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RADIOGRAPHIC IMAGING APPLICATION IN CAPTURING NEST ESCAPING MOVEMENT BY SEA TURTLE HATCHLINGS

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Abstract: Nest escaping is a vital process that takes up a significant amount of energy and time during the early life of sea turtle hatchlings. Previous studies have shown how hatchlings benefit from this aggregation behaviour while digging upward in the nested column. However, there is a lack of information on the relationship between the dimensions of nest escaping movement formation. This study explores the potential of radiographic imaging in capturing the dimension of digging formation to relate its potential with the energy conservation mechanism. This article describes the challenges and prospects of capturing hatchlings' movement while digging up and escaping their underground nest via radiographic imaging (X-ray). Several trials have been conducted to seek the most suitable approach to be applied as a standard method for future studies. We developed an open-respirometry chamber with a supply of oxygen for egg incubation according to preferred clutch sizes to meet one of the objectives, which is to determine the effect of clutch sizes on the dimension of digging formation. Exposure usage of 125 kV, 40.0 mAs, and 62.5 ms were determined to produce significant X-ray images. Additionally, iron flux has been determined to be useful in measuring hatchlings' digging progress, as it has the potential to enhance our image quality and observation. We were able to observe the position of the hatchlings with such development. Nevertheless, we do not know the accurate dimension of digging movement formation as modelled by the hatchlings.

Keywords: Sustainability, aggregation behaviour, social facilitation, endangered species, sea turtles.

Introduction

As mentioned by Carr and Hirth (1960), sea turtle hatchlings display group behaviour while digging upward in their effort out from their underground nest, which is recognised as social facilitation. The hatchlings can exhibit this group movement due to synchronous hatching of clutch mates with an average cohort size of about 50 to 150 eggs (Spotila, 2004), resulting in synchronised individual hatchlings' digging activity. The sea turtle eggs deposited in the range of 60 cm to 80 cm (Najwa-Sawawi *et al*., 2021), and hatchlings might take a long time, up to a week depending on its species for nest escape.

The digging activities require them to use their reserved energy within the residual yolk to emerge from the nest. They will apply the remaining energy to swim seawards after the nest escapes and begin their initial swimming upon entering the vast ocean for further energy expenditure. On the other hand, Rusli *et al.* (2016) reported that the larger the cohort size of the sea turtle, the lower the energy usage of individual hatchlings upon nest emergence. This resulted in a reduced metabolic rate per individual and time to escape the nest. Thus, social facilitation among hatchlings does help fasten nest emergence and lower the energetic cost per individual (Rusli & Booth, 2016; Nishizawa *et al.,* 2021). With this mechanism as their group movement, it can increase the survival rate of the hatchlings as their energy is being preserved for other activities they may encounter in the open sea. However, there is yet uncertainty about the hatchlings' digging dimension formation associated with their energetic cost.

Naturally, a Chelonian nest constructed with a flask-shaped and air-filled chamber as a nester typically deposited eggs in several layers (Packard *et al*., 1993). Such conditions could assist synchronous hatching and help shorten the time taken for nest emergence (Dehn, 1990). The sea turtle hatchlings' digging formation is believed to be clustered circularly as seen on cyclist pelotons. However, the formation dimension has yet to be measured before estimating the energy saved by individual hatchlings. Another question will be how they apply for position change or remain in their position in the formation throughout the process.

Developing a model regarding hatchlings' aggregation movement is vital to enhance our understanding of energetic conservation. There are various dimensions of formations exhibited by different groups of animals, such as flocks of birds and schools of fish. Each formation displayed, excluding the benefit of reducing predator risks when moving in-group, has another significant advantage: Increasing their energy-saving mechanism, e.g., most migratory birds move in V-formation (Lissaman & Shollenberger, 1970; Levin, 1997; Weimerskirch *et al*., 2001; Couzin *et al*., 2002). In the early life history strategy of sea turtle hatchlings, it is assumed that this would increase their survival rate due to the high energy conserved before entering the open ocean.

This study describes the challenges and prospects of capturing sea turtle hatchlings' movement while digging upward to escape from the underground nest.

