WHAT MECHANISMS UNDERLIE SYNCHRONOUS HATCHING IN LOGGERHEAD TURTLE NESTS?

by

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A Thesis Submitted to the Faculty of

Charles E. Schmidt College of Science

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, FL

May 2020

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Michael Salmon, Department of Biological Sciences, and has been approved by all members of the supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my committee members for all of their guidance and support, and a special thanks to my advisor for his persistence, patience, and encouragement throughout this project and writing of this manuscript. I am also grateful to the Save the Sea Turtle Foundation which generously contributed funding for this project. Additionally, a special thanks to Gumbo Limbo Nature Center's Sea Turtle Specialist Team which provided locations of the sea turtle nests. Last but not least, I want to thank my lab mates for all of their continued help and support over the years. All research in this study was done under FWC Permit # MTP-19-173A and IACUC Protocol #A19-21.

ABSTRACT

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Degree:	Master of Science
Year:	2020

The goal of this study was to determine if hatching synchrony occurs in loggerhead sea turtle nests and if it does, what mechanism(s) promote that synchrony. Synchrony may occur because oviposition takes place during a single evening, and because incubation temperatures within the nest show relatively little variation; thus, rates of embryonic development among the eggs are similar ("temporal synchrony hypothesis"). Alternatively, synchrony might be enhanced through embryo-to-embryo communication that stimulates and synchronizes development ("coordinated hatching hypothesis"). Experiments were designed to distinguish between these two hypotheses. I found that if only a few embryos survive, temporal synchrony occurs. However, if many embryos survive, the duration of incubation and hatching shortens, presumably because embryonic movements inside soft-shelled eggs are detected by and transmitted between eggs and stimulate development, expediting hatching synchrony.

DEDICATION

This manuscript is dedicated to my family, particularly my parents, Gilda and Scott, who have always supported me and encouraged me throughout my entire life and academic career. Thank you for believing in me and being wonderful parents.

I also dedicate this work to my best friends who I consider to be my family. Thank you for being there all these years and supporting my dreams.

Finally, Alexander. Thank you for your continued care and reassurance especially when I need it most.

WHAT MECHANISMS UNDERLIE SNYCHRONOUS HATCHING IN LOGGERHEAD TURTLE NESTS?

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INTRODUCTION

Synchronous behavior occurs when two or more individuals exhibit the same activities at the same location and time as other members of their species (Duranton and Gaunet, 2016). This behavioral adaptation is seen across a variety of marine animals. Temporal synchrony, for example, occurs in hard or stony corals. These sessile invertebrates are restricted to one location for their entire life, so they must coordinate reproduction (and external fertilization) through mass spawning events linked to seasonal changes (Keith et al., 2016; Mercier, 2010). This adaptation where thousands of gametes are dispersed into the water column allows for predator satiation, higher fertilization success, and wider dispersal (Oliver and Babcock, 1992; Levitan et al., 2004). Another example of synchronous behavior, activity synchrony, can be seen in schooling fish that use the movement cues of their neighbors to move together in a coordinated fashion (Pitcher, 2001). This behavior has evolved for foraging and predator avoidance, both increasing likelihood of survival (Pitcher, 2001). Overall, species that utilize synchronous behaviors receive significant benefits.

In marine turtles, synchrony has been well documented in the aggregated nesting of Olive Ridley (*Lepdochelys olivacea*) turtles in the Indo-Pacific and western Atlantic beaches. In this case, thousands of female turtles nest across a short interval of 2-4 days, resulting in a mass synchronized hatchling emergence after the completion of incubation (Bernado and Plotkin, 2007). Synchronized emergence is seen on a smaller numerical

scale within individual sea turtle nests; large groups of hatchlings from a single nest will exit the nest together. This results in reduction of both the amount of energy expended by each individual hatchling when digging out of the nest and the loss of hatchlings to predators during the crawl to the sea (Rusli et al., 2016; Santos et al., 2016; Erb and Wyneken, 2019). Hatching synchrony within the nest is assumed to precede emergence synchrony. However, synchronous hatching in marine turtle nests is poorly studied and rarely quantified as most previous studies have primarily examined emergence success, not embryo-to-embryo interactions (Broderick et al., 2001; Lolavar and Wyneken, 2017; Miller, 1982).

In both freshwater and marine turtles, there are temperature gradients within the nest and those gradients affect rates of development. Warmer conditions increase metabolic rates that in turn accelerate development (Miller, 1982). Freshwater turtles deposit their relatively small clutches (2-20 eggs; e.g., *Emydura macquarii*: 15 – 20 eggs, Chessman, 1978; *Chrysemys picta*: 2 - 10 eggs; Martof et al., 1980; *Carettochelys insulpta*: 3 – 12 eggs, Webb et al., 1986) in shallow egg chambers, dug only a few centimeters below the substrate surface. As a result, solar radiation affects those eggs closer to the surface, resulting in a temperature gradient of up to 6° C causing eggs in the same nest to vary in their developmental stage (Chessman, 1978; Thompson, 1988). In contrast sea turtles, which as adults are considerably larger than most freshwater turtles, dig deeper nests that are shielded from the effects of solar radiation (Tomillo et al. 2017). In addition, clutch sizes are much larger (typically 50 - 150 eggs, depending upon species). Temperature gradients do occur within the nest as a consequence of the metabolic activity of the developing embryos, with the result that there can be as much as

a 3° C difference in temperature between eggs located in the center of the egg chamber and those located at the periphery (Broderick et al., 2001).

Given that temperature gradients occur in all turtle nests, one expects that eggs should not develop at the same rate and hatching should be asynchronous. However, studies done on freshwater turtles show that hatching time can be altered by external stimuli that affect the nest toward the end of incubation. For example, premature or delayed hatching occurs in response to external cues such as predator-induced displacement of the eggs or changes in the environment that result in hypoxia, dehydration, or flooding of the nest (Webb et al., 1986; Doody et al., 2001). Premature hatching has only been observed in embryos that are developmentally mature and are in the final stages of incubation, and has been documented in *E. macquarii* (Murray River turtles; Spencer et al., 2001; Spencer et al., 2011) and *C. picta* (North American painted turtles; Colbert et al., 2010). A study done with *E. macquarii* as subjects found that when in contact with one another, embryos within unhatched eggs can alter their metabolic rate and "catch up" to their developmentally more advanced siblings (Spencer and Janzen, 2011; McGlashan et al., 2012). The mechanism involved in initiating this response in pig-nosed turtles (*C. insculpta*) appears to be vibrations associated with hatching that stimulate embryos in nearby eggs to begin hatching (Doody et al., 2001). Additionally, groups of eggs in contact with one another hatch faster than single eggs (Doody et al., 2001; McGlashan et al., 2015). These findings suggest that some form of communication by mechanical cues occurs between the embryos that promotes hatching synchrony.

Bustard (1972) examined the effects of simulating mechanical stimulation by pressing down on loggerhead turtle (*Caretta caretta*) embryos' eggshell during the last

few days on incubation. Embryos that experienced mechanical stimulation showed a similar “catch up” in development (Bustard, 1972). These findings suggest that similar mechanisms of embryo-to-embryo communication might occur within all turtle nests resulting in altered and synchronized developmental rates for the embryos in contact with one another.

Mechanical stimulation might not be the only means of embryo-to-embryo communication. In several recent studies, sounds have been recorded inside nests prior to hatching in four species of marine turtles (leatherbacks, *Dermochelys coriacea*; Ferrara et al., 2014; green turtles, *Chelonia mydas*; McKenna et al., 2019; Olive ridley, *Lepidochelys olivacea*; Ferrara et al., 2019, McKenna et al., 2019; hawksbill, *Eretmochelys imbricata*; Monteiro et al., 2019). The significance of these emissions, often characterized as "vocalizations", remains unknown but in two studies, the investigators (Ferrara et al., 2019; Monteiro et al., 2019) have proposed that the sounds could be used to synchronize hatching. However, that hypothesis was not supported by any experimental evidence and others (McKenna et al 2019) have proposed that the "vocalizations" might be a “byproduct” of the effort involved in the hatching process.

