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Sensing underground activity: Diel digging activity pattern during nest escape by sea
turtle hatchlings

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ABSTRACT

Synchronous digging activity and emergence from underground nests is important for sea turtle hatchlings, as it reduces predation risk and decreases energy consumption. However, patterns of underground hatching and diel digging activity prior to emergence is invisible and remains largely unknown. In this study, we acoustically monitored the activity of green turtle (*Chelonia mydas*) hatchlings from hatching to emergence in the field and laboratory. During the hatching period, we did not observe characteristic acoustic events corresponding to pipping and hatching. Hatchlings started digging even though there were still unhatched eggs above them, indicating that green turtles did not hatch as one synchronous group. This observation did not support our hypothesis that synchronised hatching facilitates synchronous emergence. Digging activity tended to increase at night as the hatchlings approached the sand surface and nest emergence became imminent. It is not surprising that there was diel variation in digging activity as the hatchlings approached the surface because diel fluctuations in sand temperature can provide a diel cue. Surprisingly, a diel change in digging activity was also observed when hatchlings were still at a sand depth where temperature remained relatively constant throughout the day (i.e., > 80 h before the time of emergence) and in the laboratory at a constant temperature. This study suggests that the diel activity pattern of hatchling green turtles may occur even

19 in the absence of a diel temperature fluctuation cue.

20

21 **KEYWORDS**

22 acoustic monitoring, circadian rhythm, emergence, endangered species, social facilitation,

23 Southeast Asia, synchrony

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INTRODUCTION

Synchronous group activity of animals is important for reducing predation risk and energy consumption (Duranton & Gaunet, 2016; Lehtonen & Jaatinen, 2016). In hatchlings of oviparous animals with no parental care, synchronous hatching and emergence are important for improving survival (Tucker et al., 2008; Jarrett et al., 2018). Synchronous hatching triggered by physicochemical events (e.g., inundation and hypoxia) has been reported in invertebrates (Ehlinger & Tankersley, 2003; Endo et al., 2019), fish (Bradbury et al., 2004), amphibians (Warkentin, 2011), and reptiles (Doody, 2011; Doody et al., 2012). For synchronous hatching, slower-developing embryos increase their developmental rates to catch up with more developed siblings (Colbert et al., 2010; McGlashan et al., 2012, 2015; Aubret et al., 2016). Synchronous emergence and dispersal from nests is also observed in iguanas (Burghardt, 1977) and turtles (Baker et al., 2013; Santos et al., 2016).

In most reptiles that deposit their eggs in sand, it is difficult to directly observe their activity from hatching to emergence. It is unclear whether the specific patterns of hatching and sand digging activity after hatching are formed in an underground dark environment with limited diel fluctuations in temperature (Nevo, 1979). Physiologically and ecologically significant light does not penetrate beyond 1 cm depth (Tester and Morris,

1987; Kühl et al., 1994). A hypothesis proposed in turtles is that synchronous hatching of eggs in the same clutch facilitates synchronous emergence from the nest (Spencer et al., 2001; Doody, 2011; Spencer, 2012), but this is not always supported (e.g., *Graptemys geographica*; Baker et al., 2013; Riley et al., 2020). A diel activity pattern may not begin when hatchlings are at a depth where temperature remains relatively constant throughout the day. Alternatively, hatchlings may show diel activity patterns as diel fluctuations in metabolism can exist in the developing embryos of reptiles even under constant temperature and light conditions (Dmi'el, 1969; Loudon et al., 2013; McGlashan et al., 2015).

Sea turtles are widely known for nocturnal synchronous emergence of hatchlings all at once or in a few groups (Moran et al., 1999; Santos et al., 2016). Although sea turtles spend most of their lives in the sea, their hatchlings hatch in their nests 40–80 cm below the surface of sandy beaches, dig up to the surface, and emerge from the nests as a group (Carr & Ogren, 1960; Carr & Hirth, 1961; Balazs & Ross, 1974; Rusli et al., 2016). Before emergence, hatchlings wait just below the surface and the nocturnal emergence is thought to be triggered by a decrease in temperature (Glen et al., 2006), a negative temperature gradient (Gyuris, 1993), or the temperature falling below a critical temperature threshold (Moran et al., 1999; Drake & Spotila, 2002). The synchronous digging activity towards

the surface saves hatchling energy (Rusli et al., 2016). However, our understanding of the process from hatching to emergence (e.g. patterns of hatching and diel digging activity) is far from comprehensive.

This study tested for the presence of hatching synchrony and diel patterns of synchronous digging activity before the emergence of the green turtle (*Chelonia mydas*) hatchlings. We used acoustic monitoring to investigate the activity of hatchling green turtles (typically 80–120 individuals in one nest) in their natural habitats, as well as in the laboratory. Passive acoustic monitoring using acoustic receivers has been used to investigate vocalising animals (Sousa-Lima et al., 2013) and cryptic animals (Blumstein et al., 2011). Acoustic monitoring is easier in aquatic environments because of the faster speed of sound through water and longer propagation distances than in terrestrial habitats (Ladich & Winkler, 2017). However, acoustic monitoring is also effective in other poor visibility environments such as dense forests (Mennill et al., 2006; Wrege et al., 2017); therefore, it is applicable for monitoring underground behaviour.

