.1 mm, carapace width of 33.3 mm, carapace height of 17.4 mm, plastron length of 31.6 mm, and plastron width of 25.4 mm. The results of this study provide important information to formulate conservation plan and ex-situ breeding for this endangered species.

1 Reproductive ecology of the Beal's-eyed turtle (*Sacalia bealei*)

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9
10 **ABSTRACT**

11 The Beal's-eyed turtle (*Sacalia bealei*) is endemic to southeastern China and endangered due to
12 poaching and habitat loss. Knowledge of *S. bealei* ecology is lacking and this study provides
13 baseline information of its reproduction in a natural environment. We studied the reproductive
14 ecology of *S. bealei* using X-ray, spool-and-line tracking, and direct observation. Six nesting
15 females were successfully tracked and their nesting behaviors were documented in detail.
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18 23.20 mm, and mean weight of 14.8 g. The relative clutch mass (RCM) was 9.47%, while the
19 relative egg mass (REM) was 4.60%. The mean incubation period was 94.7 days with a mean
20 nest temperature of 25.08°C. Hatchlings had a mean weight of 9.7 g, carapace length of 40.1 mm,
21 carapace width of 33.3 mm, carapace height of 17.4 mm, plastron length of 31.6 mm, and
22 plastron width of 25.4 mm. The results of this study provide important information to formulate
23 conservation plan and ex-situ breeding for this endangered species.

24
25 Subjects: Animal Behavior, Ecology, Turtle Conservation

26
27 Keywords: Beal's-eyed turtle, Reproduction, Incubation

28

29 INTRODUCTION

30 The reproductive biology of a species is an important component of its overall life history
31 strategy (Gibbons, 1982). Thus, understanding reproductive ecology is important for turtle
32 management and conservation (Tucker & Moll, 1997; Horne et al., 2003). Studies of turtle
33 reproductive ecology have revealed important direct and indirect effects on fitness and
34 demography (Bobyne & Brooks, 1994; Weisrock & Janzen, 1999; Valenzuela, 2001; Spencer and
35 Thompson, 2003; Janzen et al., 2007). For example, research on the snapping turtle (*Chelydra*
36 *serpentina*) found that initial egg mass and substrate moisture were positively correlated with
37 hatchling mass, which could then affect hatchling survival (Bobyne & Brooks, 1994).

38 The Beal's Eyed Turtle (*Sacalia bealei*), endemic to southeastern China, is distributed in
39 Guangxi, Guangdong, Fujian, Anhui, Guizhou, Jiangxi Provinces, and Hong Kong (Shi et al.,
40 2008). It is listed as endangered on the IUCN Red List (van Dijk et al., 2012) and Appendix II of
41 CITES. Poaching pressure on *S. bealei* is still strong, with the pet trade price increasing from
42 1500 RMB/kg in 2014 to 4200 RMB/kg in 2015 (Hu et al., 2016). Due to sustained illegal
43 poaching and trade, *S. bealei* has become extremely rare in the field (Shi et al., 2005; Gong et al.,
44 2017). Life history of this species is poorly understood, with only a few observations on diet and
45 breeding in captivity (Zhang et al., 1998). Since 2014, we conducted field studies focusing on
46 habitat selection, home range, and reproductive ecology through methods of radio telemetry,
47 spool-and-line tracking, and direct observation (Hu, 2016). The current study was part of this
48 work and provides baseline information on the reproductive biology of *S. bealei* in a natural
49 environment, and thus will contribute to the study and conservation of this species.

50

51 METHODS

52 Study site

53 We conducted field research in Huboliao National Nature Reserve, Fujian Province,
54 China (117° 12' 42"~117° 22'45" E; 24° 30' 05" ~ 24° 56' 20" N). The mean annual temperature
55 in Huboliao is 21.1°C, with the lowest temperature in January (mean 10.9°C) and the highest in
56 July (mean 26.7°C). The mean rainfall is 1733.5 mm, with a mean relative humidity of 81.4%.
57 The major vegetation types in this reserve are evergreen broad-leaved forest, mixed forest, and
58 bamboo forest (Fan, 2001).