The purpose of my study was to determine whether synchronous hatching occurs in loggerhead (*Caretta caretta*) turtle nests, and if so what mechanism(s) is (are) involved. To find out, I designed experiments to determine whether (i) egg-to-egg contact was required, whether (ii) rates of embryonic development were affected by the number of viable embryos in the nest, and whether (iii) eggs that were in contact with one another, but differed in developmental stage and thus amounts of external stimulation when hatching, show differences in hatching synchrony. I hypothesized that (i) contact is

required for synchronized hatching to occur since external stimulation may be necessary, (ii) that the rates of embryonic development would be accelerated with more viable embryos present in a nest due to more egg to egg external stimulation, and (iii) that embryos in contact with others that were behind in development would show longer incubation and hatch less synchronously because they would be exposed to less external stimulation near their time of hatching.

METHODS

Egg Collection, Incubation Conditions, and Measurements

I collected loggerhead eggs from 50 nests within 12 h of deposition at Boca Raton, Florida, U.S.A. (26.37° N, 80.13° W) between June and August, 2019. Within 2 h, eggs were labeled using a #2 pencil and positioned appropriately for each experiment in a styrofoam nest box of different dimensions (see below) containing sterilized (autoclaved) beach sand. Each nest box was stored inside a large "crawl-in" incubator where air temperature (31 ± 1.5° C) and volumetric water moisture (0.06 ± 0.02 m^3/m^3) were controlled. All nest boxes were equipped with temperature data loggers (HOBO U22-001; accuracy ± 0.21° C; Onset Computer Corp, Bourne, Massachusetts) that measured sand temperature every 15 minutes throughout incubation. Eggs were sprayed with a mist of de-ionized water twice daily to ensure viability. I used Soil Moisture Smart Sensors (SMC-M005; accuracy ± 0.031 m^3/m^3; Onset Computer Corp) to measure the sand moisture every 15 min to ensure that sand moisture content remained within 4-8%.

Eggs were inspected twice a day (~12 h apart) for signs of hatching. "Pipping," occurs when a fully developed embryo pierces the top of its eggshell. This was considered the first sign of hatching in all my experiments since it more accurately determines the completion of incubation than hatching time (Gutzke et al., 1984). A neonate was considered to have completed the hatching process once it fully escaped from the confines of its egg. I measured *incubation duration* for each egg in days

between the initial lay date until the first sign of pipping. I measured *hatching synchrony* either in h or days between the first embryo to pip and the last embryo to pip within a nest box or treatment group. I measured *hatching duration* in h as the time between the first embryo to pip and the last embryo to completely exit its eggshell in a treatment or group.

Experiment 1: Isolated vs. Grouped Incubation

The focus of this experiment was to examine the coordinating effects of physical contact between eggs during incubation by creating 2 treatment groups, isolated and grouped eggs. Ten eggs from 10 different nests were collected, with each clutch placed in its own styrofoam nest box (91 x 45 x 20 cm deep, inside dimensions) filled with ~ 6 cm of moist sterilized beach sand. One egg was placed in each corner of the box, separated by cardboard dividers from each other; the remaining 6 eggs were clustered in the center (Fig. 1). All eggs were covered with ~ 2 cm of sand until day 30 of incubation, approximately two weeks before hatching might occur (at a constant temperature of 31 $\pm$ 1.5° C). A small area on the top of each egg was exposed by gently removing the sand so that the onset of pipping could be observed. Nest boxes were thereafter inspected twice daily (in the morning and evening).

In this experiment, I measured incubation duration and hatching synchrony. Incubation duration was averaged for each nest box treatment, resulting in 10 mean values for the isolated embryos and 10 mean values for the grouped embryos. The hours of hatching synchrony were totaled starting from the first embryo to pip to the last embryo to pip in each treatment for each nest box. This resulted in 10 values for isolated

embryos and 10 values for grouped embryos. Comparisons between the treatment groups were done using two-factor MANOVA with 1 df (Zar 1999). The dependent variables compared in the analysis were incubation duration and hatching synchrony compared overall among all the embryos within the isolated and grouped treatments. Significance was set at $p \leq 0.05$.

Experiment 2: Vertical Nest Box Simulation

For this experiment, the focus was to examine embryo to embryo interactions in a nest-like simulation. Replicates consisted of 20 eggs from 10 different nests (total of 200 eggs), placed in a vertical oriented observation nest box (90 cm tall x 60 cm wide x 30 cm deep), fitted with a thin glass front (16 mm thick; Fig. 2). Each side of the nest box was equipped with 1 cm diameter holes to promote gas exchange during incubation. Eggs were clustered together in 4 vertical rows (4, 6, 6, 4 eggs/row) in contact with one another and with the glass ensuring all would be visible during hatching. Temperature data loggers (HOBO U22-001) were placed at the top, center, and bottom of the egg mass to record temperature changes throughout incubation and hatching (Fig. 2). One week before an anticipated pipping date, contact microphones (Luvay Piezo pickup; frequency response 20 Hz - 20 kHz) were fixed to the glass using double-stick tape and positioned adjacent to the center of the egg mass. Each microphone was connected to TASCAM recorder (DR-40X, Teac corp., Upper Saddle River, New Jersey U.S.A.), set to record sounds continuously during the last 6 days before and the first 3 days after hatching. Sound recordings were analyzed using acoustic analysis software (Raven Pro, Interactive Sound Analysis Software; Cornell Lab of Ornithology, Ithaca, New York, U.S.A.).

Additionally, infrared-sensitive game cameras (Stealth Cam™ G42 No-Glo Trail Game Cameras, model #STC-G42NG, Grand Prairie, Texas, U.S.A.) were placed ~ 30 cm in front of each nest box and programmed to take a photo every 15 s (using IR flash photography). Images were used to determine the first sign of pipping and to record when hatching was completed. I used Mann-Whitney U tests (Siegel and Castellan 1988) to statistically compare incubation and hatching durations among the replicates. Nonparametric tests were used because sample sizes were small and distributions could not be assumed to be normal (Siegal and Castellan 1988). Average daily temperatures for the last 3 days of incubation and the first 4 days of hatching were compared using two-tailed t-tests (Zar 1999).

Experiment 3: Asynchronized Development: Separating correlated factors (temperature vs. mechanical stimulation)

To distinguish between the temporal synchrony hypothesis occurring as a result of consistent incubation temperatures and coordinated hatching using embryo-to-embryo communication, this final experiment was designed to minimize temperature differences but allow for differences in mechanical stimulation. Twelve eggs from a single nest were divided into two clusters of 6 eggs each and buried in separate nest boxes (30 x 25 x 15 cm tall) within an 8 cm thick layer of autoclaved beach sand (Fig. 3). Each pair of nest boxes was placed in a crawl-in incubator where temperature was controlled at 31 $\pm$ 1.5° C. There were ten replicates, with sand temperatures recorded for 3 pairs of replicates to verify that temperature fluctuations between boxes did not differ during incubation. After two weeks of incubation, 6 eggs from another nest, deposited two weeks previously and separately incubated under identical conditions, were added to one (experimental) nest

box while 6 eggs from a third nest, deposited the previous evening, were added to the other (control) nest box. To distinguish between the nest groups, I labeled the original 12 focal eggs A_1 (6 experimental) and A_2 (6 control) eggs, the 6 eggs added to the experimental box as "B" eggs, and the 6 eggs added to the control nest box as "C" eggs (Fig. 3). On day 30 of incubation, I brushed sand away from the top of the eggs so I could determine when pipping was initiated.