Materials and Methods

Study Animal

Between August and October 2017, 105 green turtle eggs from different clutches were collected from Chagar Hutang Turtle Sanctuary (CHTS) of Pulau Redang to be transferred to Universiti Malaysia Terengganu (UMT) laboratory facilities. The Universiti Malaysia Terengganu Animal Ethics Committee approved all experiments (UMT/JKEPHT/2018/24).

Late incubation egg clutches (> 45 days of incubation) from the *in-situ* nests have been identified according to the field record at CHTS. Consequently, this period has been determined as a safe period for the incubating eggs to be periodically transferred to the mainland by boat (approximately two hours of travelling time) (Rusli *et al.*, 2016). Every single egg has been marked at the top position with a semi-permanent marker pen to maintain embryo orientation throughout transportation. Therefore, an icebox without any cooling agents has been used for transferring, requiring about three hours of journey by boat and car.

Upon arrival, the icebox container (maximum of two clutches) will be opened at room temperature for six hours before eggs continue incubating in the artificial nest column. This artificial incubating medium will serve as a respirometry chamber for trials.

It is challenging to replicate the natural digging process for sensitive species, such as green turtles, in respiratory chambers placed vertically. According to Rusli *et al*. (2016) description, several trials and errors were made to obtain the most suitable method for manipulating sea turtle eggs/hatchlings in respirometry. Hence, all precautions were taken.

Developing Respirometry Chambers

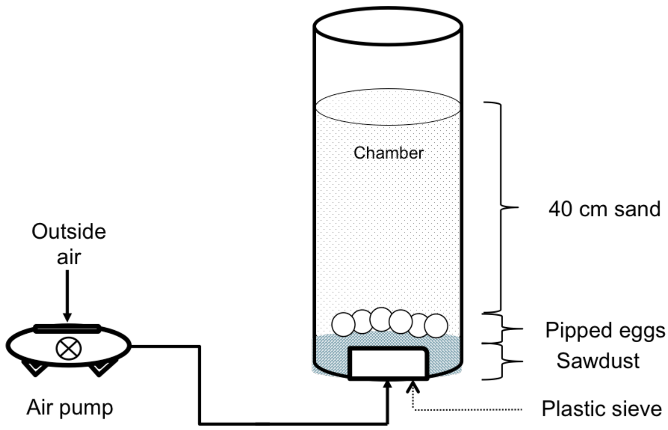


Figure 1: Schematic diagram of an open-flow respirometry chamber modified by Rusli (2016) to suit the current trial's objective

Alternatively, Rusli *et al.* (2016) have developed an open-flow respirometry, assuming that all energy consumed to determine the vertical metabolic rate of digging through a sand column is derived from aerobic metabolism. He had perfected the protocol as the open flow respirometry showed homogenous nest escaping behaviour to hatchlings in their natural nests. Therefore, current trials will be based on Rusli *et al.* (2016) with several modifications according to the suitability of this experiment.

The respirometry chambers were made from lightproof Polyvinyl Chloride (PVC) cylindrical pipe placed vertically (thickness of 0.5 cm; radius of 19.0 cm; and height of 70 cm), having the lower end of the chambers sealed with a lightproof tray. At the outer layer of each chamber, an iron flux line marker was tied around with every 10 cm interval, which assists with a measurement scale. Two holes were drilled at the bottom of each chamber to connect aeration tubes to the aquarium pump. The aquarium pump functions to draw ambient air and channel it into the chambers to provide ambient oxygen to the hatchlings. Inside each chamber, a plastic sieve was placed on the bottom of the area where the two holes separated the tubes from getting in touch with other substances and evenly distributed airflow.

Consequently, a layer of approximately 10 cm of sawdust was placed at the bottom of the chamber on top of a plastic sieve to prevent fluid retention at egg level during egg hatching. On top of the sawdust, 2 cm of sand was placed for the eggs to be put on. The pipping eggs were then buried in the chambers with 40 cm of sand from the top level. The schematic diagram of the respirometry chambers is presented in Figure 1.

