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The energetics of nest escaping by turtle hatchlings

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ABSTRACT

Turtles lay their eggs on land in underground nests and embryos take about two months to incubate to hatching. During this time, turtle embryos grow in size and consume much of the yolk that was deposited in the egg when it was laid. During the hatching process, and sometimes continuing for a period of 24-48 h after hatching, the residual yolk is absorbed into the abdominal cavity. The residual yolk acts as an energy reserve that powers the nest escape process and hatchling growth and development until feeding begins, which can be days or weeks after nest escape. Previous studies have reported the energetic cost of embryonic development across Chelonian taxa, but none has quantified the energy needed to escape the nest after hatching. This study used open-flow respirometry and bomb calorimetric analysis to measure metabolic expenditure of turtle hatchlings during simulated nest escape. Digging activity was monitored simultaneously with metabolic rate to determine the pattern of digging effort, energy expenditure, and the total energetic cost per individual during nest escape. Two species of chelonian were used in this study; the Brisbane river turtle (*Emydura macquarii signata*) and green turtle (*Chelonia mydas*).

The first part of the study quantified the energy expended by hatchlings during nest escape. The Brisbane river turtle and green turtle hatchlings were found to fuel this activity by using approximately 5-36 % and 11 – 68% respectively of their residual yolk reserve energy. This energy expenditure was then put into an energy budget context by comparing it to the energy in a freshly laid egg, the energy used during embryonic development, and the energy contained within the residual yolk of newly hatched hatchlings. The second part of the study examined the phenomenon of 'social facilitation', a term that has been coined to describe how interactions between nest mates facilitate the nest escape process. The hypothesis behind this concept, that escaping the nest in a larger group is more advantageous than in a smaller group, was tested by splitting a clutch into different sized groups and measuring the time taken to dig a set distance upwards, and calculating the per-individual metabolic cost of nest escape. Both the time taken to escape the nest and the energetic cost of nest escape decreased as the number of individuals digging together increased and thus supports the 'social facilitation' hypothesis which suggests hatchlings cooperate to share the workload of digging out of the nest among clutch mates to reduce individual energy expenditure. Lastly, because freshwater turtle can construct their nest in a wide range of soil types, the time taken and the energy required by the same number of hatchlings to dig through two different soil types was quantified. Brisbane river turtle hatchlings digging through fine sand escaped faster and spent less energy than hatchlings through coarse sand. Moreover, larger group size provided a clear energetic advantage while digging out of the nest in both soil types tested.

DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Author M. Uzair Rusli (Candidate)	Designed experiments (70%) Collected data & analysis (100%) Wrote the paper (70%)
Author David Booth	Designed experiments (30%) Wrote and edited paper (30%)

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Contributions by others to the thesis

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None.

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Keywords

Turtle hatchling, clutch size, nest escaping, aggregation behaviour, social facilitation, metabolic rate, energetic cost

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$E_{digging}$	Net energy expended on digging (kJ)
$E_{resting}$	Resting metabolic cost (kJ)
E_{total}	Total individual energy expenditure (kJ)
kJ	kilo Joule
NCOT	Net cost of transport (kJ m^{-1})
NH	Newly hatched hatchling
PD	Post digging hatchling
RQ	Research Question
SD	Standard deviation
$\dot{V}CO_2$	Rate of carbon dioxide (ml h^{-1})
$\dot{V}O_2$	Rate of oxygen consumption (ml min^{-1})
ϕ	Phi

CHAPTER 1

GENERAL INTRODUCTION

1.1 Ecological Energetics: Reproduction and Moving in Groups

Measuring energy consumption is fundamental to understanding various processes in animal ecology including behavioural, reproduction, physiological, and evolutionary ecology (Dunham et al., 1989; Schmidt-Nielsen, 1997; Spicer and Gaston, 1999; Costa and Sinervo, 2004). Energy is often cited as a major limiting factor in determining an organism's survival. Further, energy requirements at an individual level may influence the distribution and abundance of the entire population. For example, animal growth rate and reproductive outputs are dependent on energy availability, which in turn affects population structure (Brown et al., 2004).

Reproduction is usually an energetically expensive process, with energy being expended on acquiring mates, producing reproductive propagules (in the case of females, eggs) and in some species extra energy is expended in rearing young until they are independent of parents. Species that produce many offspring such as most invertebrates, parasitic animals, frogs, most fishes, and turtles typically provide no extensive parental care, and thus focus their energy expenditure on egg production. In these species, the egg must be provisioned with enough energy to enable completion of embryonic development, egg and nest escape, and to survive in the post-hatch environment until feeding can begin. Many oviparous reptiles, including turtles bury their eggs in underground nests, and hatchlings must dig their way out of the nest before they can escape to an environment where they can begin feeding.