I anticipated that because the A_1 eggs were paired with another clutch at the same stage of development, those eggs would also receive more mechanical stimulation than the A_2 eggs, paired with a clutch two weeks behind in development. Thus, if mechanical stimulation enhanced rates of development, the A_1 eggs should hatch sooner than the A_2 eggs. To determine if this happened, I compared the duration of incubation between the two groups of A eggs, using a paired one-tailed t-test (Zar 1999).

I measured sand temperatures in three experimental and three control nest boxes, then averaged them for each week of incubation. I used an ANOVA to determine if there were significant differences in weekly average temperatures between those two groups.

RESULTS

Experiment 1: Isolated vs. Grouped Incubation

The duration of incubation in the isolated eggs (mean ± sd) was 47 ± 1.23 d, with a range of 45.25 – 49.0 days while the duration of incubation in the grouped eggs was 46.72 ± 1.03 d with a range of 45.5 - 49 days. Incubation duration did not differ significantly between the isolated and grouped eggs (Table 1). Hatching synchrony of the isolated eggs was 30.78 ± 16.01 h, with a range of 12 – 60 h while the duration of incubation in the grouped eggs was 31.2 ± 15.44 h with a range of 12 – 52 hours. There were also no significant differences in hatching synchrony between those treatment groups (Table 1).

Experiment 2: Nest simulation experiment

The number of eggs completing development varied between nests, averaging 11.3 out of 20 eggs, or 57 % survival. In three of the nests only 1, 2 and 4 eggs hatched whereas in the 7 remaining nests 10 - 18 eggs hatched (Table 2). I designated the former as the low and the latter as the high hatch group. In the low hatch group, the duration of incubation in days was 48 d (in next box 1, where only one egg hatched) and a mean of 46.6 d in the nest box where 2 - 4 eggs hatched (Table 2). Among the high hatch group, the mean ranged between 42.8 – 45.1 d. Incubation duration on average was significantly shorter in the high hatch group (Mann-Whitney U test, $U = 0.0$, $p = 0.02$). Hatching

duration for the low hatch group ranged between 88 – 121 h with a mean of 101.7 h. Hatching duration in the high hatch group ranged between 58 – 81 h with a mean of 68.6 h. Hatching duration was significantly shorter in the high compared to the low hatch group (Mann-Whitney U test, $U = 0.0$, $p = 0.02$).

Incubation temperatures within the vertical nest boxes were measured in 3 different locations (top, center, bottom). However, there were no significant differences among the 3 locations within any of the nest boxes (Appendix 1). Therefore, temperature comparisons between the low and high hatch success nest boxes were based upon the data from the temperature loggers placed in the center of the egg mass and restricted to the 3 day period before and 4 day period after hatching (Fig. 4). During the first 4 days of records, there were no significant difference in temperature between the low and high hatch success nests ($p = 0.37, 0.64, 0.99, 0.49$). However, the high hatch success nests were significantly warmer during days 2 through 4 post-hatching ($t = -4.07, -2.40, -3.33$; $p = 0.004, 0.04. 0.01$)

Audio recordings in the nests were analyzed and categorized as “mechanical” sounds (consisting of "taps", "clicks" and "scratches") or “frequency modulated (FM)” sounds (Fig. 5). Mechanical sounds consisted of taps, clicks, and scratches of short (0.02 – 0.15 s) duration and broad frequencies (up to 20 kHz) whereas FM sounds ranged in duration between 0.2 – 1.25 ms and were confined to frequencies below 4 kHz in either 3 or 4 bands, separated by approximately 100 kHz (Fig. 5). The two sound categories also differed in their distribution relative to the time of hatching (Figs. 6 and 7). Mechanical sounds were recorded in the thousands/d, beginning 1 d before hatching and steadily increased in number through day 4, post-hatching (Fig. 6). The FM sounds, in contrast,

were emitted in much lower numbers, beginning as early as 6 d before hatching. They reached a peak in daily emission ($\leq$ 120 sounds/d) between two days before and the first day after hatching (Fig. 7).

Experiment 3: separating correlated variables

When comparing the group of A eggs that were split up into the experimental and control groups, the eggs from the A_1 experimental nest boxes had a mean ($\pm$ sd) incubation duration of 46.8 $\pm$ 0.55 d (range: 45.8 - 47.7 d) while the embryos from the A_2 eggs from the control nest boxes had an incubation duration of 47.4 + 1.17 d (range: 45.7 - 49.0 d). The incubation duration of the A_1 eggs was significantly shorter than the A_2 eggs (Paired t = -2.329, p = 0.023; Fig. 8).

When comparing the egg groups in contact with each other in the experimental nest boxes, there were no significant differences in incubation duration between the A_1 and B eggs, in which the latter averaged 47.0 $\pm$ 0.73 d (range: 46.2 – 48.0 d; t = - 0.708, p = 0.5). However, there was a significant difference between the eggs in contact with each other in the control nest boxes. The mean incubation duration for the C eggs (46.1 $\pm$ 0.51 d; range: 45.7 – 47.0 d) was significantly shorter than the incubation duration of the A_2 eggs (t = 2.99, p = 0.008; Fig. 8). Additionally, the C eggs had significantly shorter incubation durations compared to the B eggs (t = 2.98, p = 0.009). There was no significant difference between C and A_1 egg incubation duration. There were no significant differences in hatching synchrony among each of the egg groups (A_1, A_2, B, C).

Sand incubation temperatures were compared across 3 pairs of experimental and control boxes throughout the 7 weeks of incubation. There was no significant difference in temperature readings between any of the nest boxes (ANOVA $F = 1.098$, $p = 0.38$; Fig. 9).

DISCUSSION

Loggerhead embryos tend to hatch at about the same time if they incubate under the same temperature conditions, such as in an incubator. Isolated eggs did not show any significant difference in incubation duration or hatching synchrony (Table 1). These findings support the Temporal Synchrony Hypothesis; hatching is synchronized because embryos, especially in small numbers, when subjected to the same constant environmental conditions tend to develop at the same rate and hatch as a result of temporal synchrony without the use of external cues.

However, when a sufficient number of viable embryos were in contact and successfully hatched, both nest incubation time and hatching duration were shortened (Table 2). Recording analyses paired with photo observations show that the frequency modulated sounds increased in occurrence prior to pipping, then greatly decreased after the first day of pipping (Fig. 7). However, most occurrences of FM sounds were from nest boxes with low hatch success. Since these nests had longer incubation and hatching durations, it is likely that vocalizations do not facilitate hatching synchrony and are instead byproducts of extraneous activity such as moving within the egg and attempting to hatch (McKenna et al., 2019). It is more plausible that mechanical stimulation created by the movement of the embryos act as cues which facilitate hatching synchrony since more of these sounds were produced by high hatch success nests which had faster incubation and hatching durations (Fig. 6). Although this study did not find evidence that

vocalizations play a role in synchrony; these sounds and mechanical sounds may not be mutually exclusive cues for synchrony. Further experimental study must be done to determine the true role of FM sounds.

Due to the confounding results of whether temperature or communication accelerated hatching duration in the vertical nest experiment (Fig. 4), the final experiment was designed to eliminate any effects of temperature and examine the effects of communication alone (Fig. 9). Clutch mates showed significant differences in incubation duration depending on the group of embryos they were in contact with during development. Those in contact with other embryos at the same stage of development had significantly shorter incubation durations compared to their clutch mates in contact with embryos two weeks behind in development (Table 3, Fig 8). Additionally, this egg group that was two weeks behind in development had the shortest incubation period. It is possible that their development was affected by having contact with groups of eggs that were at a more advanced developmental stage. These findings suggest that external cues, possibly mechanical stimulation, can accelerate development, allowing for Coordinated Synchronous hatching.

