Reproductive ecology of the endangered 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 are documented in detail. Females produced a mean clutch size of 2.2 eggs (range 1–3). The 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 inform conservation plan and ex-situ breeding for this endangered species.

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11 **ABSTRACT**

12 The Beal's-eyed turtle (*Sacalia bealei*) is endemic to southeastern China and endangered due to

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18 ellipsoidal with a mean length of 45.50 mm, a mean width of 23.20 mm, and mean weight of

19 14.8 g. The relative clutch mass (RCM) was 9.47%, while the relative egg mass (REM) was

20 4.60%. The mean incubation period was 94.7 days with a mean nest temperature of 25.08°C.

21 Hatchlings had a mean weight of 9.7 g, carapace length of 40.1 mm, carapace width of 33.3 mm,

22 carapace height of 17.4 mm, plastron length of 31.6 mm, and plastron width of 25.4 mm. The

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24 breeding for this endangered species.

25

26 **INTRODUCTION**

27 The reproductive biology of a species is an important component of its overall life history

28 strategy (*Gibbons, 1982*). Thus, understanding reproductive ecology is important for turtle

29 management and conservation (*Tucker & Moll, 1997; Horne et al., 2003*). Studies of turtle

30 reproductive ecology have revealed important direct and indirect effects on fitness and

31 demography (*Bobyn & Brooks, 1994; Weisrock & Janzen, 1999; Valenzuela, 2001; Spencer and*

32 *Thompson, 2003; Janzen et al., 2007*).

33   The Beal's-eyed Turtle (*Sacalia bealei*), endemic to southeastern China, is distributed in

34 Guangxi, Guangdong, Fujian, Anhui, Guizhou, Jiangxi Provinces, and Hong Kong (*Shi et al.,*

35 *2008*). It is listed as endangered on the IUCN Red List (*van Dijk et al., 2012*) and Appendix II of

36 CITES. Poaching pressure on *S. bealei* is strong, with the pet trade price increasing from 1500

37 RMB/kg in 2014 to 4200 RMB/kg in 2015 (*Hu et al., 2016*). Due to illegal poaching and trade, *S.*

38 *bealei* has become extremely rare in the field (*Shi et al., 2005; Gong et al., 2017a*). Life history

39 of this species is poorly understood, with only a few observations on diet and reproduction in

40 captivity (*Zhang et al., 1998; Gong et al., 2017b*). This study provides baseline information on

41 the reproductive biology of *S. bealei* in a natural environment.

42

43 **METHODS**

44 **Study site**

45   We conducted field research in Huboliao National Nature Reserve, Fujian Province, China

46 (117° 12' 42"~117° 22'45" E; 24° 30' 05" ~ 24° 56' 20" N). The mean annual temperature in

47 Huboliao is 21.1°C, with the lowest temperature in January (mean 10.9°C) and the highest in

48 July (mean 26.7°C). The mean rainfall is 1733.5 mm, with a mean relative humidity of 81.4%.

49 The major vegetation types in this reserve are evergreen broad-leaved forest, mixed forest, and

50 bamboo forest (*Fan, 2001*) (Fig. 1).

51

52 **Methods**

53   In late March 2015, when turtles had completed their hibernation (from early November to

54 mid March), we used traps to capture them. When females were captured, we brought them back

55 to the field station for further inspection. We used portable X-ray radiography (BJI –UJ) to

56 confirm the presence and number of oviductal eggs. If shell-eggs were found, a plastic spool-

57 and-line tracker was attached to the carapace of the female (Fig. 2). The spool-and-line tracker

58 was composed of two parts: 1) a white box (diameter 35 mm, height 23 mm) with an internal line

59 spool (diameter 30 mm, height 19 mm), fastened to the carapace using epoxy resin, 2) a black

60 external line spool (diameter 35 mm, height 23 mm), which was attached to the line and used

61 afterwards for retrieval. The tracker weighed 8 g and contained 70 m fishing line. We designed

62 the tracker and have successfully used it to track daily movement of the red-eared slider

63 (*Trachemys scripta elegans*) during the nesting period (*Li, 2013; Yang, 2014*). In total, 10

64 females with evidence of shell-eggs were tracked. We took morphological measurements of

65 these individuals (weight, carapace length, carapace width, body height, plastron length, and

66 plastron width) following methods in *Xiao et al.* (2014).