Movement requires energy expenditure, and moving in groups may help save on the energetic cost of locomotion. Locomotion requires energy to overcome the forces of drag, friction, inertia, and gravity, and energy savings can be made if animals move together in a group in such a way that they minimise drag forces. For example, it has been suggested that birds flying in V-formation (Lissaman and Shollenberger, 1970; Cutts and Speakman, 1994) save energy by exploiting the slip-stream of the individuals in front of them. Hence it is possible that energy savings may be made by groups of hatchlings digging out from underground nests together, and that the bigger the group, the larger the energy saving might be.

1.2 Life History of Turtle Nests

In the life histories of turtles, post-egg laying maternal care is typically absent (but see Agha et al., 2013 for a few exceptions), so maternal care is confined to choosing an appropriate nest location. The nest provides protection against predation, insect infestation, buffers the incubating eggs against changes in temperature and provides a relatively high humidity environment for development (Miller, 1985). Incubation requires several months and successful embryonic development is determined by the interaction of several factors, including appropriate concentrations of respiratory gases (oxygen and carbon dioxide), temperature, salinity, humidity, and avoidance of water inundation (floods in freshwater species, tidal inundation in sea turtles), nest erosion and predation (Miller, 1985; Booth, 1998a; Price et al., 2007; Wang and Weathers, 2009; Reid et al., 2009).

Once embryonic development is completed and the eggs hatched, turtle hatchlings must dig out of the nest without assistance, and frequently some hatchlings successfully hatch but fail to reach the nest's surface (Drake and Spotila, 2002; Matsuzawa et al., 2002). Despite some factors that can cause asynchronous development rates within a clutch (e.g. order of ovulation and variation of the thermal environments within a nest) (Andrews, 2004) turtle embryos have strategies that allow them to hatch and emerge from a nest more or less synchronously (Doody et al., 2001; Spencer et al., 2001). A more recent study has demonstrated that the mechanisms causing synchronous hatching can vary between different species (Spencer and Janzen, 2011).

Near synchronous hatching of turtles probably increases the fitness of hatchlings by two different mechanisms: (i) by assisting each other to dig out of the nest, and (ii) in species that have large clutches, by swamping predators. Hence, synchronous hatching is significant for the survival of turtle offspring during their early life. A consequence of large female body size such as occurs in sea turtles is the ability to dig deep nests and lay a large number of eggs. Hence sea turtle hatchlings are able to share the workload of digging upward, and this process has been described as a form of 'social facilitation' (Carr and Ogren, 1959). Indeed, Carr and Ogren (1959) suggested that a single sea turtle hatchling is unlikely to reach the nest surface if not assisted by its clutch mates. In contrast, smaller species produce fewer offspring per nest, but also construct shallower nests. Shallow nests imply that less digging work is required to escape the nest. It is not known if the work required per individual may be similar in species that have large clutches and deep nests and species that have small clutches and shallow nests.

Nest depth of turtles is dependent on nest construction behaviour and hindlimb length. Because of their relatively small size freshwater turtles construct shallower nests compared to larger body sized

sea turtles. In loggerhead turtles (*Caretta caretta*) nest depth is correlated with female size (Carthy et al., 2003). Some freshwater turtles maximize the reach of their hind limbs by propping up the body with their front limbs (Booth, 2010), and many sea turtles dig a body pit with their front and hind limbs before beginning to dig the nest chamber with their hind limbs which also increases nest depth (Hailman and Elowson, 1992). Hence, differences in body size and nest digging behaviour among different species result in different nest depths and thus variation in the distance hatchlings need to dig to escape the nest.

1.3 Nest Escape by Hatchling Turtles

After hatching, turtles spend some time in the nest before they emerge onto the surface. This is a critical stage in the natural history of turtles because hatchlings typically emerge from eggs in a premature condition, with residual yolk not fully incorporated into the abdominal cavity and the carapace still curved as a result of being enclosed in the egg (Kraemer and Bennett, 1981, Spencer and Janzen, 2011). Subsequent to hatching, the residual yolk sac is retracted into the abdominal cavity through the umbilical opening on the plastron, probably by the contraction of the ruptured amniotic membrane as occurs in lizards (Pezaro et al., 2013). Most sea turtle hatchlings found on the nest surface have completely straightened carapaces and completely absorbed residual yolk (Godfrey and Mrosovsky, 1997), so carapace straightening and yolk internalization take place between hatching and nest emergence.