59

60 **Methods**

61 In October 2014, we began a radio telemetry study to understand the habitat selection,
62 daily rhythm, and home range of *S. bealei*. In total, 19 individuals, including 13 females and 6
63 males, were tracked using radio transmitters. In 2015, when turtles had completed their
64 hibernation in late March, we captured them and brought females back to the field station for
65 further inspection. We used portable X-ray radiography (BJI –UJ) to confirm the presence of
66 oviductal eggs. If shell-eggs were found inside plastron, the radio-transmitter was removed and
67 replaced with a plastic spool-and-line tracker. The spool-and-line tracker was 8 g total weight
68 and contained 70 m fishing line. We designed the tracker and have successfully used to track
69 daily turtle movement during reproduction period (Li, 2013; Yang, 2014). Ten of the 13 females
70 were gravid, and took six morphological measurements of the individuals (weight, carapace
71 length, carapace width, body height, plastron length, and plastron width) following methods in
72 *Xiao et al. (2014)*.

73 Every morning, we followed the fishing line of each of the 10 females to track its
74 movement. The presence of fishing line on land indicated that the individual was attempting to
75 nest. When we encountered such a situation, we took notes on the nesting and egg-laying
76 behaviors, minimizing disturbance by observing using binoculars (8–10 m distance) during the
77 day and headlamps with red light during the night. After the turtle laid eggs and returned back to
78 the stream, we checked the nest, recording (1) nest chamber characters (*e.g.* size and nest
79 materials), (2) clutch size and egg measurements (length, width, and weight), (2) distance
80 crawled by the female before nesting, and (4) straight distance between the nest and the stream
81 bank. Fertilized and unfertilized eggs were distinguished by the presence of a white spot on the
82 eggshell. Relative egg mass (REM) (mean egg weight/body weight before oviposition) and
83 relative clutch mass (RCM) (clutch weight/body weight before oviposition = REM x clutch size)
84 were calculated for each gravid female as estimates of reproductive effort (*Wang et al., 2011*).
85 To monitor the temperature and humidity of the nest during incubation, we buried a data logger
86 (HOBO U23-001, Onset Computer Inc., USA) near the nest, at the same depth and using the
87 same materials. To prevent predation, we placed a wire net above the nest.

88 We checked the nests at least twice a week during incubation. When hatchling turtles
89 emerged from the nests, we measured their weight, carapace length, carapace width, plastron
90 length, and body height. Hatchlings were taken back to the field station and released back to the
91 stream when the yolk sac was completely absorbed. Data loggers were retrieved after hatchling
92 emergence, and the data were downloaded by HOBOWare Graphing & Analysis Software (Onset
93 Computer Inc., USA).

94 **Data analysis**

95 Statistical analyses were performed in SPSS 19.0. Descriptive statistics were expressed
96 by mean \pm standard error. Significance level was set to 0.05. We used linear regression to
97 investigate the relationship between female body size and clutch or egg size

98

99 **RESULTS**

100 **Timing and behavior sequence of nesting**

101 Among the ten gravid females, six individuals were successfully tracked, of which we
102 also collected detailed nesting behavior from individuals. The remaining four individuals were
103 lost because the fishing line broke. The nesting activities lasted from May 3–31. For the six
104 females, we recorded 10 nesting activities—four failed attempts and six successful nesting. All
105 observed nesting activities happened on rainy days and egg laying only happened at night. The
106 behavior sequences of nesting could be categorized into five successive steps: 1) nest-site
107 selection, 2) chamber excavation, 3) egg laying, 4) nest covering, and 5) returning to water.

108 During the nest-site selection, females emerged from water and made their way into the
109 forest. Movement was not continuous, but instead occurred in stops and starts. During the pauses,
110 individuals often elevated their head a few centimeters above the carapace and move side-to-side
111 a few times, possibly as a vigilance behavior. While moving, the ground substrate did not impede
112 their movement—they crawled along rocks, fallen wood, thick leaves, and gentle slopes. Often,
113 females would attempt to dig nests 1–2 times (which we called “empty holes”) before they chose
114 the final site.