As CHTS beach sand has been used throughout this experiment and has been analysed earlier by the dry sieving method and graded as medium sand, the phi (ϕ) value of mean grain size is 1.05 to 1.24 (Folk & Ward, 1957). This study employed mitigation methods to reduce afitation during the transport of the chambers using high-density foam while planning and following a route with smooth roads, maintaining a steady speed, and avoiding sudden manoeuvres while continuously monitoring environmental conditions and vibration levels inside the transport vehicle. Note that all chambers were placed on a hand trolley equipped with high-density foam to ease moving them from the incubation room at 26°C to 28°C to the radiographic imaging room for daily X-ray observations.

Radiography on Hatchlings

The study was conducted at the Health Unit of UMT, which provides X-ray services for both human health and academic research. The facility's X-ray machine is stationary, and all X-ray sessions were overseen and guided by the university's health unit radiologist. Throughout the nest escaping process by the hatchlings, the chambers will have pictures taken using the X-ray technique to observe the dimension formation of the digging activity.

Several trials had to be done on dummies beforehand to determine the most suitable exposure for the X-ray being radiated. The properties of the X-ray will be chosen with guidance from the radiographer. Correspondingly, the precautions taken during the X-ray process are for researchers not to be in the same room as the subject upon the X-ray radiation being emitted. The room is covered with a layer of barium plaster with a thickness of one centimetre and made of concrete to prevent leakages. Other than that, it can stop the X-ray radiation from further penetration, which acts as a shield while one of the chambers is undergoing the X-ray process.

Several trials were done using hatchling dummies to determine the appropriate exposure factors that can be used. These dummies were buried according to their escaping position in the nest with cluster formation. This practice has been done to reduce radiation risk towards the live hatchlings. Furthermore, it has been assumed that hatchlings will receive less radiation as the X-ray radiation will be absorbed about 50% by the sand medium in the chambers (Knoll, 2010).

In addition to chamber thickness, the distance from the X-ray source to the chamber was 1.21 m. Normally, in the standard radiographic procedure, the standard distance from the X-ray source to the object is 1.0 m, which is used to preserve the geometric image sharpness and optimum radiation dose. However, the distances must be increased to cover all the tube samples included in single images.

Data Analysis

To determine the digging progress of hatchlings, the X-ray images taken will be analysed by comparing the distances and saturation density between hatchlings daily using Adobe Photoshop and Matlab software. Upon comparing the distances, we would see the possible differences made by the hatchlings, which means they might be moving. Meanwhile, for saturation density, we could differentiate the possible whereabouts of hatchlings in the chambers based on the images by comparing the saturation density of hatchlings, sands, and air.

Results and Discussion

Preliminary System

Figure 2: An image of an X-ray of exposure 102 kV, 50.0 mAs, and 63.7 ms shows turtle hatchling dummies inside a trial chamber

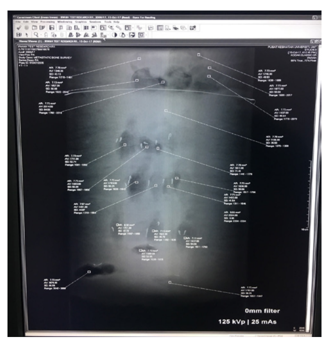


Figure 3: An X-ray image shows sea turtle hatchling dummies inside the chamber and eggs on the upper part with labels of the image's properties

The pipped eggs were brought to the X-ray theatre using the chamber constructed as in Figure 1. The eggs could be seen in X-ray images after three preliminary tests to find suitable exposure by the radiographer. However, the movements of hatchlings could not be detected by X-ray due to several possible flaws the chamber had at the experiment's beginning. Several flaws are associated, such as space constraints, where the rigidity and fixed dimensions of PVC chambers might restrict the natural movement of hatchlings and lead to reduced activity that is less detectable by the X-ray.

Besides that, X-ray imaging challenges like depth penetration and resolution limitations would struggle to differentiate small movements of hatchlings within the dense environment of the artificial chamber, mainly due to the attenuation properties of the material. Nevertheless, the hatchlings emerged from the chamber early in the morning a few days later, even though no movement was captured during the X-ray.