Only a few studies have investigated the nest escape process in freshwater turtles (Spencer et al., 2001; Pignati et al., 2013), as a consequence, most of what is known about the nest escape process in Chelonians comes from studies of sea turtles. Complete absorption of the residual yolk and the straightening of the carapace occur within 24 – 48 hours of hatching (Reid et al. 2009). Several studies (Carr and Ogren, 1959; Carr and Hirth, 1961; Dial, 1987; Christens, 1990; Godfrey and Mrosovsky, 1997; The Chu et al., 2008) indicate that sea turtle hatchlings spend one to seven days in the nest before emerging onto the surface. Little is known about the nest emergence period in freshwater turtles, but a recent study on the yellow-spotted river turtle, (*Podocnemis unifilis*) found hatchlings of this species took at least seven days to emerge (Pignati et al., 2013). Overall, there is no clear detailed description of within nest activities of hatchling turtles during the hatching-emergence interval in either sea or freshwater turtles.

It has been suggested that in sea turtles approximately half of the residual yolk is utilized during the period from hatching to nest emergence and the used yolk is thought to fuel energy metabolism rather than being used to synthesize new tissue of being converted into abdominal fat storage bodies

(Kraemer and Bennett, 1981). Hence, a considerable quantity of energy reserve in the residual yolk is used during the hatch to nest emergence period, and most of this is probably used for the digging out effort. Hence, the greater the digging effort, the more energy reserve is used leaving less available for the crawl to the water and in-water activities until feeding begins.

In sea turtle nests, periodic intense digging bouts by hatchlings are fuelled by both aerobic and anaerobic metabolic pathways. Anaerobiosis is necessary because of the intense muscular activity associated with digging, and in deep nests with many hatchlings slower rates of oxygen diffusion may cause nest conditions to become hypoxic (Prange and Ackerman, 1974; Ackerman, 1997; Miller, 2008) which may hamper the uptake of oxygen by hatchlings from the nest environment. In vertebrates such as sea turtles, anaerobic metabolism results in lactate production which accumulates in muscle and causes fatigue and exhaustion (Rees et al., 2009) and also results in increased lactate concentration in the blood (Baldwin et al., 1989). In sea turtle hatchlings, Dial (1987) found that five minutes of exercise can significantly increase the whole body lactate content, and Baldwin et al. (1989) demonstrated that blood lactate concentration starts to decrease within five minutes of ceasing activity in hatchlings that have high blood lactate concentration. In a more recent study Hamann et al. (2007) found that the blood lactate concentrations of hatchlings were elevated during nest digging compared to just-hatched hatchlings. Hence, upward digging during nest escape is a discontinuous process, with short intense activity being separated by inactive rest periods which enable aerobic metabolism to convert lactate back to pyruvate which then is used in gluconeogenesis so that muscles can be prepared for the next bout of intense digging. The use of anaerobic pathways, and the need to recover from lactate accumulation before commencing a new digging bout might explain why the time between hatching and nest emergence (termed emergence lag) takes several days in turtle hatchlings.

In sea turtles, the final move to the nest surface usually occurs in the late evening or at night. Once hatchlings reach the nest surface, they typically remain slightly buried in the sand with heads partially exposed for a period of time (Dial, 1987). This resting time presumably allows hatchlings to reduce their blood lactate as discussed above. Predominantly nocturnal emergence has long been considered an adaptation of hatchlings to increase their early survivorship (Mrosovsky, 1968; Witherington et al., 1990). Nest emergence during the day would expose hatchlings to lethally high body temperatures and dehydration (Glen et al., 2006). Sea turtle hatchling locomotion activity becomes disorganized at body temperatures of 33.5 ˚C, 33.7 ˚C and 35.8 ˚C, in *Chelonia mydas*, *Dermochelys coriacea* and *Lepidochelys olivacea* respectively (Drake and Spotila, 2002), and at such body temperatures, hatchling may have difficulty reaching the water. Apart from avoiding heat stress, nocturnal emergence is also believed to reduce avian predation as hatchlings move from the

nest to the sea (Stancyk, 1982), and fish predation in water (Gyuris, 1994). Unlike sea turtles, the immediate pre-emergence behaviour of freshwater turtles from natural nests are poorly known (Kuchling 1999), but in artificial nests of smooth softshell turtle (*Apalone mutica*) hatchlings typically emerged synchronously at sunset (Plummer, 2007).