MATERIALS AND METHODS

We conducted three field experiments and one laboratory experiment.

80 *Field experiments*

81 Field experiments were conducted at Chagar Hutang beach (5°48.778' N, 103°0.502' E),
82 Redang Island, Terengganu, Malaysia (Fig. 1). Chagar Hutang beach is one of the major
83 nesting beaches of green turtles in Malaysia (Chan, 2006). The beach was patrolled by
84 rangers, interns, and volunteers of the Sea Turtle Research Unit in Universiti Malaysia
85 Terengganu (SEATRU) every night from March to October. Nesting sea turtles were
86 identified by flipper tags (Chan, 2013) and almost all sea turtle nests were marked by
87 sticks and a string inserted into the clutch of eggs at laying and incubated *in situ*. Nests
88 were checked by excavation to the top of the eggs or hatchlings on the 45th day of
89 incubation in order to monitor the incubation and hatching status. If the eggs were
90 unhatched, the nest was re-checked every three days thereafter.

91 In 2018 and 2019, microphones connected with sound recorders, Song Meter
92 SM4 with an SMM-A2 Acoustic Microphone (Wildlife Acoustics, Inc.) or Roland R-05
93 (Roland Corporation) with an AT9905 microphone (Audio-Technica Corporation), were
94 placed in the column of sand above the eggs at the periphery of nests of green turtles
95 incubated *in situ* at Chagar Hutang during the nest check by excavation. The frequency
96 responses of the SMM-A2 and AT9905 was 60 Hz–2 kHz and 20 Hz–2 kHz, respectively.
97 The sampling frequency was 44.1 kHz with a 16-bit resolution. In addition, sand

temperature was recorded every 10 min using temperature data loggers, HOBO Pro v2 U23-003 and Tidbit v2 UTBI-001 (Onset Computer Corporation). The comparative accuracy of loggers was 0.15 °C. Our experiments involved the following three types of settings (Fig. 2).

Type 1 ($N = 5$ nests): A microphone was placed at a depth of 50–70 cm from the sand surface, just above the top of the eggs (Fig. 2), after the confirmation that the eggs (at least at the top of the nest) had not hatched. Temperature data loggers were placed beside the microphone, and at 10 cm below the surface (Fig. 2). Every 24 h from the start of recording, we checked for hatching by excavating the nests. During the excavation, we quickly dug the minimum possible space (usually within 5 min) and refilled the nests to avoid disturbing the sand temperature profile (inner sites were refilled with sand displaced from the inner site and the surface was filled with surface sand). We recorded the sound and temperature continuously until the green turtles hatched. We retrieved all the devices after the hatching was confirmed.

Type 2 ($N = 13$ nests): We placed the microphones approximately 10 cm below the surface (Fig. 2) after it was confirmed that the top hatchlings had dug to approximately

30 cm below the surface. In addition, temperature data loggers recorded the sand temperature at depths of 10 cm and 30 cm (Fig. 2). After starting the recording, we visually monitored hatchling emergence without excavating nests every 24 hours. We recorded the sound and the temperature continuously until the green turtle hatchlings emerged. For three out of the 13 nests, a second sound recorder with a microphone was set 1 m away from the nest at a depth of 10 cm. These second recorders acted as controls to ensure that we would only use sounds directly attributable to digging hatchlings.

Type 3 ($N = 3$ nests): Two sets of microphones connected with sound recorders were placed in each of the nests that were unhatched on the day of excavation. One microphone was placed just above the top of the eggs at a depth of 70–80 cm from the sand surface (as done in Type 1), and another one was placed at a depth of 10 cm in the sand (as done in Type 2) (Fig. 2). Temperature data loggers were placed with microphones (Fig. 2). In two nests, we placed two additional temperature data loggers at intermediate depths (20 cm and 45 cm, or 40 cm and 60 cm). We recorded the sound and temperature continuously until the green turtle hatchlings emerged. Once recording began, we checked every 24 h for hatching in the same manner as Type 1. After hatching, we checked for emergence in the same manner as Type 2. The recording continued throughout hatching to emergence.

Laboratory experiment

In 2018, we randomly collected 40 eggs each from three green turtle clutches that had been incubated *in situ* for approximately 50 days at Chagar Hutang beach. Each egg was marked at the top by a marker pen to maintain egg orientation and reduce the risk of disconnecting the embryonic membrane. Eggs were placed on the egg rack within the cooler box to avoid sudden changes in temperature that might harm the incubation process. The eggs were then taken to the laboratory of SEATRU in The Taaras Beach & Spa Resort (Fig. 1). The transfer took approximately 20 min from Chagar Hutang by a motorboat. We buried eggs of each nest in the sand at a depth of 50 cm in a chamber. The chamber consisted of a vertically placed cylindrical pipe (31.5 cm diameter and 1 m height) with a layer of vermiculite at the bottom. We used two types of beach sand, fine sand and coarse sand, collected from Chagar Hutang beach. The temperature in the chamber was maintained at 29 °C. The microphone connected to the Song Meter SM4 was placed at a depth of 10 cm in the sand. We recorded sounds continuously until the green turtle hatchlings emerged. The only break in recording occurred when we replaced the battery or memory card.