In the context of behavioral studies, there are advantages and disadvantages to performing experiments in the field or in the laboratory. In the case of this study, experiments were conducted in a laboratory setting using incubators. This approach was advantageous because the incubators allowed for controlled nesting environments for the embryos where temperature and moisture were tightly regulated. Natural nests experience extreme temperature fluctuations, differences in moisture content due to unpredictable weather patterns, and are also susceptible to disturbance or predation (Matsuzawa et al.,

2002; Booth and Astill, 2001; Wyneken and Lolavar, 2015; Fowler, 1979). With all these variables, it would be difficult to differentiate what was causing an effect on incubation or hatching, and thus the experiment would yield uninformative results. Currently, little is known about sea turtle hatching behaviors. Therefore, it is useful to examine this behavior under controlled conditions to begin obtaining more information that can be expanded upon in the future with complimentary field research. In this study, the use of incubators eliminated the influence of these variables and allowed a test of whether external stimulation alone had any effect on incubation duration or hatching synchrony.

Previous studies that recorded vocalizations failed to determine if these sounds play a role in hatching, due to several problems with their methods. These studies involved inserting or placing a microphone onto the top portion of a natural nest for short periods of time during different times of incubation and hatching (Ferrara et al., 2019; Monteiro et al., 2019). The first problem with this method is that there currently is not an accurate way to observe pipping of an entire nest in its natural environment. Since the recorded sounds were not correlated with accurate hatching times, these findings do not provide evidence that the sounds facilitate hatching synchrony. The second problem is that sounds were recorded in short intervals not allowing for a whole analysis on the emission patterns of these sounds prior to and during hatching. Lastly, by repeatedly inserting a microphone into the sand surrounding a nest, this seemingly miniscule movement may have stimulated nearby embryos resulting in altered behaviors. These issues from previous studies were avoided with improved experimental designs that allowed for nondisruptive, continuous, and controlled observation of embryos throughout the entirety of incubation and hatching.

Most of the previous research examining hatching synchrony in turtles has been focused on freshwater species. Temperature differences within a nest should cause some embryos to develop faster than others, consequently promoting asynchronous hatching (Chessman, 1978; Thompson, 1988). However, studies have shown that different freshwater turtles use differing methods to achieve coordinated synchronous hatching. different species have evolved varying responses to external stimuli which affecting pipping time and alter the duration of incubation (Spencer and Janzen, 2011). Species such as *C. insculpta* enter into embryonic aestivation after incubation is complete and delay hatching until they are exposed to anoxic stimuli which occur when the nest is flooded (Georges et al., 2008; Doody et al., 2001).

Other species such as *E. macquarii* and *C. picta* coordinate early hatching when exposed to more advanced embryos that may produce external stimulation towards the end of their incubation (Colbert et al., 2010; McGlashan et al., 2015; Spencer et al., 2001; Spencer et al., 2011*).* Hatching early can be a result of shortening developmental time (Colbert et al., 2010; McGlashan et al., 2018) or metabolically compensating and "catching up" developmentally (Spencer and Janzen, 2011; McGlashan et al., 2012). These examples show that across freshwater turtle species, there is variation on how synchronized hatching is achieved. Although this variation exists, it appears that some form of information is transmitted among the embryos through physical contact of the eggs within a clutch. The vibrations associated with hatching have been shown to stimulate hatching of eggs within contact (Doody et al., 2001). Additionally, previous experiments have shown that hatching movement vibrations have a function in synchrony since larger groups hatch faster than single embryos (Georges et al., 2008).

Since both marine and freshwater nesting ecologies promote asynchronized hatching, it is not surprising that there seem to be similarities in their hatching behaviors. Results from these experiments suggest that loggerhead embryos coordinate synchronous hatching through behavioral responses to mechanical stimuli produced by their clutch mates prior to and during hatching. This supports previous experimental research where simulated mechanical stimulation resulted in accelerated development of embryos (Bustard, 1972). Additionally, I found that larger groups of embryos hatch faster than smaller groups; similar trends have been seen in freshwater turtles (Doody et al., 2001; McGlashan et al., 2015). These findings suggest that social facilitation is utilized during hatching; this adaptation has been seen in sea turtles to decrease the energy expended in digging out of the nest (Carr and Hirth, 1961; Rusli et al., 2016; Santos et al., 2016). It is possible that when hatching, embryos in close contact indirectly assist each other with escaping from their eggs. In this study, observations of hatching behavior revealed that embryos in nests with low hatch success struggled for prolonged periods of time when attempting to escape their eggs*;* this resulted in longer hatching durations for the low hatch nests. Furthermore, synchronous hatching in marine turtles is thought to occur along with synchronized nest emergence as a survival strategy. Synchronized mass emergence is advantageous by swamping potential predators, thus increasing the likelihood that more hatchlings would survive (Rusli et al., 2016; Santos et al., 2016; Erb and Wyneken, 2019).

Other than this study, there are not many others that focus on the embryo-to-embryo communication and hatching behaviors of sea turtles (Bustard, 1972). The design of Experiment 1 can be improved upon to determine the true role of contact with larger

numbers of embryos within each treatment group. Further experimental studies can be done with vocalizations to determine if they are truly a byproduct of exercise or play any role in hatching or emergence synchrony. The results of this study do not provide any evidence that vocalizations play a role in hatching however, the effects of vocalizations alone were not examined. A playback experiment using previously recorded vocalizations may yield more conclusive results. Lastly, other experimental research is required to further support the findings that mechanical stimuli transferred through physical contact facilitates synchronous hatching across turtle species. Such studies would further our understanding on the phenomenon of synchronized hatching across both freshwater and marine species.

This novel study examines the hatching behaviors of marine turtles under controlled laboratory conditions with three separate experiments. The overall findings of this study provide reliable conclusions on embryo-to-embryo communication in marine turtles. Temporal synchrony was observed; regardless of isolation, the embryos still hatched synchronously with the grouped embryos. This occurred because development began at the same time under similar incubation conditions. However, subsequent experiments show that the embryos have an ability to adapt their behaviors to hatch synchronously when stimulated (either mechanically or vocally), showing that coordinated hatching also occurs. These findings suggest that temporal and coordinated synchrony are not mutually exclusive in marine turtles. Results suggest that mechanical stimulation may be the cue that facilitates synchrony however, the role of vocalizations in hatching synchrony has yet to be determined. These cues may not be mutually exclusive in facilitating hatching synchrony. Overall, these findings suggest that social facilitation

may be a conserved adaptation across turtle species and is utilized in the hatching process allowing for synchronized emergence. Mass emergence from the nest is an important survival tactic in turtles since this swamps potential predators, allowing for more hatchlings to survive (Rusli et al., 2016; Santos et al., 2016; Erb and Wyneken, 2019). Additionally, sea turtle hatchlings work together to dig out of their nests which decreases energy expenditures (Henderickson, 1980). Thus, synchronous hatching would be advantageous for survival in turtle species.

TABLES

Table. 1. Summary of MANOVA analysis comparing the differences between incubation duration and hatching synchrony for the isolated and grouped eggs in Experiment 1. There was no significant difference between the isolated and grouped eggs for both incubation duration and hatching synchrony.

Variable	F	*df*	*p*	Partial Eta Squared
Incubation Dur.	0.306	1	0.587	0.017
Hatching Syn.	0.084	1	0.775	0.005

Table 2. Summary of the results from the nest simulation experiment. Data include the number of eggs that hatched (out of a total of 20 in each nest), the duration of incubation (in days) for each nest, and the hatching duration (in hours).

Nest Box	No. Eggs Hatched	Incubation Duration (d) (Mean ± sd)	Hatching Duration (h)
1	1	48	96
2	2	48.0 ± 1.41	121
3	4	45.3 ± 0.50	88
4	10	44.6 ± 0.70	68
5	12	45.1 ± 0.51	68
6	15	42.8 ± 0.49	81
7	16	44.1 ± 0.48	58
8	17	44.1 ± 0.61	58
9	18	43.6 ± 0.46	77
10	18	45.1 ± 0.83	70

Table 3. Summary Table of the incubation duration (in days) and the hatching synchrony (in hours) for the "A" eggs. There were 10 nest replicates of the pair, each from a different female.