1.4 Aims and Hypotheses of the Current Research

I had four major aims in my study. Firstly, the theory of reproductive allocation dictates that the proportion of an organism's energy budget allocated to their offspring must be balanced against opposing expenditures (Congdon, 1989). In chelonians, females have to trade-off between the number of eggs produced and the amount of energy allocated to each egg. This energy allocation must be sufficient for embryonic development, nest escape and transport during early crawling and swimming phases before food sources are found. Hence the energy available to fuel nest escape and the early post-nest stage is the difference between the energy contained in the freshly laid egg and the energy used during embryonic development. I hypothesized that the fuel for nest escape comes from the residual yolk and as a consequence, the mass of residual yolk is reduced during nest escape. This hypothesis was tested by measuring the mass and the energy density of the residual yolk present in hatchlings using bomb calorimetry. These values were then compared between newly hatched hatchlings and post-digging hatchlings.

Secondly, to my knowledge, the only study to examine the energetics of nest escape in turtles used the doubly-labelled water (DLW) technique in olive ridley turtles (*Lepidochelys olivacea*) (Clusella Trullas et al., 2006), and none has attempted to quantify the energetics of nest escape in freshwater turtles. The DLW technique uses multiple blood sampling from the same animal over a period of time (Nagy, 1989). However, this multiple blood sampling disturbs the natural digging behaviour of turtles and also only gives a single integrated estimate of metabolic rate between blood sampling intervals. According to Speakman (1998), determination of the time–energy budgets requires detailed observations of animal behaviour while simultaneously measuring the energy expenditure preferably through indirect calorimetry (respirometry). Hence, I used open-flow respirometry to quantify the energetics of nest escape in hatchling turtles.

Thirdly, I investigated the influence of the number of clutch mates on nest escape process because turtles typically hatch synchronously within a clutch and as a consequence experience incidental association among clutch mates. The term 'social facilitation' has been used to describe within nest hatchling interaction during the digging out process in sea turtles as it requires the combined digging effort of many individuals to successful escape the nest (Hendrickson, 1958; Carr and

Hirth, 1961). Accordingly, the work of digging is shared amongst the individuals within a group so the energetic cost should also be shared. Therefore, if the group size is reduced, the workload per individual will increase and as a consequence, the energetic cost per individual should also increase. Given that in a normal group size, a substantial part of the energy reserve in the residual yolk is used to escape the nest, decreased group size should reduce this energy reserve further. This hypothesis was tested by splitting a clutch into different sized groups and measuring the time and combined metabolic cost of nest escape. This combined metabolic cost was then divided by the number of hatchlings in each group so that the average cost of nest escape per individual could be compared amongst different group sizes.

Fourthly, unlike sea turtles which typically construct their nest in beach sand, freshwater turtles can construct their nest in a wide range of soil types, ranging from fine sand to heavy clay (Vestjens, 1969; Ratterman and Ackerman, 1989; Kuchling, 1999; Hughes and Brooks, 2006; Booth, 2010). However, it is unknown if soil type influences the digging out process and the energy expenditure associated with nest escape, but it is anticipated that soil type will affect this process. Hence, I quantified the energy required by the same number of hatchlings to dig through two different soil types.

1.5 The Study Species

1.5.1 Brisbane river turtle

The Brisbane river turtle (*Emydura macquarii signata*) is commonly found in waterways of Southeast Queensland and northern New South Wales and is restricted to the eastern coastal plain due to the Great Dividing Range (Cann, 1998). The turtles used in the current study were sampled from a population inhabiting artificial lakes on the UQ St Lucia campus. Females typically nest between September and January during and after rainfall events. They nest at any time of the day but prefer to nest at night. At the St Lucia Campus, they normally construct their nest within 2 –10 m of the water's edge, but some may nest 50 m from the water (McCosker, 2004). They can construct nests in a wide range of soil type ranging from sandy loam to heavy clay (Booth, 2010) making them an ideal species in which to study the effect of soil types on the energetics of nest escape.

According to Booth (2010), the mean bottom nest depth of Brisbane river turtles averages 12.5 ± 0.5 cm deep (range 8-16 cm) and is constructed with the hind feet. Brisbane river turtle eggs are calciferous, ellipsoid in shape and white in colour upon deposition. A clutch of Brisbane river turtle may consist of between 12 and 30 eggs (mean clutch size 18 eggs)

(Booth, 2010). Within a clutch egg mass is relatively uniform but can vary from 4.7 g to 10.6 g between clutches (Booth, 1999). The incubation period is temperature dependent and varies between 42 ± 0.2 days at 31 ºC and 77.4 ± 0.4 days at 24 ºC (Booth, 1998b). However, incubation temperature does not affect hatchlings' phenotypes and the energetics of embryonic development in this species (Booth, 1998b).