Data analysis

We downloaded the audio data from each sound recorder for the analyses. Each data included wideband sound events with large amplitudes that lasted for more than 5 s (Fig. 3). We detected these sounds by listening and visually inspecting the waveform and frequency spectrum using Adobe Audition (Adobe Systems Inc.). These events were not detected in the control and were therefore considered to be generated by the synchronous digging activity of multiple hatchlings (Balazs & Ross, 1974). We defined this event as ‘synchronous digging’ and quantified the duration of these synchronous digging events.

In addition to synchronous digging events, the time of emergence was determined using acoustic data. The emergence sound was a wideband sound event with a duration of more than a few minutes of sequential synchronous digging events on the date of emergence (determined by the beach patrol). We defined the time of emergence as the start of this prolonged sound event. We were unable to clearly define the time of hatching from acoustic data (see Results).

Hatchling activity using hourly number of synchronous digging events was modelled by a generalised additive mixed model (GAMM) using the package ‘mgcv’ (Wood, 2017) in R ver. 4.0 (R Core Team, 2020).

For the field data during the hatching period, we analysed the data from the Type

1 and Type 3 experiments. In the Type 3 experiment, we included data before hatching at the top of the nest (i.e., data that includes hatching events). We calculated the number of synchronous digging events and average sand temperatures every hour using the data recorded just above the eggs. The relationship of the number of synchronous digging events with temperature, hour of day, and their interaction was modelled using GAMM assuming a Poisson distribution. We used a thin plate regression spline and cyclic cubic regression spline as smoothing functions for temperature and hour of day, respectively. We considered differences among nests as simple random effects using the argument `bs = "re"`.

For the field data before emergence, we used the data from the Type 2 and Type 3 experiments. The hourly number of synchronous digging events was analysed using Poisson GAMM, similar to the hatching data analysis, but the explanatory variables were hour of day and time before emergence. Sand temperature was not included in the explanatory variables because we could not quantify the temperature at the depth of the hatchlings. Instead, hourly fluctuations in sand temperature were modelled by Gaussian GAMM using all sand temperature data obtained in Type 1, Type 2, and Type 3 experiments. Tensor product smooths of depth and hour of day were included in the model. The smoothing bases for depth was thin plate regression spline and for hour of day was

cyclic cubic regression spline. We considered differences among nests as simple random effects.

The hourly number of synchronous digging events in laboratory data was analysed using Poisson GAMM. As an analysis of the field data before emergence, the explanatory variables included hour of day and time before emergence. We did not measure sand temperature and include it in the model because we assumed it was constant as the laboratory was air-conditioned.

Ethical note

This study appropriately took care of animal welfare and was conducted under the required permissions and approval. The study protocol was approved by the Animal Experimentation Committee of Kyoto University (Inf-K18012 and Inf-K19010). Green turtle eggs were collected under permit no: Prk. Tr. 2213 Jld 3 granted by the Terengganu State Fisheries Office.

RESULTS

Activity during the hatching period in field nests

We detected no characteristic events representing pipping or hatching, but 378

synchronous digging events occurred from 182 hours (21–32 hours per nest) of audio data from seven nests. We excluded data from one nest of Type 1 because most eggs in the nest did not hatch. The duration of the synchronous digging events (average $\pm$ standard deviation) was 27.1 ± 8.1 s. GAMM analysis indicated that there were no significant effects of temperature ($edf = 2.520$, $\chi^2 = 6.372$, $P = 0.096$), hour of day ($edf < 0.0001$, $\chi^2 < 0.001$, $P = 0.709$), and their interaction ($edf = 1.311$, $\chi^2 = 1.922$, $P = 0.194$) on the occurrence of synchronous digging events.

Activity before emergence in field nests

We successfully obtained audio data from 11 nests. We excluded data from five nests of Type 2 because of the loud noise of heavy rains or unexpected stops in recording when newly landed nesting female green turtles swept away the device. The detected time of emergence was between 20:12–8:52, and most emerged during the night (mean: 23:24, 95% confidence interval: 22:15–1:15; Appendix Fig. A1). In three nests, hatchlings emerged in two or three groups within one to three hours.