Nest Box	No. Eggs Hatched	Incubation Duration (d) (Mean ± sd)	Hatching Synchrony (h) (Mean)
Exp. 1	6	46.5 ± 0.55	18
Cont. 1	6	47.7 ± 0.52	35
Exp. 2	4	46.3 ± 0.5	24
Cont. 2	6	46.3 ± 0.55	24
Exp. 3	6	45.8 ± 0.41	18
Cont. 3	6	45.7 ± 0.52	20
Exp. 4	6	46.8 ± 0.75	36
Cont. 4	4	46.8 ± 0.41	24
Exp. 5	5	47.0 ± 0.71	37
Cont. 5	6	49.0 ± 1.41	92
Exp. 6	6	46.8 ± 0.41	18
Cont. 6	6	46.7 ± 0.52	30
Exp. 7	5	46.6 ± 0.55	29
Cont. 7	4	47.5 ± 1.29	83
Exp. 8	6	47.3 ± 0.52	24
Cont. 8	6	47.0 ± 0.63	36
Exp. 9	3	47.3 ± 0.58	30
Cont. 9	4	48.8 ± 0.50	20
Exp. 10	6	47.7 ± 0.52	25
Cont. 10	6	49.0 ± 1.41	90

Exp. = Experimental "A_1" Eggs, Cont. = Control "A_2" Eggs

FIGURES

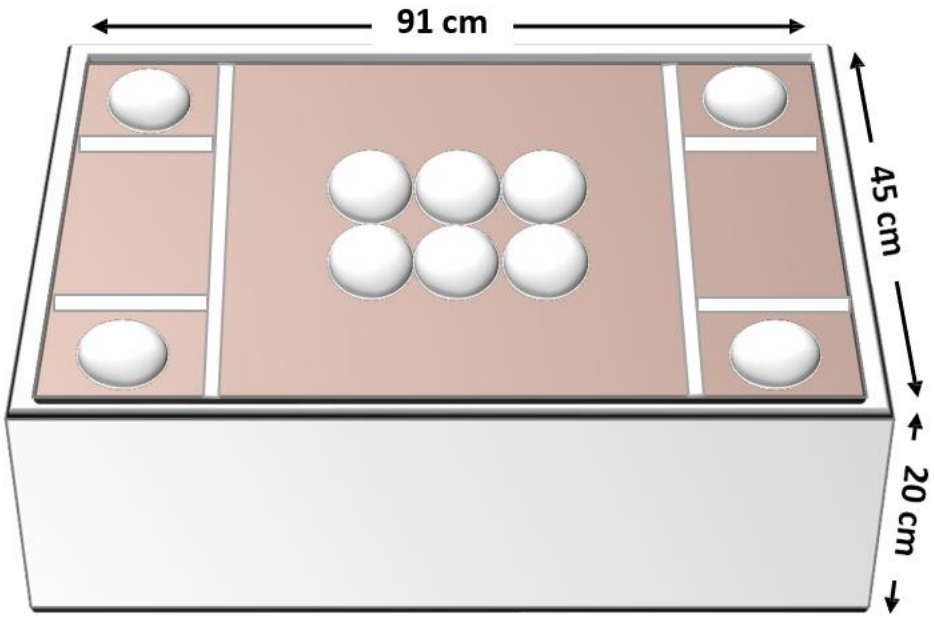

Figure 1. Ten eggs from a single nest are separated into 4 isolated and 6 grouped eggs clustered in contact with one another. Eggs are buried within a ~8 cm layer of autoclaved beach sand inside a large styrofoam nest box. Each of 10 such replicate coolers are placed inside of an incubator set at 31 $\pm$ 0.51° C until hatching occurs. Eggs are further isolated from one another by (cardboard) barriers**.** See the text for further details.

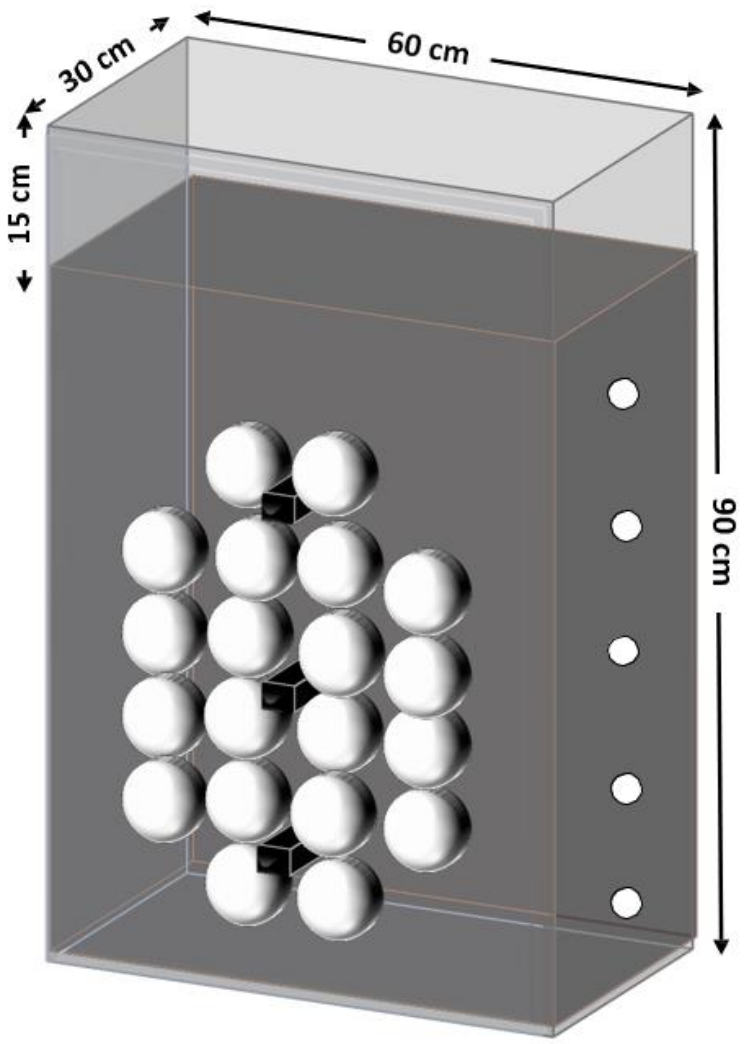

Figure 2: Vertical Next Box Experiment setup. This was the observation nest box that was used to observe the hatching behaviors of loggerhead eggs (represented by the spheres). The eggs are stacked against a thin glass pane. The small black rectangles represent data loggers that recorded the temperature at different locations inside the group of eggs. The darkened portion of the nest box represented the sand level. A 15 cm deep area on the top of the sand represented the space reserved for the emerging hatchlings. The sides, and bottom of the nest box had holes to promote gas exchange.