1.5.2 Green turtle

The green turtle (*Chelonia mydas*) is found in tropical and subtropical oceans throughout the world (Seminoff, 2004). Female green turtles only nest once every 2-8 years, but typically produce 4-8 clutches of approximately 100 eggs in the season that they do breed (reviewed by Hirth, 1997). Eggs of green turtle are soft, spherical shaped and white in colour upon oviposition. Booth and Astill (2001) reported that incubation period of green turtle from Heron Island, Great Barrier Reef was dependent on incubation temperature taking 79 and 53 days to hatch at incubation temperatures of 26 ºC and 30 ºC respectively.

Green turtles dig substantial body pits before constructing their egg chamber, and nest depth averages 65.1 ± 8.6 cm (Rusli, 2011). Most of the nesting beaches used by green turtles are characterized by moderately sorted sand (mean particle size 0.2-1.0 mm) with low levels of organic carbon (Mortimer, 1990). Hence, unlike Brisbane freshwater turtle, green turtles nest in a relatively homogenous range of sands, and therefore it would not be ecologically relevant to conduct nest escape experiments in different soil types.

1.6 Significance of the Study

The biology of incubation in chelonian can be divided into a sequence of events: embryonic development, hatching, and nest escape. However, the measurement of the energy requirements of these early life events has been limited to embryonic development and hatching while the nest escape process has not been studied. In this study, open-flow respirometry and bomb calorimetric analysis were used to fill the knowledge gap on the energetics of nest escape process in chelonians. Further, I put the energetic cost of nest escape into an ecological context by comparing it to the energy in the freshly laid egg, energy used during embryonic development, quiescence period (time between hatching and beginning the digging out process) and the early activities after emergence from the nest (i.e. off-shore swim in sea turtles).

In efforts to increase hatchling production through active hatchery rehabilitation programs, the embryonic development of chelonians has received more attention than possibly any other aspect of their reproductive life-history. However, few studies have addressed the question of how turtle

hatchlings manage to escape from their underground nest. It has been two decades since the first evaluation of the effect of splitting sea turtle clutches under hatchery conditions has on nest emergence success (Mortimer et al., 1994). This study found that splitting clutches increased hatching success and has been the basis for hatcheries in Malaysia and other regions of the world routinely splitting clutches before incubation in managed hatcheries. Even though more than half a million turtle hatchlings are released from split clutches from sea turtle rookery in Malaysia annually (reviewed in Shanker and Pilcher, 2003), the question of whether or not this practice is producing the high-quality hatchlings needed to sustain a stable population of sea turtle is unknown. Hence, measuring the energetics of nest escape to evaluate the energy used by hatchlings to escape the nest was investigated in green sea turtle hatchlings (*Chelonia mydas*) to discover if group size affects this process so it could be related to the practice of splitting clutches in managed hatcheries.

Factors likely to affect nest escape time and energy expenditure include nest depth and clutch size, both of which depend on nesting female size, and nest soil characteristics which determine how difficult it is to dig through the substrate (Figure 1.1). Determining how these factors affect nest escape time and consequently, the energy expended by each hatchling in the digging out process could assist hatchery managers to develop methods to minimize energy expended by hatchlings in the nest escape process and thus produce higher quality hatchlings to release into the wild. Energy not expended during the nest escape process is available for post-nest activities such as the beach crawl, and frenzy swim used to escape the beach and transport hatchlings to the relative safety of off-shore waters. As such energy saved during the nest escape process may increase the chances of a hatchling's survival during the first few weeks after nest emergence.