Synchronous digging events occurred from the start of recording until emergence in all 11 nests. We detected a total number of 2115 synchronous digging events from 437 hours (5–132 hours per nest) of audio data. The duration was 13.4 ± 5.2 s. In the following

GAMM, we used 96 hours or less of data before emergence because only one nest from the Type 3 experiment contained data of 97–132 hours before the emergence. GAMM analysis indicated that synchronous digging events were significantly influenced by the time before emergence ($edf = 8.80$, $\chi^2 = 498.8$, $P < 0.0001$), hour of day ($edf = 7.30$, $\chi^2 = 213.0$, $P < 0.0001$), and their interaction ($edf = 11.26$, $\chi^2 = 528.5$, $P < 0.0001$). Synchronous digging events of hatchlings were more common at night (Fig. 4a). The occurrence of synchronous digging events tended to increase as the time of emergence neared (Fig. 4b), particularly in the morning preceding emergence from 2:00 to 8:00 (Fig. 4c). In addition, there were several peaks of synchronous digging events at approximately 20 h, 45 h, and 85 h before the time of emergence (Fig. 4b, c).

Sand temperature in the field

Sand temperature varied with depth and hour of the day ($edf = 17.34$, $F = 342.3$, $P < 0.0001$). At a depth of 40 cm or deeper, particularly ≥ 60 cm, daily fluctuations in the sand temperature were small, resulting in a relatively constant sand temperature (Fig. 5). At the ground surface at 10 cm, the sand temperature was relatively low from 1:00 to 8:00 (≤ 28.0 °C in the model, ranging 24.6–30.2 °C in actual measurement), whereas it was relatively high from 14:00 to 19:00 (≥ 30.0 °C in the model, ranging 25.8–35.0 °C in

actual measurement) (Fig. 5).

Activity in the laboratory

In total, we detected 1868 synchronous digging events from 434 hours (144–146 hours per nest) of audio data from three nests. The duration of synchronous digging events averaged 27.2 ± 11.2 s. We detected synchronous digging events as soon as we began recording until emergence. There were significant effects of time before emergence ($edf = 6.55$, $\chi^2 = 228.0$, $P < 0.0001$), hour of the day ($edf = 5.49$, $\chi^2 = 260.4$, $P < 0.0001$), and their interaction ($edf = 9.38$, $\chi^2 = 211.7$, $P < 0.0001$) on the occurrence of synchronous digging events. The number of events increased during the night (Fig. 6a), and as the time of emergence became closer (Fig. 6b). As indicated by the field experiment, the occurrence of synchronous digging events tended to be greater between 2:00 and 8:00 on the morning of emergence (Fig. 6c).

DISCUSSION

We hypothesised that hatching is synchronised in sea turtles to facilitate synchronous digging and emergence (Spencer et al., 2001; Colbert et al., 2010; Spencer, 2012). However, we detected no characteristic acoustic events representing pipping and hatching

in this study; this result is in contrast with Balazs and Ross (1974). Synchronous digging events occurred prior to hatching of all the eggs (notably, those on the top). Synchronous digging events intermittently occurred with intervals of more than 10 s of silence. This indicates that green turtle hatchlings were digging and resting synchronously, as previously suggested (Carr & Ogren, 1960; Balazs & Ross, 1974; Rusli et al., 2016). Greater hatchling activity produces a greater amount of lactate that would require resting periods before activity can recommence (Dial, 1987). It is unclear whether the synchronous digging events detected during the hatching period relate to hatching, but there was no significantly intense one-off event. This indicates that green turtle hatchlings did not hatch synchronously within a short period of time, as was observed for nest emergence events. Possibly, the eggs in the centre of the nest developed and hatched faster than the surrounding eggs because of the higher temperature caused by metabolic heating (Godfrey et al., 1997; Booth & Astill, 2001).

Our suggestion that green turtle eggs may hatch asynchronously indicates that green turtle embryos lack a strong catch-up mechanism, as has been suggested for some freshwater species of turtles (Spencer et al., 2001; McGlashan et al., 2015). However, even in freshwater turtles that have catch-up mechanisms, hatching of all eggs within a nest may vary by 1–2 days (Spencer et al., 2001; McGlashan et al., 2015). In addition,

synchrony has often been evaluated at the time of pipping (Colbert et al., 2010; Spencer, 2012; McGlashan et al., 2012, 2015), but the hatching timeframe is often more variable than the time of pipping (Doody et al., 2012). Synchronisation of hatching may not be a problem in green turtles because they can wait for their siblings after they hatch and early hatching to achieve synchrony may entail a cost of inferior neuromuscular development (Colbert et al., 2010). Therefore, full development of the neuromuscular system may overweigh rigorous synchrony in hatching to ensure that sea turtles can efficiently perform digging activities.

During the hatching period, there were no significant effects of temperature and hour of day on the number of synchronous digging events. In the day or two after hatching, the digging activity of hatchlings occurred less frequently but relatively constantly throughout the day and night, as expected in the relatively stable underground environment. In fact, the diel variation in sand temperature at the depth of eggs was small, as reported previously for green turtle nests (Hendrickson, 1958; Booth & Astill, 2001; Drake & Spotila, 2002; Glen et al., 2006).