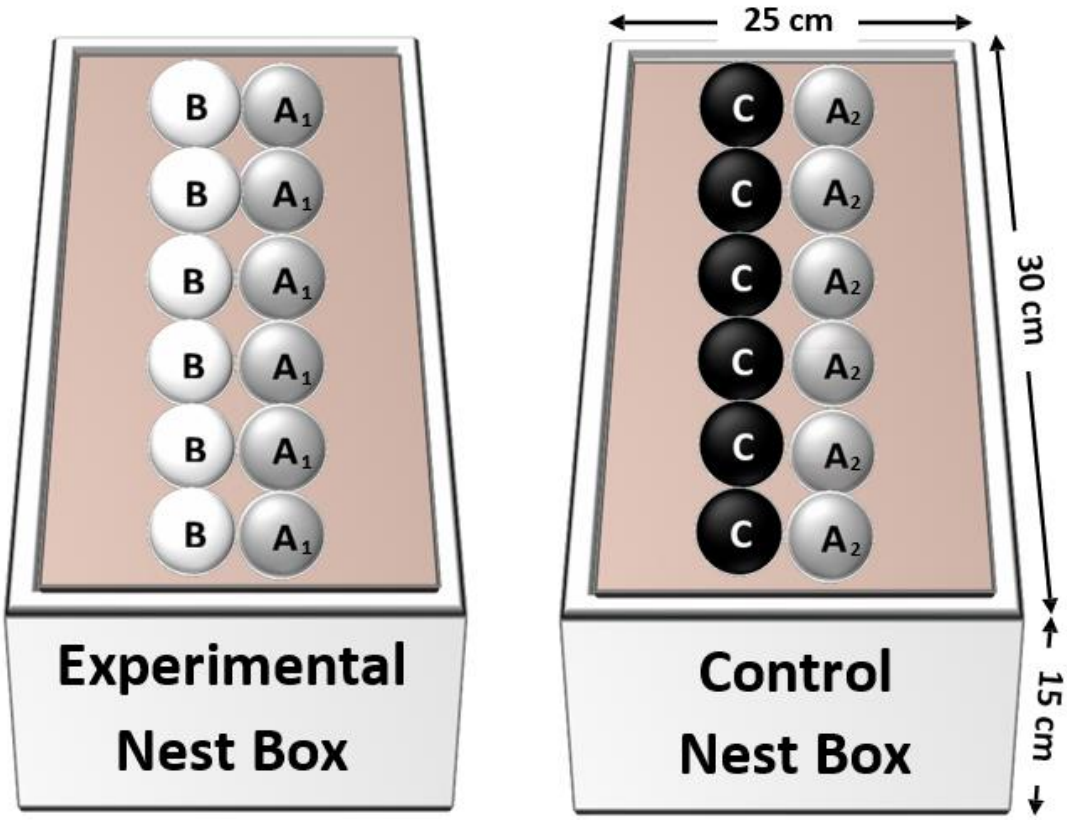

Figure 3. Experiment designed to determine if differences in mechanical stimulation affect the duration of incubation when temperature is held constant at 31 $\pm$ 1.5° C. To find out, 12 eggs from the same nest are divided into two groups of 6 (A_1) experimental and 6 (A_2) control eggs, buried in separate nest boxes under 8 cm of autoclaved beach sand. Two weeks later, 6 (B) eggs from another nest, *deposited two weeks earlier*, are placed in contact with the A_1 eggs while 6 (C) eggs from another nest, *deposited the night before*, are placed in contact with the A_2 eggs. The duration of incubation shown by the two groups of A eggs is subsequently compared to determine if the A_1 eggs hatch sooner than the A_2 eggs. See the text for additional details.

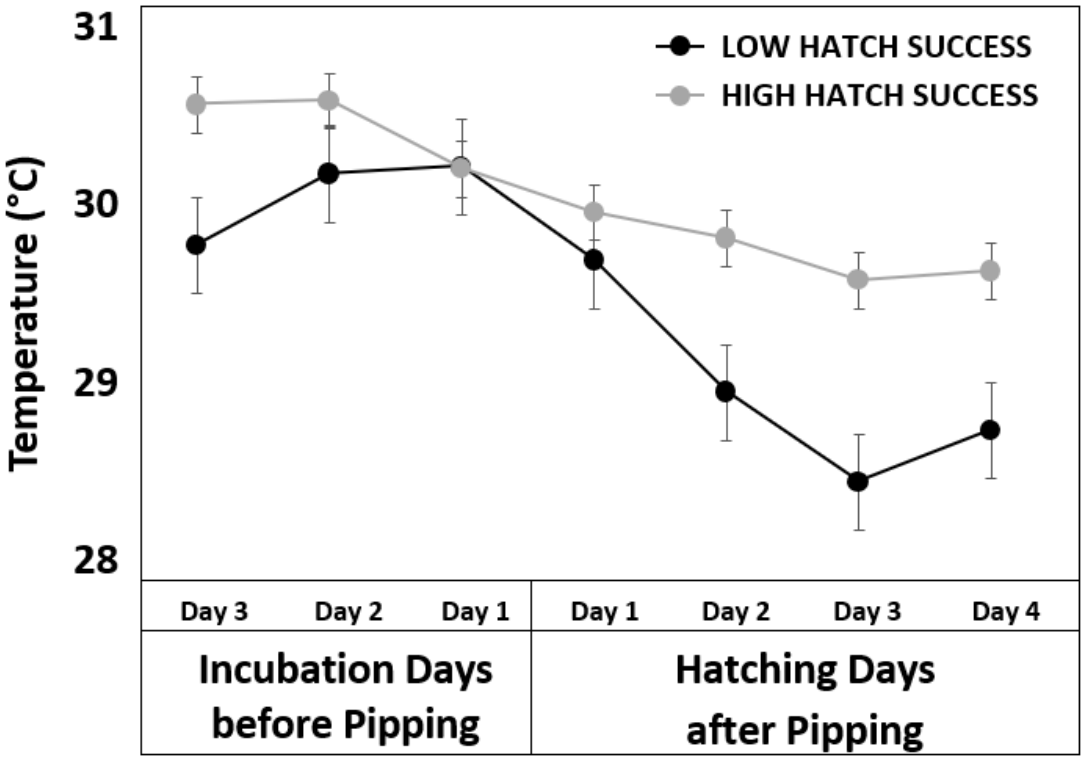

Figure 4. Vertical Nest Box Experiment: values show changes in mean temperature (± sd**)** in the two hatch success groups over the last 3 days of incubation, and over the first 4 days after hatching. Two to 4 days after hatching, the high hatch success nests show a statistically significant increase in mean temperature.

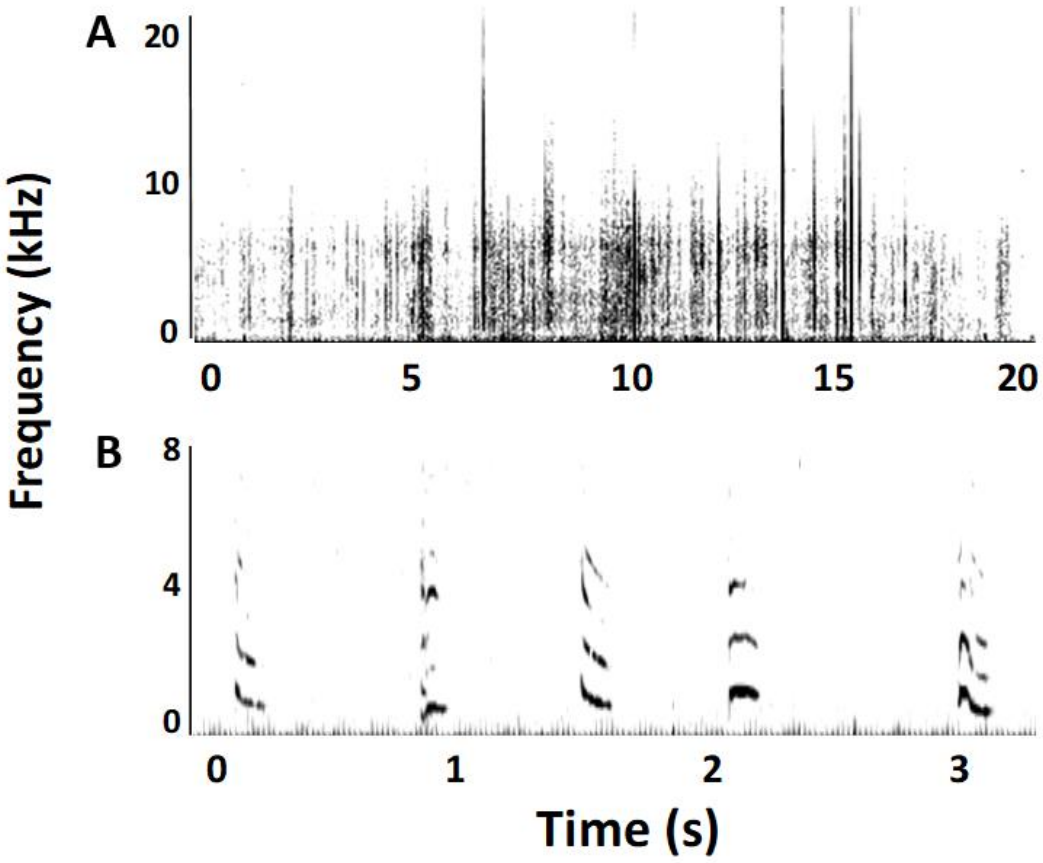

Figure 5. Spectrographs of the two sound types. Above, mechanical sounds (A); below, frequency modulated sounds (B). The former consist of temporally brief taps, clicks, and scratches that range over a broad range of frequencies, up to 20 kHz. The latter contain harmonics, are of longer duration, and encompass a smaller range of frequencies up to ~ 5 kHz.