67   From 8:00 am each day, we followed the fishing line of the 10 females to track their

68 movement. The fishing line was retrieved carefully and spooled back into the white box every

69 day to ensure the line was not used up and so the turtles could move freely. The presence of

70 fishing line on land indicated that the individual was attempting to nest. When we encountered

71 such a situation, we took notes on the nesting and egg-laying behavioral patterns, minimizing

72 disturbance by observing using binoculars from 8–10 m away. After the turtle laid eggs and

73 returned back to the stream, we checked the nest, recording (1) nest chamber characters (*e.g.* size

74 and nest materials), (2) clutch size and egg measurements (length, width, and weight), (2)

75 distance crawled by the female before nesting (following the fishing line), and (4) straight

76 distance between the nest and the stream bank. Developing and undeveloping eggs were

77 distinguished by the presence of a white spot on the eggshell. Relative egg mass (REM) (mean

78 egg weight/body weight before oviposition) and relative clutch mass (RCM) (clutch weight/body

79 weight before oviposition = REM x clutch size) were calculated for each gravid female as

80 estimates of reproductive effort (*Wang et al., 2011*). To monitor the temperature and humidity of

81 the nest during incubation, we buried a data logger (HOBO U23-001, Onset Computer Inc., USA)

82 at 10 cm distance to the nest and at the same depth. To prevent predation, we placed a wire net

83 above the nest.

84   We checked the nests at least twice a week during incubation. When hatchling turtles emerged

85 from the nests, we measured their weight, carapace length, carapace width, plastron length, and

86 body height. Hatchlings were taken back to the field station and released back to the stream

87 when the yolk sac was completely absorbed. Data loggers were retrieved after hatchling

88 emergence, and the data were downloaded by HOBOware Graphing & Analysis Software (Onset

89 Computer Inc., USA).

90   The field study was approved by Administration and Services Centre of Nanjing County,

91 Fujian Provinc (No. 20141203NJ0173). The animal ethics was approved by Animal Research

92 Ethics Committee of Hainan Provincial Education Center for Ecology and Environment, Hainan

93 Normal University (No. HNECEE-2014-003).

94

95 **Data analysis**

96   Statistical analyses were performed in SPSS 19.0. Descriptive statistics were expressed by

97 mean ± standard error. Significance level was set to 0.05. We used linear regression to

98 investigate the relationship between female body size and clutch or egg size.

99

100 **RESULTS**

101 **Timing and behavior sequence of nesting**

102   The ten gravid females captured in this study had a mean weight of 329.6 ± 11.8 g, carapace

103 length of 136.3 ± 1.5 mm, carapace width of 94.4 ± 1.1 mm, carapace height of 51.3 ± 0.8 mm,

104 plastron length of 121.7 ± 1.5 mm, and plastron width of 78.8 ± 0.9 mm (n=10). Among them,

105 six individuals were successfully tracked. The remaining four individuals were lost because the

106 fishing line broke. The nesting activities lasted from May 3–31. For the six females, we recorded

107 10 nesting activities—four failed attempts and six successful nesting. All observed nesting

108 activities happened on rainy days and egg laying only happened at night. The behavior sequences

109 of nesting could be categorized into five successive steps: 1) nest-site selection, 2) chamber

110 excavation, 3) egg laying, 4) nest covering, and 5) returning to water.

111   During the nest-site selection, females emerged from water and made their way into the forest.

112 Movement was not continuous, but instead occurred in stops and starts. During the pauses,

113 individuals often elevated their head a few centimeters above the carapace and move side-to-side

114 a few times, possibly as a vigilance behavior. While moving, the ground substrate did not impede

115 their movement—they crawled along rocks, fallen wood, thick leaves, and gentle slopes. Often,

116 females would attempt to dig nests 1–2 times before they chose the final site.

117 After finding the right site, females excavated the nest chamber, alternating between the two

118 hindlimbs. After completing excavation, females used the rear portion of its carapace to hit the

119 nest 4–6 times. The chamber excavation lasted 40–60 minutes.