Conversely, the number of synchronous digging events tended to increase during the night as hatchlings approached the surface and prepared for nest emergence. It is reasonable to assume that the diel variation in hatchling digging activity begins as

hatchlings reach depths of ~40 cm from the surface where diel fluctuations in temperature become more pronounced. The peaks of the occurrence of synchronous digging events at about 20 and 45 hours before the emergence indicate a circadian rhythm of activity in correspondence with diel fluctuations in temperature. Surprisingly, however, a diel change in activity was also observed when hatchlings were assumed to be at a depth with relatively constant sand temperatures (i.e., > 80 h before the time of emergence) and in the laboratory where the temperature was kept constant.

Metabolic circadian rhythms occur during the development of embryos of freshwater turtles (Loudon et al., 2013; McGlashan et al., 2015) and snakes (Dmi'el, 1969), even under constant temperature and light conditions. The diel metabolism fluctuations did not correlate with the time of day in freshwater turtles (Loudon et al., 2013; McGlashan et al., 2015), but the correlation was observed in snakes (Dmi'el, 1969). Therefore, sea turtle embryos may have a diel metabolism rhythm that results in diel digging activity post hatching. The correlation with time of day may have resulted from the time of laying that breaks embryonic arrest (i.e., preovipositional arrest of development in the hypoxic oviduct) (Rafferty & Reina, 2012; Williamson et al., 2017) at night.

Alternatively, the diel cycle may be established by unobserved environmental

cues, including anthropogenic effects. For example, excavation for setting the devices and checking the hatching status may have facilitated the early establishment of the diel cycle in the field experiments. In the laboratory experiments, transportation of the eggs from nests to the laboratory or external sound stimuli (Balazs & Ross, 1974; Rusli et al., 2016) could have triggered the diel cycle of activity, although we took care to minimise these effects.

Nonetheless, the effects of sand temperature on synchronous digging events could be possible in the field experiment. Sand temperatures ranging from 28.5 °C to 33 °C may inhibit green turtle hatchling activity (Hendrickson, 1958; Mrosovsky, 1980). The average sand temperature recorded in this study was relatively constant at ~30 °C at a depth of 40–60 cm, and was > 30 °C at the subsurface from 14:00 to 19:00. The relatively low subsurface sand temperature from 1:00 to 8:00 corresponded with the higher occurrence of synchronous digging events just before the emergence. In this study, emergence occurred mainly at night, as reported in previous studies (Gyuris, 1993; Glen et al., 2006; Santos et al., 2016). Therefore, in most cases, hatchlings moved up to near the surface in the morning, decreased their activity in the afternoon, and emerged at night when the sand temperature decreased (Glen et al., 2006) or became lower than the critical threshold temperature (Moran et al., 1999; Drake & Spotila, 2002).

This study demonstrates the usefulness of acoustic monitoring for investigating underground hatchling behaviours from hatching to emergence in natural habitats. Masking by noises (e.g., heavy rain) and undetected quiet events are challenges that require future improvement. However, this non-invasive method is a prospective method for monitoring hatchlings without exposing them to unnatural light and temperature conditions. Acoustic monitoring will also contribute to revealing vocal communication among hatchlings and embryos that may be important in reptiles (Vergne & Mathevon, 2008; Ferrara et al., 2013), including sea turtles (Ferrara et al., 2014a, 2014b, 2019; McKenna et al., 2019; Monteiro et al., 2019). In addition to synchronous digging events, our recordings detected narrow-band harmonic sounds that seemed to be vocalisations, but these were not analysed in the current study. Further vocalisation studies should also assist us in further understanding sea turtle biology and animal communication.

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Figure Legends

Fig. 1. Location of Chagar Hutang beach and laboratory in Redang Island, Malaysia

Fig. 2. Diagram of the three types of experimental settings

Fig. 3. Spectrogram of two synchronous digging events (arrows). Colours represent sound intensity.

Fig. 4. Effects of (a) hour of day, (b) time before emergence, and (c) their combination on the number of synchronous digging events in the field. Shaded areas in (a) and (b) represent 95% confidence intervals. The combined effects in (c) include the interactions of variables and the contour lines indicate the number of synchronous digging events.

Fig. 5. (a) Sand temperature measured at the depth of 10 cm (green), 30 cm (orange), and ≥ 50 cm (violet). Thick lines indicate averages and shaded areas indicate ± 1 standard deviation. (b) Average sand temperature in the field as contours in relation to depth and hour of day predicted by modelling.

Fig. 6. Effects of (a) hour of day, (b) time before emergence, and (c) their combination on the number of synchronous digging events in the laboratory. Shaded areas in (a) and (b) represent 95% confidence intervals. The combined effects in (c) include the interactions of variables and the contour lines indicate the number of synchronous digging events.

Appendix Fig. A1. Temporal distribution and circular histogram of the time of emergence. Each dot represents the time of each nest. The arrow and dotted lines indicate the mean and 95% confidence interval, respectively.