115 After finding the right site, females excavated the nest chamber, alternating between the
116 two hindlimbs. After completing excavation, females would use the rear portion of its body to hit
117 the nest 4–6 times. The chamber excavation lasted 40–60 minutes.

118 After resting approximately 20 minutes, females would start laying eggs with hindlimbs
119 straddling the chamber opening. To expel eggs, forelimbs and head would completely extend
120 then simultaneously withdraw. Body tremors were obvious when the eggs were released into the
121 nest. In total, egg laying lasted 8–15 minutes. No vigilance behaviors were observed during this
122 sequence.

123 Nest covering began immediately after laying the last egg. Soil and leaves were packed
124 down using alternate, backward movements of the hindlimbs, followed by a bobbing motion of
125 the body that further compressed the soil and leaves into the nest chamber. Nest covering lasted
126 40–70 minutes.

127 After covering the nest, females immediately returned to water. While returning to the
128 water, similar behaviors to those when choosing a nest-site were exhibited, such as movement in
129 stops and starts, and crawling along shelters like rocks and fallen wood.

130

131 **Distance of nests from the stream**

132 Straight distance from nest to the stream bank was 8.55 ± 1.23 m (n=6), while straight
133 distance from nest to the sites where they emerged from water and returned to water, was 11.47
134 ± 1.31 m and 16.55 ± 2.93 m (n=6), respectively. Females crawled a distance of 42.67 ± 7.18 m
135 (n=6) to choose a nesting site.(see Table 1).

136

137 **Characteristics of nests and eggs**

138 Nests were well camouflaged by leaves and soil, making it difficult to find nests without
139 the help of fishing line. The shape of nests was hemispherical, with a diameter of 70–100 mm
140 and depth of 55–70 mm. Due to the relatively shallow depth, eggs were often half-buried by soil
141 then covered by leaves. Clutch size was 2.2 ± 0.3 (range 1–3, n=6). The white, hard-shell eggs

142 were ellipsoidal, and weighed 14.8 ± 0.8 g, with a length of 45.5 ± 1.0 mm and width of $23.2 \pm$
143 0.5 mm ($n=13$). A negative, linear correlation was found between mean egg length and female
144 plastron width (Pearson correlation coefficient= 0.934, $R^2 = 0.934$, $P < 0.05$), and a positive,
145 linear correlation found between mean egg width and female carapace height (Pearson
146 correlation coefficient = 0.847, $R^2 = 0.718$, $P < 0.05$). The mean RCM was $9.47 \pm 1.01\%$ (5.14 –
147 12.21% , $n=6$) and the mean REM $4.60 \pm 0.44\%$ (2.98 – 6.11% , $n=6$) (see Table 2).

148

149 **Incubation period and temperature**

150 Among the 13 eggs inside six nests, 10 were fertilized (76.9%, 33.3%–100% per nest) and
151 7 successfully hatched (70%, 0–100% per nest). The incubation period was 85–108 days (mean =
152 94.7 ± 2.5 days; $n=7$) and the temperature range during incubation was 17.51–29.64 °C (mean =
153 25.08 ± 0.13 °C; $n=7$). (see Table 3).

154

155 **Hatchling turtles**

156 Of the seven hatchlings, two from the same clutch died two days after hatching. Their
157 deaths were probably due to ant infestation, as we found ants on their body when the nest was
158 opened. The remaining five hatchlings were kept in the laboratory and their yolk sac was
159 absorbed, after which we released them to the stream.

160 The measurements of the hatchlings are found in Table 3. Hatchlings had soft, reddish-
161 brown carapaces with curled margins that gradually flattened after several days, orange plastron
162 and skin, and bright yellow stripes on their neck. Four bright-green eye-spots were obvious on
163 the head, with the two spots on the same side linked together. A black dot could be seen in every
164 eye-spot.