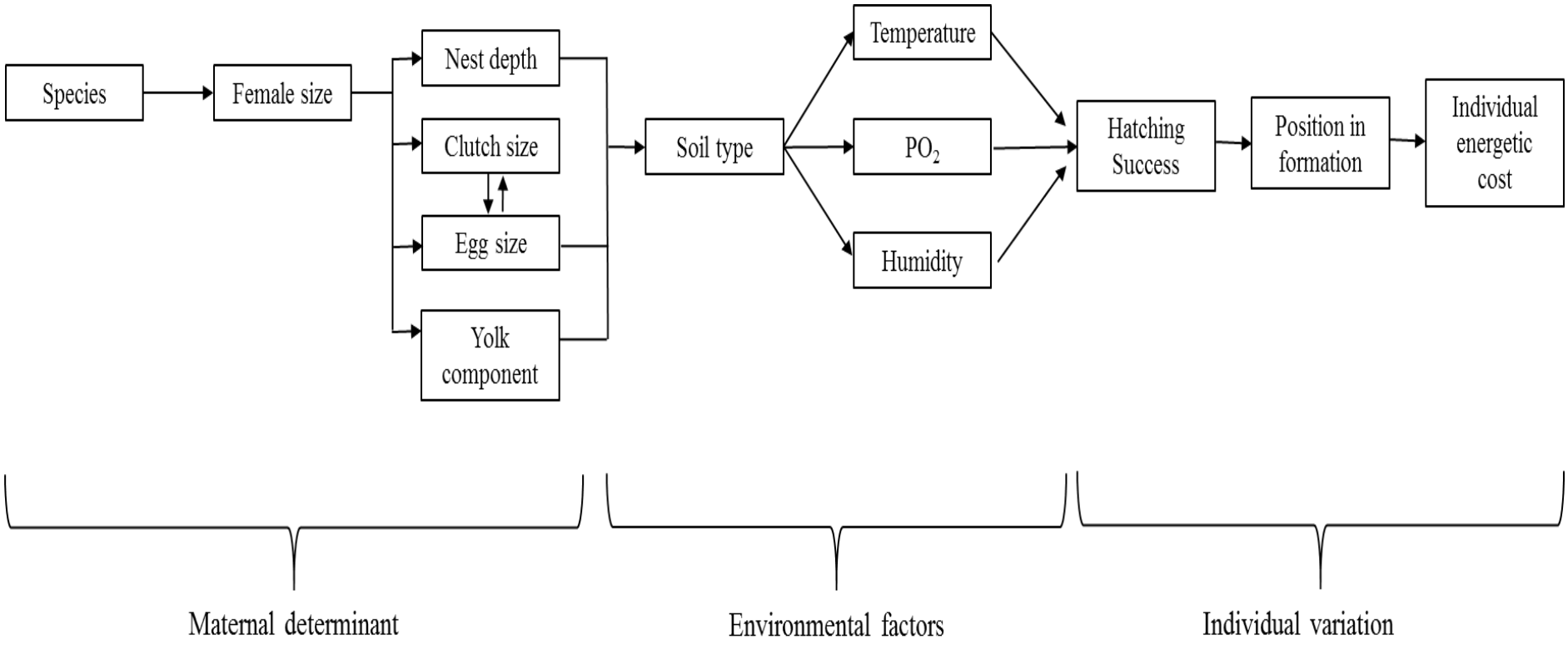

Figure 1.1. Potential drivers of variation in the energetics of nest escaping by turtle hatchlings. The key variables in determining nest escaping energetics of turtle hatchlings can be divided into three categories; (i) maternal factors (nest depth, clutch size and egg size), (ii) incubation environment (gas partial pressure, humidity, and temperature), and (iii) individual variation (hatching success, position in nest and during nest escape).

1.7 Thesis Structure

This thesis is structured as a series of data chapters, each designed to be published as a standalone research paper (Chapters 2-5), bookended by a general introduction chapter (Chapter 1) and a general discussion and future studies chapter (Chapter 6). As such there is some repetition in general themes in the introduction and discussion sections of some chapters, and also the methods sections.

The central theme of my study was to further understanding of the behaviour and energetics of nest escape in chelonian hatchlings. To achieve this aim, I asked three research questions (RQs):

I. How much energy do hatchlings use during nest escape, and how does this compare with energy used during embryonic development, and as a proportion of energy in the freshly laid egg and residual yolk of freshly hatched hatchlings?
II. Does synchronous hatching and subsequent group digging activity lower the per-individual energetic cost of nest escape?
III. What are the effects of soil types on the digging activity and energetic cost of nest escape in hatchling freshwater turtles?

I answered these questions in Chapters 2, 3 and 4:

Chapter 2 investigates the nest escaping behaviour and energetic expenditure of Brisbane river turtle hatchlings. In these experiments, Brisbane river turtle hatchlings were buried in a respirometry chamber in different group sizes and then their energy expenditure was measured as they dug out of fine river sand. The proportion of energy used from the residual yolk of freshly hatch hatchlings was also calculated (RQ I). This study is the first to test directly the long-standing interest in the hypothesis of 'social facilitation' in emergence from nest by turtle hatchlings (RQ II). This work has been published as: Rusli, M. U. and Booth, D. T. (2016). Bigger clutch sizes save offspring energy during nest escapes. Behavioral Ecology and Sociology, 70(4): 607-616.