Figure 2 illustrates an X-ray image on film with exposure of 102 kV, 50.0 mAs, and 63.7 ms. This X-ray image showed dummies' hatchlings in the sand column, which is done to assess the ability of X-ray to detect objects buried in a chamber filled with sand.

Figure 3 depicts the image of an X-ray film exposed with X-ray properties of 125 kV, 40.0 mAs, and 62.5 ms. The exposure did not provide a satisfactory X-ray image of the dummies or eggs within the sand column. There is no clear observation of their orientation, so the iron flux attached to each carapace was added to the development. This is due to the ability of iron flux to be seen clearly on the X-ray film, which made it easy to determine their position within the chamber. Note that turtle eggs on the upper part of the chamber were placed to determine their ranges of saturation density and sand turtle hatchlings (Table 1) to help guide further experiments.

Table 1: Ranges of observed saturation density of sand, eggs, and hatchlings inside the chamber

Subject	Ranges of Saturation Density
Sand	1070 – 1309 (middle) 2334 – 2334 (side)
Eggs	1219 – 1461 * 1622 – 2079 ** 1782 – 2218 (overlap)
Hatchlings	1311 – 1858 **(vertically oriented) 3542 – 3888 (horizontally oriented)

* It is due to the presence of air/gas.

** The saturation density ranges from the lowest to the highest amounts from all the samples presented.

Final System

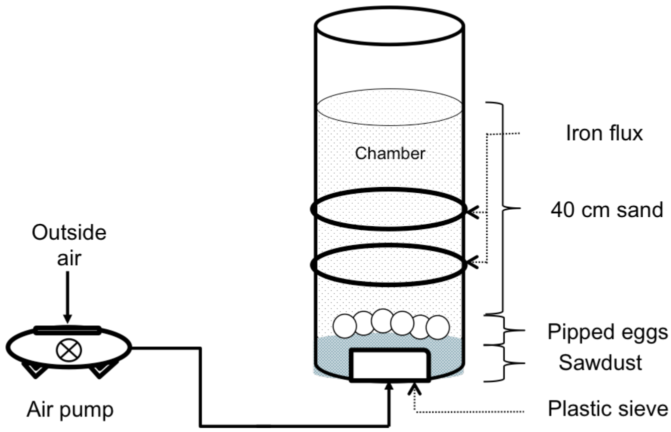


Figure 4: A diagram of an open-flow respirometry chamber (final system)

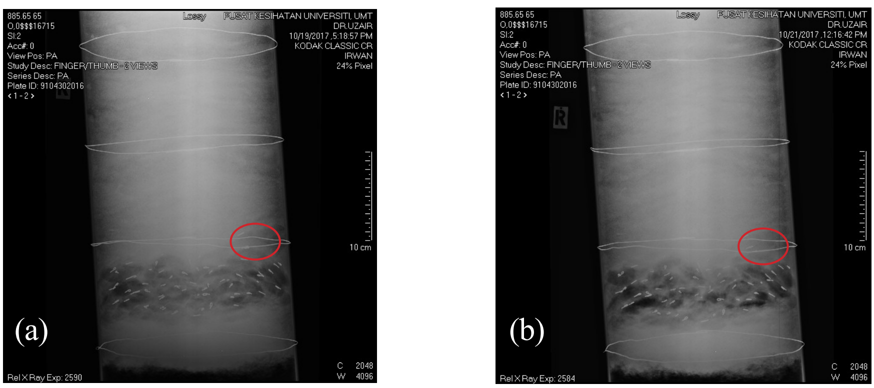
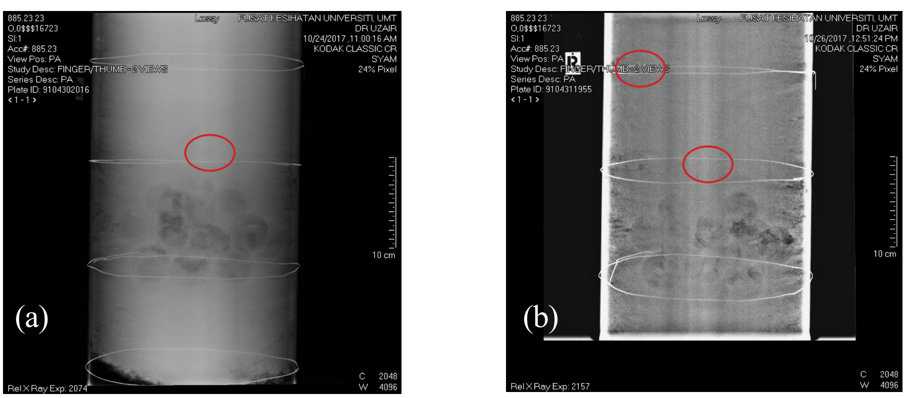