165

166 **DISCUSSION**

167 The process of selecting a nesting site is important to females in an immediate sense
168 because they are more vulnerable to terrestrial predators at this time (*Spencer, 2002*). Therefore,

169 many turtle species spend less than three hours out of water during the nesting process,
170 especially for those species nesting mainly on sandy beaches and banks (*Doody et al., 2009;*
171 *Booth, 2010*). However, we found *S. bealei* could spend 4 – 10 hours out of water when nesting.
172 We believe this is mainly due to the environment, where heavy forest and shrubs are present and
173 offer protection from predators. In addition, small body size and cryptic coloration (dark-brown
174 carapace) might also contribute their safety.

175 Females constructed shallow, bowl-shaped nests covered by soil and leaves. This is
176 similar to two other forest species in Asia, *Platysternon megacephalum* (*Sung et al., 2014*) and
177 *Cuora mouhotii* (*Wang et al., 2011*), but different from the flask-shaped, deeper nests of
178 *Trachemys scripta elegans* (*Li, 2013*), *Carettochelys insculpta* (*Doody et al., 2009*), and
179 *Phrynops tuberosus* (*Rodrigues & Silva, 2014*) that all nest in open areas. Nests that are flask-
180 shaped, compared to bowl-shaped, are advantageous in that they hold more eggs and daily
181 temperature fluctuations inside the nest are reduced (*Booth, 2006; Booth, 2010*). Sea turtles and
182 larger freshwater turtle species tend to construct such flask-shaped nests are more commonly
183 constructed by sea turtles and larger freshwater species as these turtles have stronger and longer
184 hindlimbs (*Parris et al., 2002; Corra-H et al., 2010; Booth, 2010*). *Sacalia bealei* may be
185 constrained to constructing bowl-shaped nests due to their relatively small body size.
186 Additionally, temperatures may not fluctuate greatly in *S. bealei* nests as nest sites are often
187 inside heavy forest where temperatures are moderated by shade.

188 Turtle body size has been shown to influence reproductive potential in female turtles
189 (*Valenzuela, 2001*), as the area of the pelvic girdle is correlated with female size and may
190 constrain the size and number of eggs an individual can oviposit (*Bowden et al., 2004*). Though
191 considered a small-size turtle species, *S. bealei* produces larger eggs (length 45.5 mm, width 23.2
192 mm, weight 14.8 g) than some larger freshwater species, such as *T. s. elegans* (egg length 35.4
193 mm, width 22.1 mm, weight 10.36 g, *Tucker & Janzen, 1998*). However, *S. bealei* produces
194 smaller clutches (average 2.2 eggs) of larger eggs, while *T. s. elegans* produces larger clutches
195 (average 12.5 eggs) of smaller eggs. Consequently, the RCM of *S. bealei* (5.14–12.21%) was

196 similar to other freshwater turtle species: *C. mouhotii* (6.9–14.6%, Wang et al., 2011), *C.*
197 *flavomarginata* (2.3–9.2%, Chen & Lue, 1999) and *T. scripta* (3–17%, Ernst & Lovich 2009).
198 Therefore, the total volume for eggs is relatively similar, but different species take different
199 approaches to eggs—many small or few big. No correlations were found in our study between
200 female body size and clutch size or egg size, however this may be due to small sample size.

201 The incubation period of *S. bealei* (94.7 ± 2.5 days at mean 25.1°C) was relatively longer
202 when compared to *T. s. elegans* (62.25 days at mean 27.4°C , Yang, 2014) and Magdalena River
203 Turtle (*Podocnemis lewyana*) (59.1 days at mean 32.8°C , Corra-H et al., 2010). This is likely
204 due to the nest conditions—*S. bealei* nests in cooler, shaded forests, while the other two species
205 nest in open areas with higher nest temperatures.