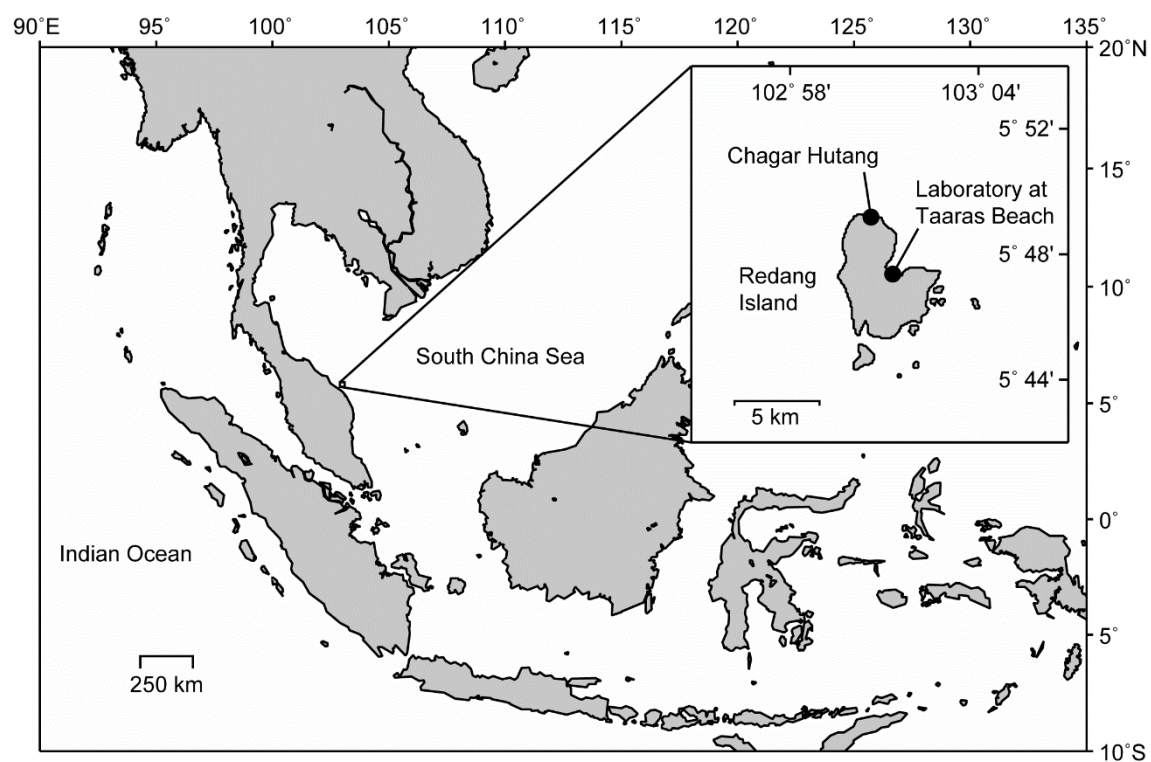


Fig. 1.

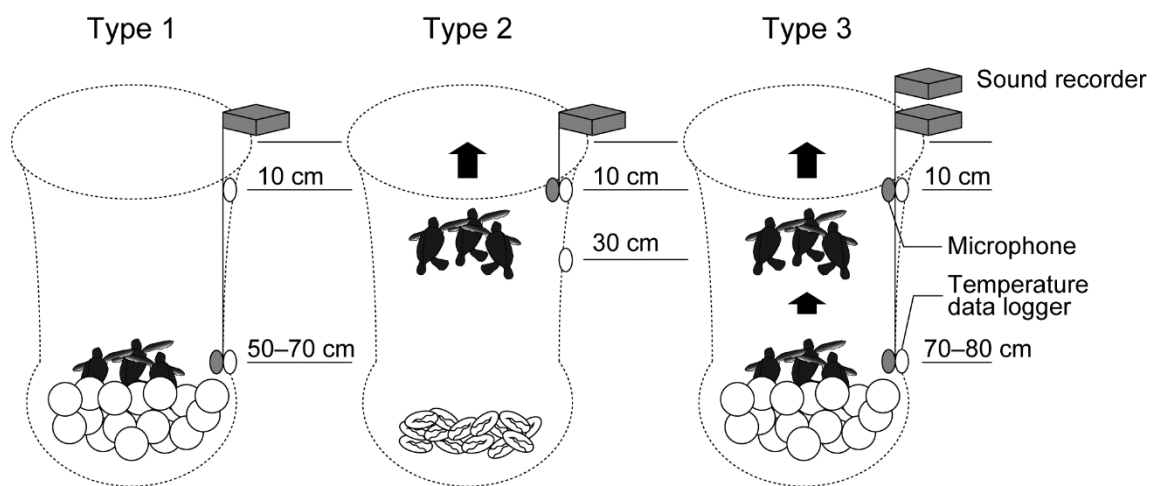
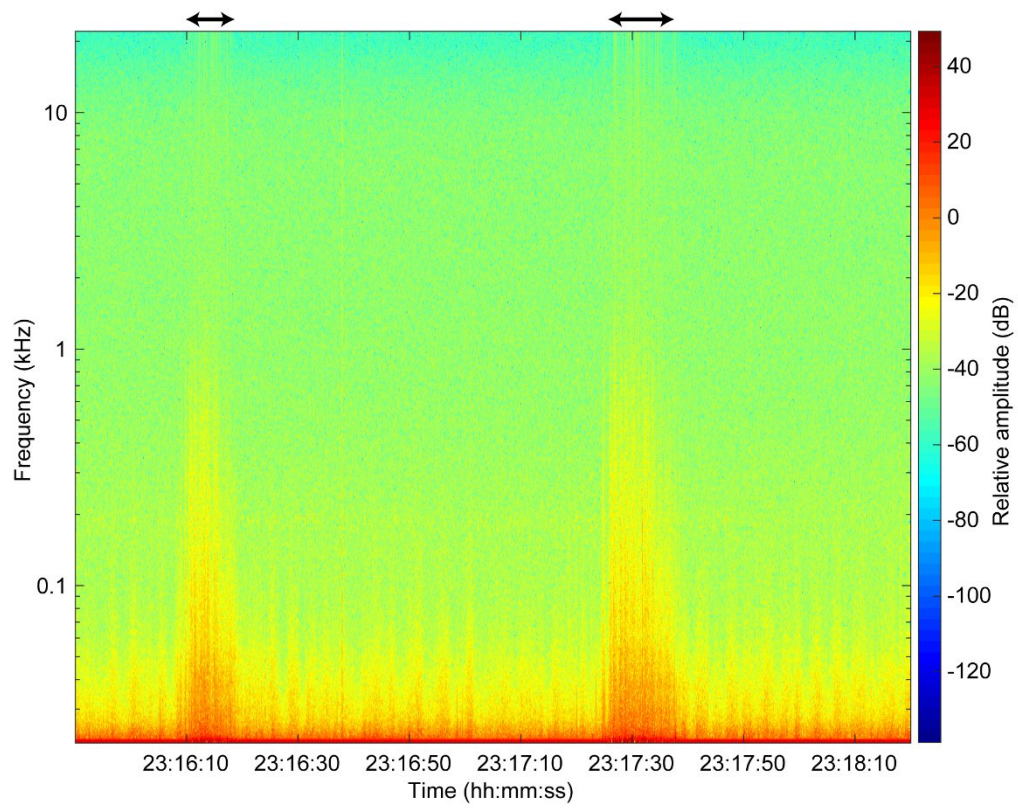