Figure 5: Images show a chamber comprising 65 hatchlings reburied into the chamber of the same view: (a) Image dated 19th October 2017 and (b) Image dated 21st October 2017. Red circles indicated a possible slight movement



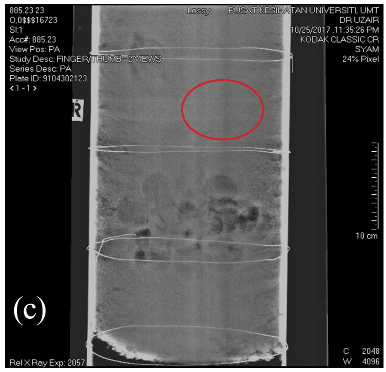


Figure 6: Images show a chamber consisting of 23 eggs of the same view: (a) Image dated 24th October 2017, (b) image dated 25th October 2017, and (c) image dated 26th October 2017. Red circles indicate the presence of the hatchling and how it moves inside (upwards; towards the surface) the chamber upon hatching

According to Smith and Taylor (2009) and Knoll (2010), the radiographer concluded this study that the most suitable exposure for the hatchlings and eggs was 125 kV, 40.0 mAs, and 62.5 ms. Note that 65 hatchlings were reburied into the chamber in stack form like the eggs were stacked in nature, with iron flux attached to each carapace using medical tape. The X-ray image's saturation density was adjusted on the spot to observe the presence of the eggs and possible movement from the hatchlings inside the chamber as clearly as possible. Small movements were detected in Figure 5. A minor difference in distances might be exhibited by several hatchlings upon X-ray of the chamber twice daily: Morning (1100 hours) and evening (1700 hours). A few days later, the hatchlings' position remained the same, and they had an unpleasant smell, indicating that the hatchlings had ceased.

Three chambers were constructed with the same properties as the trial in Figure 2 but continued using eggs with different clutch sizes of 6, 11, and 23. The X-ray image's saturation density was adjusted on the spot, similar to the previous step. The chambers were taken for X-rays twice daily at similar timing preferences as doing X-rays for hatchlings. Based on Figure 6, a few possible small movements were detected upon X-ray of the chambers of 23 eggs exhibited by the hatchlings, which were indicated by differences in their positions of each X-ray image by comparing the distances with previous images.

However, there were only a few hatchlings whose possible movement could be detected. Upon the first detection, the chambers were shot for X-ray in two views as the chambers are three-dimensional and consist of sand. Perhaps the X-rays are unable to emit through the thickness of the sand. Nonetheless, images of chambers with eggs 6 and 11 were not provided as they did not show a significant difference in terms of movement. Most of the hatchlings possibly emerged from the chamber early in the morning, between 0600 and 0800 hours. This ended the experiment due to different timing preferences regarding when the chambers would be X-rayed.

Figure 2 illustrates an image of hatchling dummies in the chamber upon being X-rayed during the first trial of the preliminary system. As it is being exposed to such an amount mentioned before, the image quality shown could have been better for us to observe hatchlings due to the low saturation density presented. It is determined by the amount of grain scales of the image presented on the X-ray film. Such a phenomenon occurred due to the penetration of the X-ray itself. A higher amount of electrons penetrated through a subject's body and reached the X-ray detector in a given time, and we could observe more darkness on the X-ray film (Smith & Taylor, 2009; Knoll, 2010).

Meanwhile, the higher X-ray attenuation through a subject's body indicates that the subject's body absorbs the X-ray emitted. Thus, it will produce more whiteness in the images. Furthermore, in the second trial of the preliminary system (Figure 3), we used a different amount of exposure. The exposure used for this experiment had to be increased to improve the penetration of the sand, preserve the image's sharpness, and reduce the radiation dose.