120 After resting approximately 20 minutes, females would start laying eggs with hindlimbs

121 straddling the chamber opening. To expel eggs, forelimbs and head would completely extend

122 then simultaneously withdraw. Body tremors were obvious when the eggs were released into the

123 nest. In total, egg laying lasted 8–15 minutes. No vigilance behaviors were observed during this

124 sequence.

125 Nest covering began immediately after laying the last egg. Soil and leaves were packed down

126 using alternate, backward movements of the hindlimbs, followed by a bobbing motion of the

127 plastron that further compressed the soil and leaves into the nest chamber. Nest covering lasted

128 40–70 minutes.

129 After covering the nest, females immediately returned to water. While returning to the water,

130 similar behaviors to those when choosing a nest-site were exhibited, such as movement in stops

131 and starts, and crawling along rocks and fallen wood.

132

133 **Nest and egg characteristics**

134 Each of the six tracked females laid eggs in a nest. The number of eggs in each nest was

135 consistent with the number of oviductal eggs seen in the x-ray, showing all eggs were laid at

136 once. Straight distance from nest to the stream bank was 8.55 ± 1.23 m (n=6), while straight

137 distance from nest to the sites where they emerged from and returned to water, was 11.47 ± 1.31

138 m and 16.55 ± 2.93 m (n=6), respectively. Females crawled a distance of $42.671.47 \pm 7.18$ m

139 (n=6) to choose a nesting site.

140 Nests were well camouflaged by leaves and soil (Fig. 3), making it difficult to find nests

141 without the help of fishing line. The shape of nests was hemispherical, with a diameter of 70–100

142 mm and depth of 55–70 mm. Due to the relatively shallow depth, eggs were often half-buried by

143 soil then covered by leaves.

144 The mean clutch size was 2.2 ± 0.3 (range 1–3, n=6). The hard-shell eggs were ellipsoidal, and

145 weighed 14.8 ± 0.8 g, with a length of 45.5 ± 1.0 mm and width of 23.2 ± 0.5 mm (n=13). A

146 negative, linear correlation was found between mean egg length and female plastron width

147 (Pearson correlation coefficient= 0.934, R^2 = 0.934, P <0.05), and a positive, linear correlation

148 found between mean egg width and female carapace height (Pearson correlation coefficient =

149 0.847, R^2 = 0.718, P < 0.05). The mean RCM was 9.47±1.01% (5.14–12.21%, n=6) and the

150 mean REM 4.60 ± 0.44% (2.98–6.11%, n=6).

151

152

153 **Incubation and hatchling characteristics**

154 Among the 13 eggs inside six nests, 10 were developed (76.9%, 33.3%-100% per nest) and 7

155 successfully hatched (70%, 0-100% per nest). The incubation period was 85–108 days (mean =

156 94.7 ± 2.5 days; n=7) and the temperature range during incubation was 17.51–29.64 °C (mean =

157 25.08 ± 0.13 °C; n=7).

158 The seven hatchlings had a mean weight of 9.7 ± 0.5 g, carapace length of 40.1 ± 0.5 mm,

159 carapace width of 33.3 ± 1.4 mm, carapace height of 17.4 ± 0.5 mm, plastron length of 31.6 ± 0.5

160 mm, and plastron width of 25.4 ± 0.7 mm. Hatchlings had soft, reddish-brown carapaces with

161 curled margins that gradually flattened after several days, orange plastron and skin, and bright

162 yellow stripes on their neck. Four bright-green eye-spots were obvious on the head, with the two

163 spots on the same side linked together. A black dot could be seen in every eye-spot. (Fig. 4)

164 Two hatchings from the same clutch died two days after hatching, probably due to an ant

165 infestation, as we found many ants on their body when the nest was opened. The remaining five

166 hatchlings were kept in the laboratory until their yolk sac was absorbed, after which we released

167 them to the stream in two days.

168

169 **DISCUSSION**

170 The process of selecting a nesting site is important to females because they are more vulnerable

171 to terrestrial predators at this time (*Spencer, 2002*). Therefore, many turtle species spend less

172 than three hours out of water during the nesting process (*Doody et al., 2009; Booth, 2010*).