Chapter 3 investigates the nest escaping behaviour and energetic expenditure of green turtle hatchlings during nest escape (RQ I). I provide an estimation of how much energy is spent by hatchlings throughout embryonic development and nest escape and compare this to the amount of energy in the fresh eggs and residual yolk of freshly hatched hatchlings (RQ I). Here I also test the hypothesis of 'social facilitation' but in a species that has a larger clutch size (RQ II). This work has been published as: Rusli, M. U., Booth, D. T. and Joseph, J. (2016). Synchronous activity lowers the energetic cost of nest escape for sea turtle hatchlings. Journal of Experimental Biology, 219:1505-1513.

Chapter 4 investigates differences in digging performance and energetic expenditure of Brisbane river turtle hatchlings digging through different soil types (fine sand vs. course sand) (RQ III). This work will be submitted to Australian Journal of Zoology for consideration of publication.

Chapter 5. During my work with green turtle hatchlings, I discovered a 'tonic immobility' behaviour which may prove to be a useful tool in future studies requiring the temporal immobilization of hatchlings. This chapter has been published as: Rusli, M. U., Wu, N. C. and Booth, D. T. (2016) Tonic immobility in newly emerged sea turtle hatchlings, Chelonian Conservation and Biology, 15(1): 143-147.

Chapter 6. In this chapter, I summarize my major research findings and their limitations, highlight the challenges encountered while conducting this research and suggest some directions for future research in the nesting biology of reptiles.

CHAPTER 2

Bigger clutch sizes save offspring energy during nest escapes

2.1 Introduction

Research on social facilitation while moving in a group originated with an observation of competitive cyclists riding faster by 25 % when accompanied by front line pacemakers (Triplett, 1899). In the animal kingdom, at least ten different patterns of moving formation have been identified and described with mathematical models (Eftimie et al., 2007). Some of the most remarkable examples of how moving in groups can reduce drag resistance around the adjacent individuals and thus save on the energetic cost of transport are observed in flying bird flocks and fish school (Hansell, 1993; Fish, 1995; Ebensperger and Bozinovic, 2000). The concept of energy saving by associating in groups is not restricted to locomotion effort, it also benefits stationary aggregations such as huddling penguins during freezing weather by reducing the cost of thermoregulation (Gilbert et al., 2008, 2010; Zitterbart et al., 2011).

Energy saving on one aspect of life history such as locomotion allows animals to spend energy on other functions, such as growth and reproduction thus potentially improving their survival and fitness. As chelonian hatchlings typically emerge from their underground nest simultaneously (as reviewed in Salmon and Reising, 2014) the term ‘social facilitation’ has been used to describe how synchronous hatching and nest emergence might enhance hatchling fitness in this taxa (Carr and Ogren, 1959; Carr and Hirth, 1961; Koch et al., 2007; Spencer and Janzen, 2011; Pignati et al., 2013). Sharing the work required to dig out of the nest across nestmates might also be a strategy to reduce the energy expended by individual hatchlings during the nest escape process. In this hypothesis, the larger the clutch size, the smaller the per-individual energetic cost will become.

Prolonged intense physical activity results in high energy demands and this could be challenging to vertebrate neonates if it happens immediately after hatching. Chelonian eggs are macrolecithal (i.e. they have a relatively large amount of yolk in their eggs) and the energy in the residual yolk after hatching provides sufficient energy to engage in post-hatching activities such as digging, crawling and swimming (Kraemer and Bennett, 1981; Booth and Astill, 2001; Clusella Trullas et al., 2006). Given that the amount of energy in the residual yolk is finite, the energy used during nest escape will detract from energy available to hatchlings for post-nest activities such as crawling to water or swimming once the water is reached. However, the nest escape process has been reported to last

about a week in turtle hatchlings, during which periods of intense digging activity are separated by rest periods (Bustard, 1967; Mrosovsky, 1968; Moran et al., 1999; Pignati et al., 2013).

Theoretically, hatchlings have to dig upward against gravity and they are not morphologically specialized as a digger, so their energetic cost of digging is likely to be greater than specialist burrowers (for a review, see Dorgan, 2015). Hence, the nest escape process in chelonian hatchlings might be energetically expensive, being fuelled principally by residual yolk which typically protrudes through the plastron in newly hatched turtles but is absorbed into the abdomen during the nest escape process. Because the cost of digging out from a nest is potentially expensive, females have to allocate energy to offspring beyond that required for embryonic development to fuel this and other post-hatching activities.