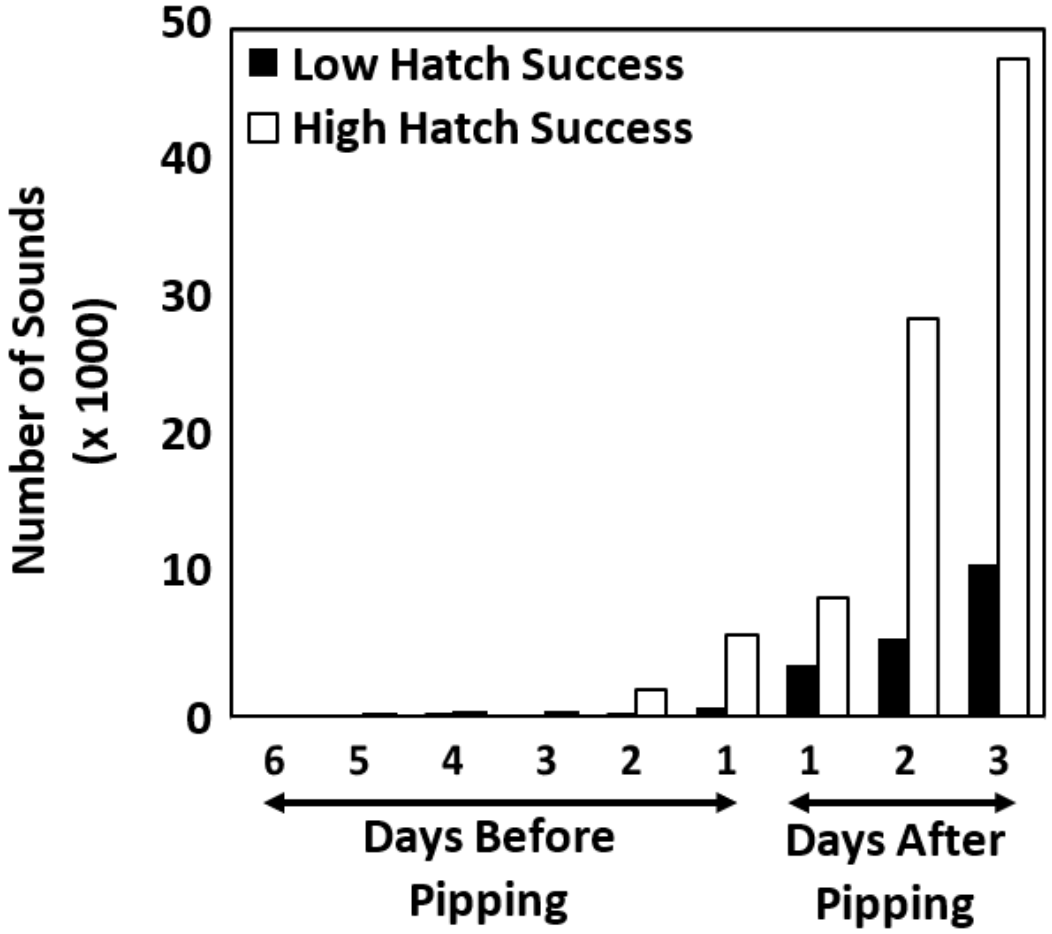

Figure 6. Temporal distribution of the mechanical sounds, shown relative to the days of incubation before pipping and the days of hatching after pipping. Open bars, high hatch success group; filled bars, low hatch success group. More sounds are produced just before and increasingly, after pipping.

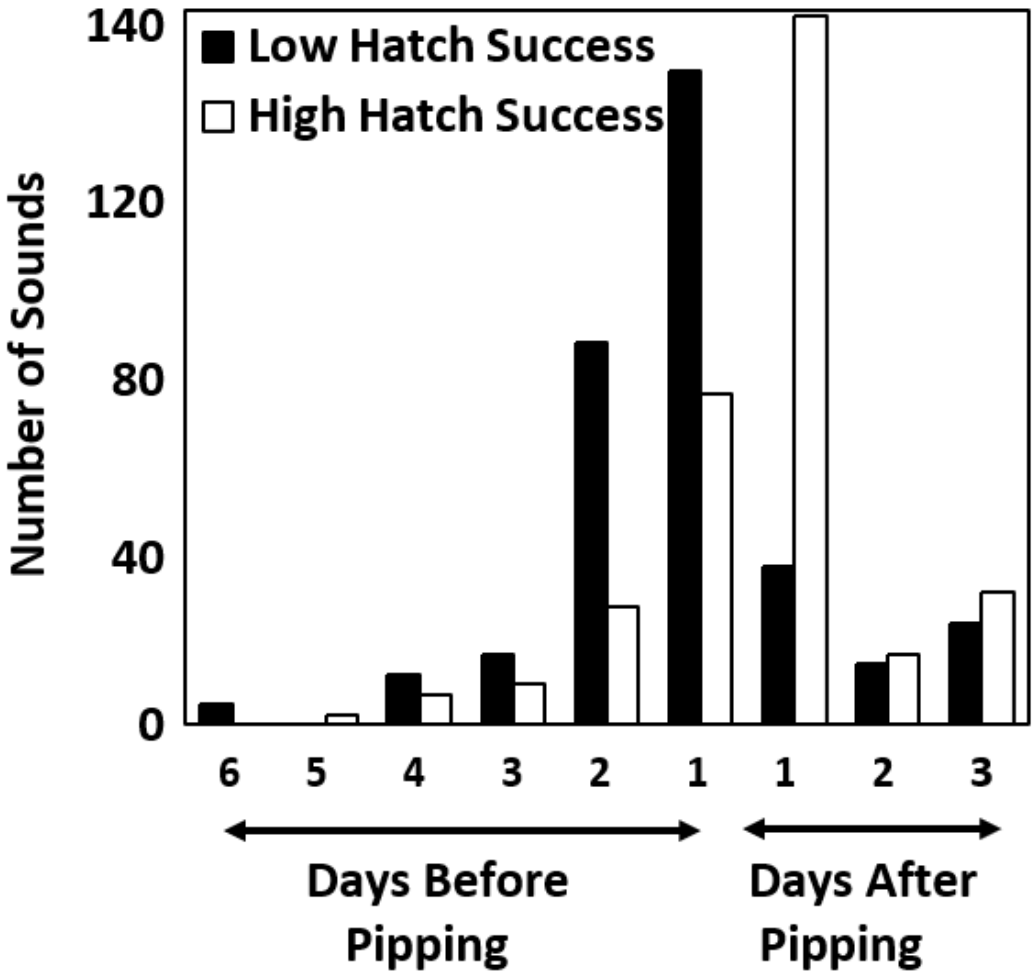

Figure 7. Temporal distribution of the FM sounds relative to the days of incubation before pipping and the days of hatching after pipping. Open bars, high hatch success group; filled bars, low hatch success group. These sounds peaked between 2 days before and 1 day after pipping.