173 However, we found *S. bealei* would spend 4–10 hours out of water when nesting. Perhaps the

174 presence of dense forest and shrubs offers protection from predators. Additionally, the small

175 body size and cryptic coloration (dark-brown carapace) of *S. bealei* may contribute to their safety.

176 Turtle body size has been shown to influence reproductive potential in female turtles

177 (*Valenzuela, 2001*), as the area of the pelvic girdle is correlated with female size and may

178 constrain the size of eggs an individual can oviposit (*Bowden et al., 2004*). Though considered a

179 small-sized turtle species, *S. bealei* produces larger eggs (mean length 45.5 mm, width 23.2 mm,

180 weight 14.8 g) than some larger freshwater species, such as *T. s. elegans* (egg length 35.4 mm,

181 width 22.1 mm, weight 10.36 g, *Tucker & Janzen, 1998*). Continuing the comparison with *T. s.*

182 *elegans*, *S. bealei* produces smaller clutches (average 2.2 eggs) of larger eggs, while *T. s. elegans*

183 produces larger clutches (average 12.5 eggs) of smaller eggs. Consequently, the RCM of *S.*

184 *bealei* (5.14–12.21%) was similar to other freshwater turtle species: *C. mouhotii* (6.9–14.6%,

185 *Wang et al., 2011*), *C. flavomarginata* (2.3–9.2%, *Chen & Lue, 1999*) and *T. s. scripta* (3–17%,

186 *Ernst & Lovich 2009*). Therefore, the total volume for eggs is relatively similar, but different

187 species take different approaches to eggs—many small or few big. No correlations were found in

188 our study between female body size and clutch size or egg size, however this may be due to

189 small sample size. As we did not continue tracking turtles after nesting, we do not know whether

190 females nested a second time during the breeding season. However, in captivity, females usually

191 produced only one clutch per year (*Gong et al., 2017b*), implying that *S. bealei* has a low

192 intrinsic rate of population increase.

193 The incubation period of *S. bealei* (94.7 ± 2.5 days at mean 25.1°C) was relatively long when

194 compared to *T. s. elegans* (62.25 days at mean 27.4 °C, *Yang, 2014*) and Magdalena River Turtle

195 (*Podocnemis lewyana*) (59.1 days at mean 32.8 °C, *Corra-H et al., 2010*). This is likely due to

196 the nest conditions—*S. bealei* nests in cooler, shaded forests, while the other two species nest in

197 open areas with higher nest temperatures.

198   Both development and hatching rate of eggs (76.9% and 70%, respectively) in our study were

199 higher than *S. bealei* in captivity (29.6% and 62.5%, respectively; *Gong et al., 2017b*). The

200 natural habitat with lower anthropogenic disturbance may contribute the higher success of

201 reproduction in field. Ant predation likely contributed to the death of two hatchling turtles. Ant

202 predation is commonly reported in many other turtle species (*Parris et al., 2002; Ferreira Júnior*

203 *et al., 2011; Buhlmann & Offman, 2001; Correa-H et al., 2010; Yang, 2014; Erickson &*

204 *Baccaro, 2016*). In some cases, invertebrates including ants, flies, and beetles could infest more

205 than 50% nests (*Baran et al., 2001*). No evidence of vertebrate nest-predators was found in our

206 study, probably because of the nest protection we constructed.

207   Successful conservation management of the endangered *S. bealei* will likely involve a

208 combination of in-situ and ex-situ approaches. We believe the information on the reproductive

209 ecology from our study (habitat use, nesting, breeding behavior) will help guide habitat

210 protection and captive breeding of this rare turtle species endemic to China.

211

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215

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Figure 1

Natural habitat of *Sacalia bealei*

(A) An ideal stream habitat with many big stones; (B) A typical nesting site in the nearby forest, covered by heavy canopy. Photos credit: Liu Lin.

Figure 2

Tracked female turtles with a spool-and-line tracker

(A) The components of the tracker. 1, The white box with an internal line spool; 2, the fishing line; 3, the black external line spool for retrieving the line; (B) A female turtle in the wild with the tracker on her carapace.Photos credit: Qingru Hu(A), Liu Lin(B).