206 Both fertilization and hatching rate of *S. bealei* (76.9% and 70%, respectively) in our
207 study were higher than *S. quadriocellata* in captivity (22.6% and 47.4%, respectively; He et al.,
208 2010) and *T. scripta elegans* in the field in Hainan Province (33.7% and 36.7%, respectively; Li,
209 2013). Hatching rate for *S. bealei* may have been aided by caging of the nests. Captive stress and
210 lack of male individuals could cause the low fertilization rate of female turtles in captivity or
211 wild (Cadi et al., 2004; He et al., 2010). Based on the high fertilization and hatching rate of *S.*
212 *bealei*, we believe the population in our research area is healthy, with low stress levels and a
213 proper sex ratio.

214 Ant predation probably caused the death of two hatchling turtles in our study. This
215 occurrence is commonly reported in many other turtle species (Parris et al., 2002; Ferreira
216 Júnior et al., 2011; Buhlmann & Offman, 2001; Correa-H et al., 2010; Yang, 2014; Erickson &
217 Baccaro, 2016). In some cases, invertebrates including ants, flies, and beetles could infest more
218 than 50% nests (Baran et al., 2001). No evidence of vertebrate nest-predators was found in our
219 study, probably because of the nest protection we constructed.

220

221 CONCLUSIONS

222 Like other turtle species in China, *S. bealei* is facing critical crisis due to heavy poaching.

223 During our work, we found more than 30 illegal turtle traps in the streams. Huge demand in
224 cities is a major driver of turtle poaching in reserves (Gong *et al.*, 2017). Even just a few years
225 ago, species in the genus *Sacalia*, (*S. bealei* and *S. quadriocellata*) were not considered valuable
226 as they emit a musk odor. However, the rarity of other turtle species has made *Sacalia* species
227 more popular (Linh *et al.*, 2016), and price in local markets has sharply increased to 4200
228 RMB/kg (Hu *et al.*, 2016).

229 Poaching, along with low fecundity and habitat encroachment, threaten the survival of *S.*
230 *bealei* across its range. Low fecundity (only one clutch with 1–3 eggs per year) and long
231 incubation period imply that *S. bealei* has a low intrinsic rate of population increase. Thus, the
232 survival of populations should be more difficult under high poaching pressure. Habitat
233 encroachment also threatens the survival of *S. bealei*. *S. bealei* inhabits medium elevation of
234 276–389 m, which overlaps with banana and grapefruit plantations (Hu *et al.*, 2016). We believe
235 that effective conservation measures should include eliminating poaching and preserving native
236 forest and stream habitats. Meanwhile, no successful captive breeding of *S. bealei* has been
237 reported and we hope the information yielded from our study could improve methods for its
238 captive breeding and ex-situ conservation.

239

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243

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248 design, data collection and analysis, decision to publish, or preparation of the manuscript.

249 **Competing Interests**

250 There are no competing interests

251 **Author Contributions**

252 ● Liu Lin, Qingru Hu and Haitao Shi conceived and designed the experiments, performed the
253 fieldwork, analyzed the data, contributed materials/analysis tools, and wrote the paper.

254 ● Jonathan J. Fong analyzed the data, wrote, and reviewed the drafts of the paper.

255 ● Jiangbo Yang, Zhongdong Chen and Feiyu Zhou conceived and designed the experiments,
256 performed the fieldwork, and contributed materials/analysis tools.

257 ● Jichao Wang and Fanrong Xiao contributed materials/analysis tools and reviewed drafts of
258 the paper.

259 **Animal Ethics**

260 This work was approved by Animal Research Ethics Committee of Hainan Provincial Education
261 Center for Ecology and Environment, Hainan Normal University (HNECEE-2014-001).

262 **Field Study Permissions**

263 The study was approved by Administration and Services Centre of Nanjing County, Fujian
264 Province, and conducted under Licenses ID 20141203NJ0173.