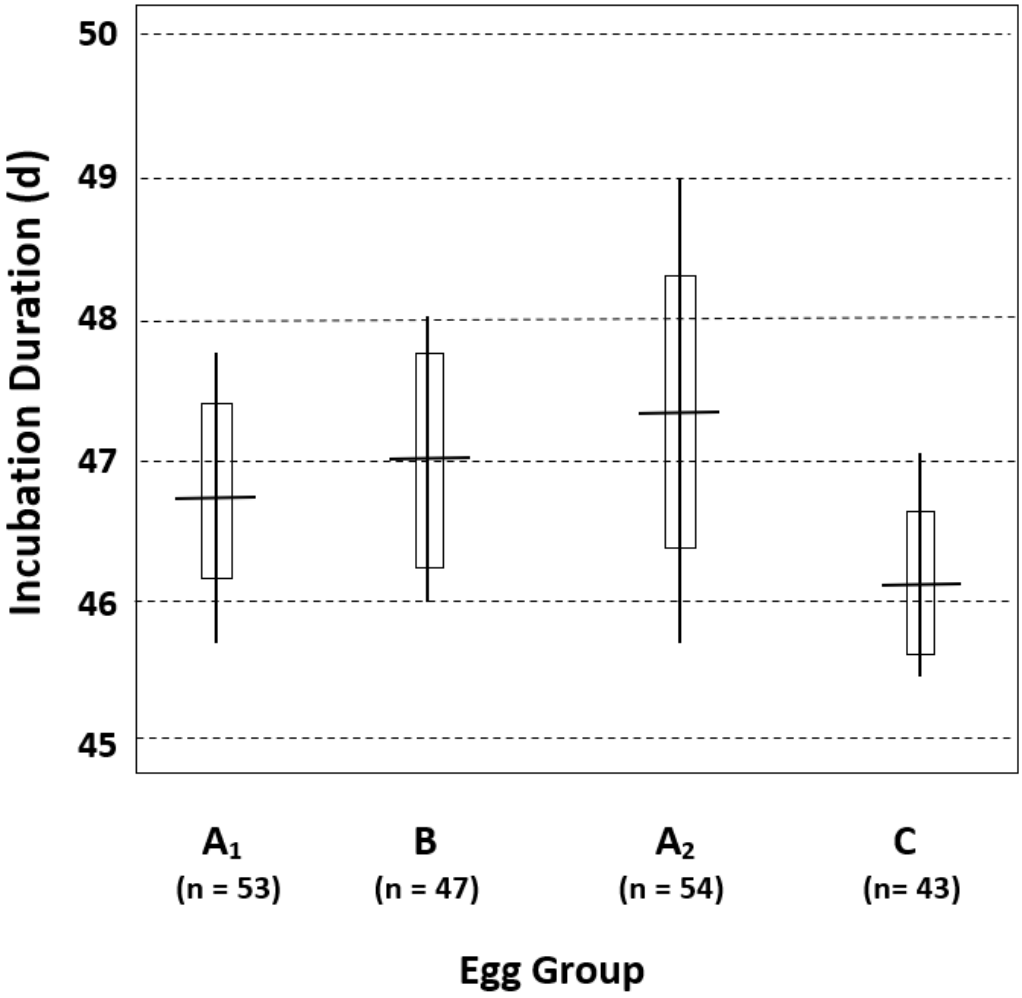

Figure 8. Box plots showing the mean (horizontal line, range (vertical line) and sd (vertical box) for the incubation duration (in days) of the 4 egg groups. Values under each group show the number (out of 60 eggs) that hatched. See Table 3 for the individual means shown by the A_1 and A_2 groups. For those two groups, n = 10 replicates but for the B and C egg groups, no eggs hatched in one nest box (n = 9 replicates). Mean incubation times were significantly shorter for the A_1 vs. the A_2 eggs and for the C vs. the A_2 and B egg groups. There was no significant difference for C vs A_1 egg group.

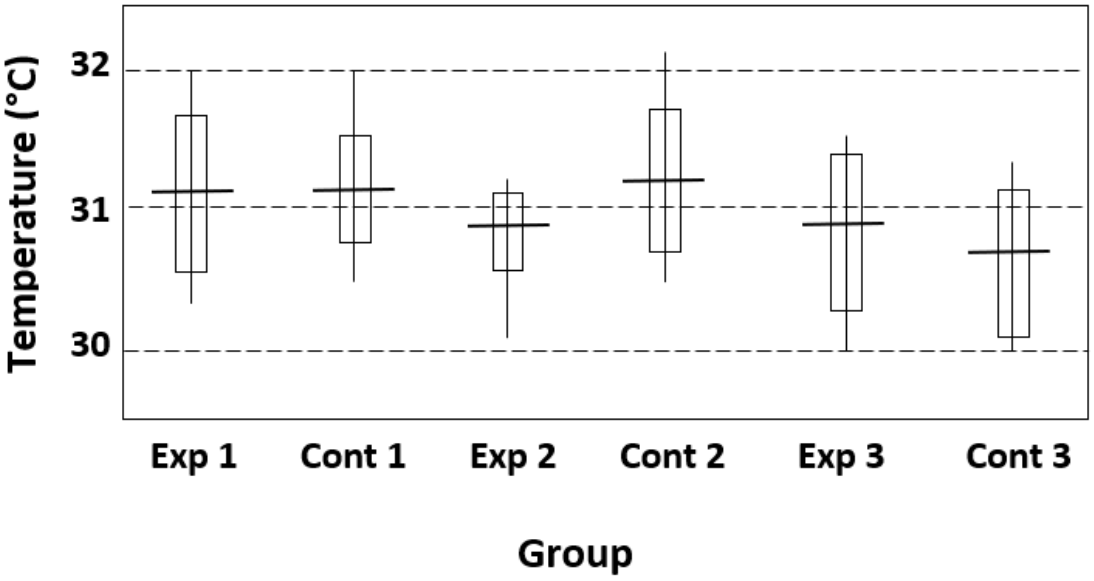

Figure 9. Asynchronized Development Experiment: Whisker plots show the temperature records throughout incubation for three pairs of boxes. Format, as in Figure 8. There were no significant differences between the mean temperatures among the pairs of boxes.

APPENDIX

Appendix A. Summary of the incubation temperature for the vertical nest box experiment. Comparisons for each nest box was done using two-way ANOVA. The Data Logger Position refers to the placement in the egg mass (T=top, M=middle, B=bottom).

Nest Box	Data Logger Position	Incubation Temperature (Mean ± sd)	F	*p* value
1	T	31.42 ± 1.00	0.530	0.590
	M	31.75 ± 1.05		
	B	31.67 ± 1.59		
2	T	32.09 ± 1.07	2.646	0.074
	M	32.35 ± 0.87		
	B	32.87 ± 0.72		
3	T	31.91 ± 0.95	1.217	0.299
	M	32.17 ± 1.09		
	B	32.22 ± 1.02		
4	T	31.77 ± 0.95	2.646	0.075
	M	32.19 ± 0.98		

	B	32.01 ± 1.02		
5	T	31.87 ± 1.34	0.985	0.376
	M	32.21 ± 1.57		
	B	32.24 ± 1.46		
6	T	31.40 ± 0.67	2.726	0.069
	M	31.59 ± 0.93		
	B	31.43 ± 0.96		
7	T	31.85 ± 1.17	2.053	0.132
	M	32.03 ± 1.26		
	B	32.00 ± 1.33		
8	T	31.60 ± 0.19	2.000	0.140
	M	31.77 ± 0.25		
	B	31.86 ± 0.21		
9	T	31.89 ± 0.58	2.423	0.093
	M	32.17 ± 0.72		
	B	32.19 ± 0.68		
10	T	31.10 ± 0.82	2.668	0.073
	M	31.47 ± 0.82		
	B	31.44 ± 0.85		

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