Figure 3

Well-camouflaged nest and eggs of *Sacalia bealei.*

(A) the nest (denoted by a circle and arrow) covered by leaves and soil; (B) two eggs inside the nest indicated by the arrow.Photos credit: Qingru Hu.
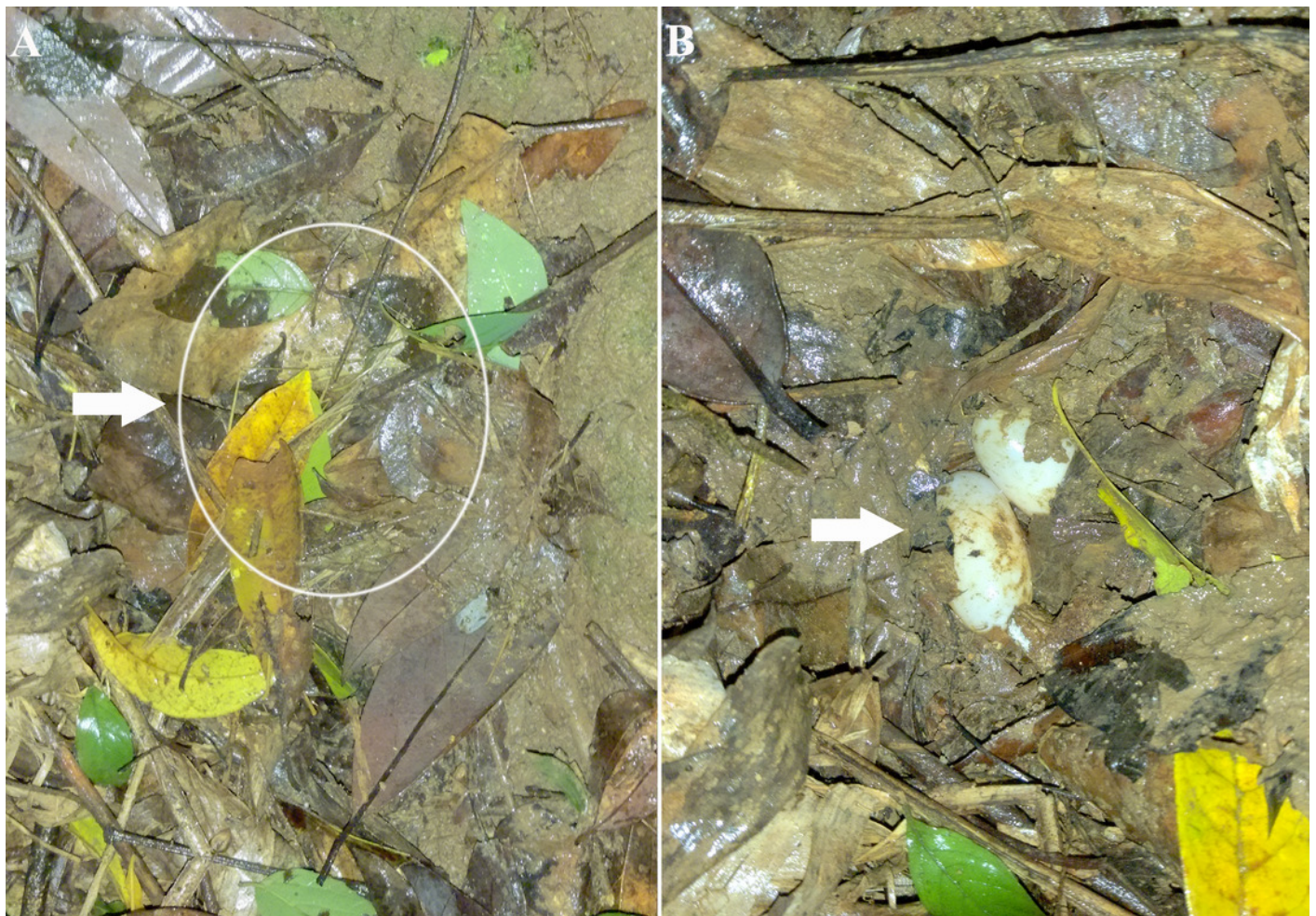
Figure 4

Hatchlings of *Sacalia bealei.*

(A) One hatchling emerging from the nest; (B) One-week old hatchlings.Photos credit: Qingru Hu (A), Liu Lin (B).