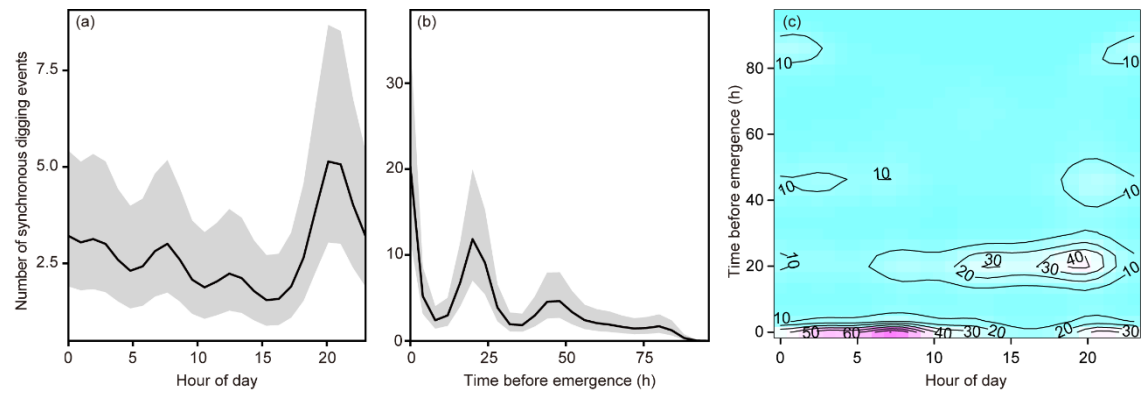
Fig. 2.



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578 Fig. 3.

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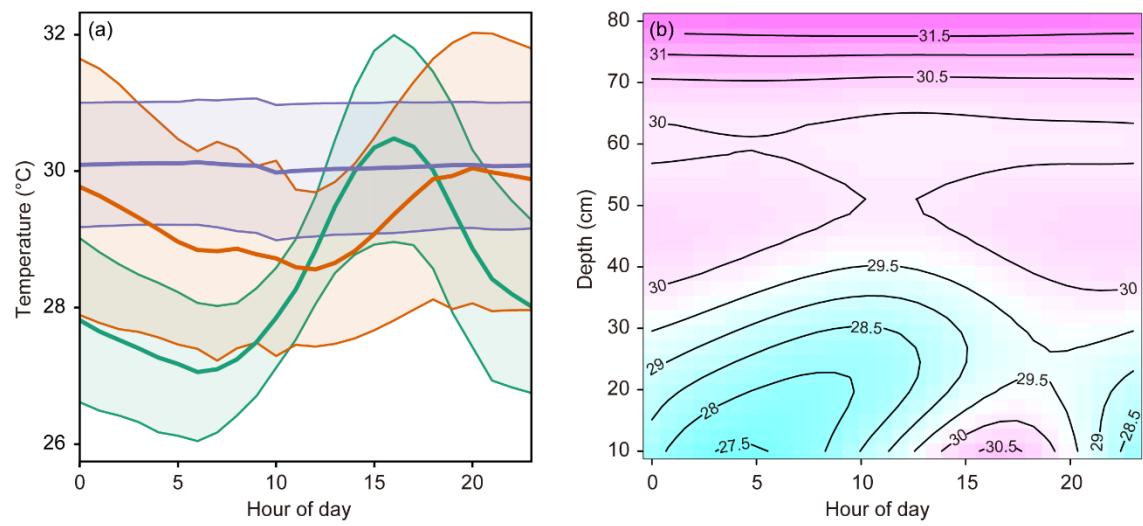
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Fig. 4.