Based on Figure 4, the chamber was constructed with an additional 10 cm mark apart. Iron flux was tied around the outer part of the chamber as a new development to the trial. The iron fluxes tied around the chambers were used as a distance indicator for the digging progress. This is due to the ability of iron flux to be seen clearly on the X-ray film, which made it easy for us to determine the possible distance travelled by the hatchlings inside the chamber. This higher exposure [within the ranges stated in Smith and Taylor (2009) and Knoll (2010)] aimed to improve the image quality of the hatchling dummies within the chamber, and the attached iron flux on each dummy's carapace was used to observe their orientation. Comparing two images from each trial shows that higher X-ray exposure produces a better image quality than lower exposure. Thus, chambers further developed into the final system will use the same exposure as trial 2.

Figure 3 also depicts the labels of each item presented in the film. The labels indicated their amount of saturation density, which is presented in Table 1. The presence of the values enables us to determine the range of saturation density, which will be referred to in the future. Two saturation densities of sand were detected upon X-ray, in which the middle indicated the middle part of the chamber and the side indicated the side of the chamber.

Sand in the middle part has a higher saturation density. It might be harder for the X-ray radiation to penetrate through the sand as the chamber is cylindrical compared to the side part of the chamber, which has a low saturation density (Smith & Taylor, 2009). For the eggs' saturation density, the value of saturation density that detected air/gas in the egg has the lowest range compared to others. This has helped us differentiate the contents of the eggs. In addition, the normal range of saturation density for the eggs is 1,622 to 2,079. However, the value of 1,782 to 2,218 is the highest saturation density due to the possibly overlapping position of the eggs.

Thus, a darker image might be produced in certain overlapping areas. This could also occur because some eggs are positioned closer to the X-ray source than others inside the chamber. Due to the higher exposure (higher in kV and lower in mAs) used for the second trial chamber, along with the suitable distance between the subject and the X-ray source (1.21 m), we were able to observe a better marginal outline of the image and clearly see the overlapping positions of the eggs. When the subject is closer to the source, with sufficient kV and mAs, the image produced will have a good marginal outline. The X-ray radiation had better penetration than subjects positioned further away from the source inside the chamber.

Additionally, the X-ray machine blasted the beam from one side only and not at a 360° angle, which might have contributed to variations in image clarity and darkness across different chamber areas (Baker, 2010). Hence, the positions of the eggs play a considerable role in determining the range of their saturation density. The ability of X-ray radiation to penetrate each egg differs depending on their proximity to the source during exposure.

As for turtle hatchling's saturation density, most hatchlings positioned horizontally inside the chamber have values ranging from 3542 to 3888. In contrast, those vertically had lower saturation density (Table 1). These are because they were not at the same distance from the chamber wall. Nevertheless, a possible reason the hatchlings in a horizontal position within the chamber showed the highest saturation density in the image is that the hatchling itself might have low body density. The hatchling has low body density due to a sea turtle's body mainly consisting of soft tissues as their body parts.

Meanwhile, the green turtle hatchlings' carapace or shell has a high density due to being made from calcium. The carapace itself might exhibit a high saturation density value due to the calcium's properties by absorbing the electrons emitted by the X-ray. Theoretically, calcium would absorb the X-ray and exhibit more whiteness on the X-ray film (Harrington, 2004). Meanwhile, lowering an object's density due to soft tissues will let the X-ray pass through it and exhibit more darkness on the X-ray's film, indicating a higher saturation density of the image.

The contrast of an image is determined by the amount of kV used upon it. The higher kV increases its electrons' speed for better image quality. Note that higher speed will result in more direct darkness on the X-ray film, indicating a good penetration between the X-ray emitted and a subject's body (Kei Ma *et al.*, 2014). In addition, it also exhibits a good marginal of the image upon seeing the images. Correspondingly, decreasing the quantity of its electrons from 50.0 mAs to 40.0 mAs helps lower the dose. Due to the subject being exposed to higher kV, the amount of mAs needs to be lowered because higher mAs will also cause higher image darkness. This will affect the subject's contrast and generate the image of the film produced not to have a good marginal.