265

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Table 1 (on next page)

Table1 Distance of nests from the stream

1

2 Table1 Distance of nests from the stream

Turtle ID	Straight Distance from nest to stream bank (m)	Straight Distance from nest to site emerging from water (m)	Straight Distance from nest to site returning to water (m)	Whole crawling distance during nest selection (m)
13	7.00	9.00	13.00	30.50
14	7.80	11.50	18.00	38.00
15	11.00	13.00	17.00	67.00
17	13.00	17.00	20.50	47.50
18	8.00	10.00	26.00	55.00
20	4.50	8.30	4.80	18.00
Mean±SE	8.55±1.23	11.47±1.31	16.55±2.93	42.67±7.18

3

4

Table 2 (on next page)

Table 2 Characters of nesting female *S. bealei* and their fecundity

1 Table 2 Characters of nesting female *S. bealei* and their fecundity

Turtle ID	Body Weight (g)	Carapace Length (mm)	Carapace Width (mm)	Plastron Length (mm)	Plastron Width (mm)	Body Height (mm)	Clutch size	No. of Fertilized Eggs	Egg length (mm)	Egg width (mm)	Egg weight (g)	RCM (%)	REM (%)
13	340	133.9	94.6	121.2	78.6	50.3	3	2	42.1	24	14	11.47	3.82
									46	23.6	12		
									46.7	24.5	13		
14	378	143	96.8	126.9	77.3	53.9	2	2	49.4	25	18.7	9.76	4.89
									49.5	24.9	18.2		
15	298	128.3	93.3	117.8	77.3	57.1	2	2	49.5	24.9	18.2	12.21	6.11
									46.5	26.1	18.2		
17	290	132.8	92	115.1	76.5	52.1	1	1	49	23.3	14.9	5.14	5.14
18	342	140.6	91.8	124.1	79.8	49.4	2	1	45.6	20	15.6	9.27	4.65
									45.9	20	16.1		
20	373	142.8	103.8	131.5	84.4	50.4	3	2	41.1	22.7	11.8	8.95	2.98
									42.5	21.8	11.5		

									38.1	21.4	10.1		
11	370	138.5	93.2	121.8	80	52	/	/	/	/	/	/	/
12	341	156	95.8	123.5	82.5	51.4	/	/	/	/	/	/	/
16	299	133.1	92.8	120.1	76.7	48.7	/	/	/	/	/	/	/
19	265	131.2	90.3	115.3	74.8	47.2	/	/	/	/	/	/	/
Mean±SE	329.6±11.8	136.3±1.5	94.4±1.1	121.7±1.5	78.8±0.9	51.3±0.8	2.2±0.3	1.7±0.2	45.5±1.0	23.2±0.5	14.8±0.8	9.47±1.01	4.60±0.44

Table 3 (on next page)

Table 3 Information on hatchling.

1 Table 3 Information on hatchling

Hatchling ID	Female ID	Egg-laying date	Hatching Date	Incubation Period (Day)	Mean							
					incubation temperature (°C)	Body Weight (g)	Carapace Length (mm)	Carapace Width (mm)	Body Height (mm)	Plastron Length (mm)	Plastron Width (mm)	
1	13	19-May	19-Aug	92	25.17	8.0	39.0	31.9	20.3	29.9	24.9	
2	13	19-May	24-Aug	97	25.22	8.0	37.6	25.7	15.9	29.3	22.1	
3	14	20-May	22-Aug	94	25.09	10.0	40.5	38.0	17.5	31.9	27.3	
4	14	20-May	24-Aug	96	25.11	11.0	40.1	33.5	17.4	31.3	24.0	
5	15	31-May	24-Aug	85	25.32	10.0	39.9	35.4	17.0	32.6	27.2	
6	15	31-May	30-Aug	91	25.29	12.0	42.1	36.7	16.0	33.5	27.8	
7	18	03-May	19-Aug	108	24.33	9.0	41.5	32.1	17.5	32.6	24.3	
				Mean±SE	94.7±2.5	25.08±0.12	9.7±0.5	40.1±0.5	33.3±1.4	17.4±0.5	31.6±0.5	25.4±0.7

2