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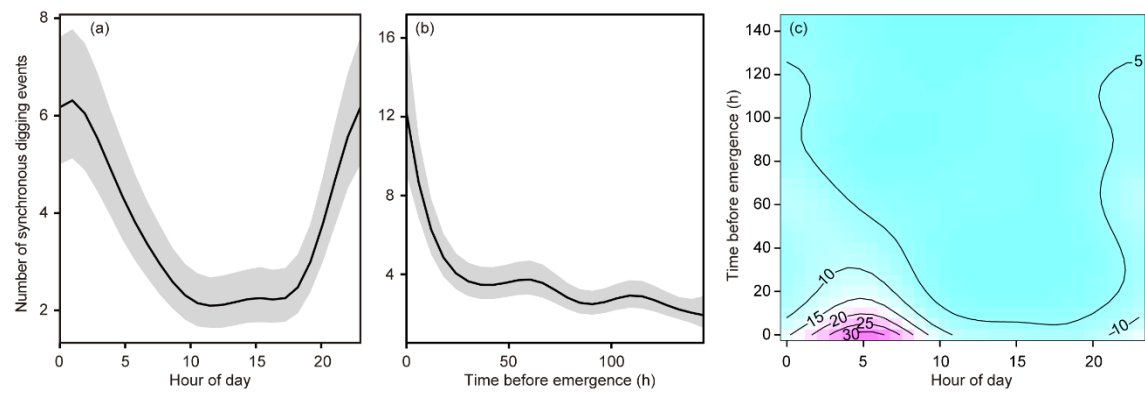
585 Fig. 5.

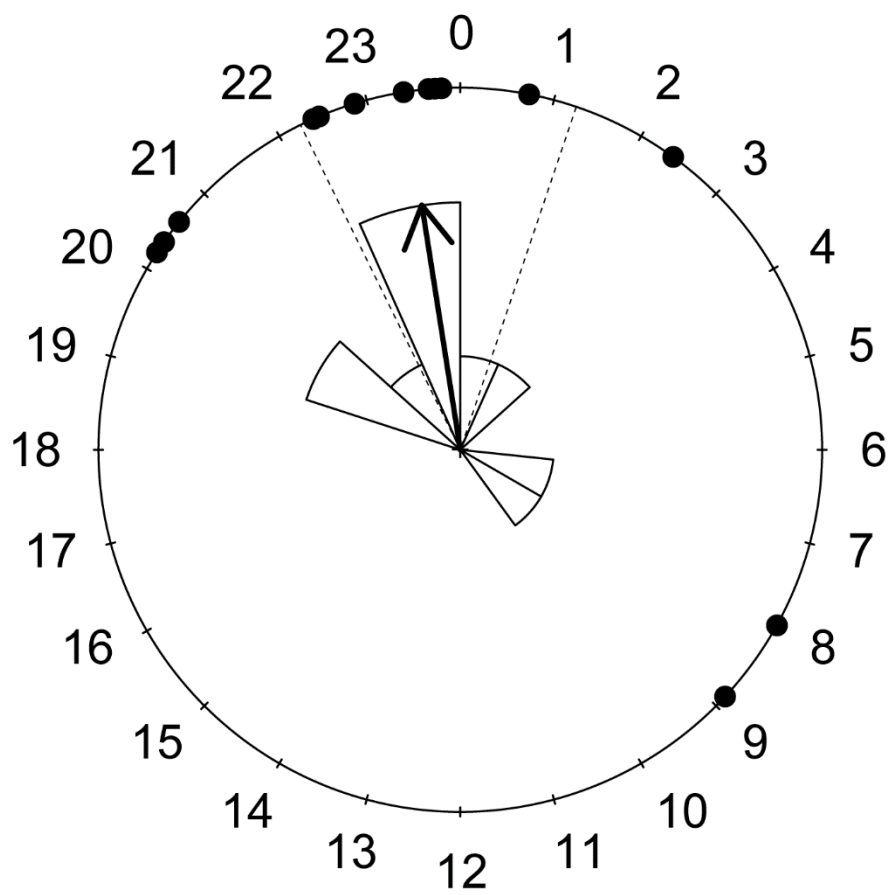
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589 Fig. 6.





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591 Appendix Fig. A1.

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