In addition, applying the Inverse Square Law in radiography (Shafiei *et al.*, 2012; Rehani, 2013) explained that the intensity of a radiation source is inversely proportional to the square of the distance, which plays another vital role in determining a good marginal upon the image produced on the X-ray film. Hence, the further the distance of the subject's body from the X-ray source, the lower the darkness portrayed in the film, resulting in a low marginal image. Such an event could occur due to other properties such as kV, mAs, and ms (duration of X-ray emitted from the source) not reaching the subject's body for penetration and exhibit on the X-ray film.

For the final trial, live turtle hatchlings and eggs were used. Based on Figure 5, the chamber was developed according to Figure 4. Unfortunately, it resulted in the hatchlings' death as we reburied them into the chamber by attaching a small iron flux piece. Hatchling mortalities may arise due to the substantial pressure exerted by densely packed sand. When the sand in the chamber is tightly compacted, it forms a substantial obstacle for the hatchlings, impeding their ability to crawl and reach the surface. This sand compacting factor diminishes the amount of space surrounding the hatchlings, confining them and impeding their mobility.

Moreover, studies have demonstrated that the sand compaction level substantially affects the likelihood of hatchling emergence (Miller *et al.*, 2003). Excessive sand compaction can result from multiple sources, including intense precipitation, human presence on the beach, or natural sedimentation processes (Ackerman, 1997).

Thus, it is vital to ensure that nesting sites have suitably porous and well-ventilated sand to facilitate the successful emergence of sea turtle hatchlings. This possibility could be assumed since there were no significant changes after a few days in their position and unpleasant smells. Therefore, we decided to end the trial by reburying the new hatchlings.

Since the previous development caused the hatchlings to die, we furthered our study by excluding the attachment of iron flux on hatchlings and carrying on with burying the eggs in a manner similar to that of the natural nest. Figure 6 illustrates the X-ray images of 23 eggs despite having two other chambers with different clutch sizes (6 and 11 eggs). This was the most significant event between the three chambers. The other chambers could not portray such events due to the absence of iron flux on their carapace and their positions, which perhaps made it harder for the X-ray radiation to penetrate and be observed on the X-ray image.

Meanwhile, even with the unavailability of the iron flux, which might hinder our observations of the images produced, the 23 eggs' chamber captured several possible movements by the hatchlings (Figure 6). This is because the hatchlings' position was perhaps nearer to the source, enabling the radiation emitted to penetrate them and be shown on the film. Hence, with this development, we assume that the possible causes of our inability to see the possible movements exhibited by the hatchlings are (i) there was a lack of marker to indicate their position upon hatching within the artificial chamber and (ii) their positions within the chamber from the source.

The condition of sand within the chamber will experience moisture due to surrounding factors such as temperature and humidity. The incubation period of eggs within the chambers will take approximately five to seven days. However, within several hours of incubation, sand can retain moisture. Thus, a gradient of moisture occurs from bottom to top. Due to the moisture gradient, the dry sand on top can exhibit higher pressure on the hatchlings until it creates no ambient space for them to move or dig upward.

The dry sand can presumably dehydrate the hatchlings, which might result in their death. This event is crucial in altering the sand's moisture and may negatively impact the hatchling's movement. Therefore, it is vital to retain the moisture of the sand to maintain its shape of the sand and not cause any severe complications in the hatchling's movement while escaping the nest.

Conclusions

The ability of the X-ray imaging technique to penetrate through a thick and opaque object has helped us observe the possible formation of the hatchlings inside the artificial nest known as the open-flow respirometry chamber. This method can be used as proven by this experiment, in which we observed the hatchlings, which ultimately emerged from the chambers throughout the experiments. In addition, the absorption of radiation emitted by the source would be halved as the radiation penetrates through the subject's body due to the thickness of the material that made the chamber and the sand medium that filled the chamber. Hence, the subject's body, the hatchlings in this experiment, appeared healthy by the end of each trial using the X-ray method to study the development of the nest escaping movement formation in sea turtle hatchlings.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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