This study investigated the proportion of energy in residual yolk that is used during nest escape in the Brisbane river turtle *Emydura macquarii signata* by comparing the size and energy content of residual yolk in newly hatched hatchlings to that of hatchlings that have dug their way through a column of sand similar in depth to that of natural nests. I hypothesised that the fuel for nest escape comes from the residual yolk and as a consequence, the mass of residual yolk is reduced during nest escape. A second aim was to explore the effects of clutch size on an individual's energetic cost of nest escape. Because of the number of eggs laid in a clutch of the Brisbane river turtle can vary between 9 and 25 (Booth, 1999), I hypothesized that the greater the number of hatchlings in a nest, the greater the effect of the benefits of social facilitation would be, so that the individual energetic cost of nest escape would be reduced in larger clutches. This hypothesis was tested by comparing the time required to escape the nest and combined metabolic cost of nest escape across different sized groups of digging hatchlings. The combined metabolic cost from each group size was divided by the number of hatchlings in each group to determine the average individual energetic cost of nest escape.

2.2 Materials and Methods

Obtaining hatchlings

This study was approved by The University of Queensland Animal Ethics Committee (AEC approval number: SBS/133/13/URG). Ten gravid Brisbane River turtle (*Emydura macquarii signata)* were captured from The University of Queensland (St. Lucia Campus) Lakes during December 2013 and induced to lay eggs by intramuscular inject of synthetic oxytocin (activity = 10 iu ml^{-1}) at a dose of 2 ml kg^{-1}. Females were then placed in a plastic bin container (80 cm x 30 cm x

40 cm) in 10 cm depth water. Once eggs were laid, they were immediately removed from the water and labelled with a pencil before being buried in moist river sand for incubation. Females were returned to the lakes after oviposition. Eggs were incubated at a constant temperature of 28 ˚C but some clutches were initially incubated for one to two week at 24 ˚C to ensure that different clutches hatch at different times. The newly hatched hatchlings were weighed and marked prior to experiments and individuals marked by notching peripheral scutes with a nail clipper.

Measuring energy expenditure during nest escape

The newly hatched turtles (6-8 hours after hatching) were buried under a column of fine moist sand in a clear perspex cylindrical respiratory chamber 2.85 cm in radius and 30 cm in height. The cylinder was placed vertically as hatchlings naturally dig upward to escape from their nest. Because the total number of hatchlings used in each digging out trial differed, the depth of sand from the uppermost hatchling to the surface was standardized to 15 cm to ensure that hatchlings dug through the same volume of sand to reach the surface. Open-flow respirometry was used to measure the rate of carbon dioxide production ($\dot{V}CO_2$, ml h^{-1}) throughout the experiment. Outside air was pumped sequentially through a series of absorbent tubes (Soda Lime and Drierite; to scrub CO_2 and water vapour, respectively) and a mass flow controller (OMEGA, FMA5400/5500) regulated at an air flow of 100 ml min^{-1}. The dry CO_2 free air was supplied through the base of the respirometry chamber containing a group of hatchlings. The outflow air from the top end of the chamber was then directed through another drying column of drierite before entering a CO_2 analyser (PP Systems, SBA-5). The voltage output of the CO_2 analyser was connected to a computer via an analogue/digital converter (ADInstrumens, PowerLab 4/30). The ADInstruments Lab Chart 7 data acquisition software was used to sample the voltage output every 30 seconds. The CO_2 analyser was calibrated every three-hours with CO_2 free air and a precision CO_2 gas mixture.

The CO_2 production was calculated using equation 10.5 of Lighton (2008):

$$\dot{V}CO_2 = FR_i\,(F'_e\,CO_2 - F'_i\,CO_2) \,/\, \{1 - F'_e\,CO_2\,[1 - (1/RQ)]\}$$

where FR_i is the incurrent mass flow, $F'_i\,CO_2$ denote the fractional CO_2 concentration of the incurrent gas (in this case zero), $F'_e\,CO_2$ denote the fractional CO_2 concentration of the dry excurrent gas, and RQ which is the respiratory quotient. A RQ of 0.72 was used because lipid was assumed to the substrate metabolized during respiration. To record hatchling activity and their emergence time, two webcams, on opposite sides of the respirometry chamber were utilized so that hatchlings could be observed whenever they were near the clear wall of the respirometry. To minimize observer bias, blinded methods were used when all behavioural data were recorded and

analysed. Once individuals reached the surface, hatchling/s were removed from the chamber immediately and weighed. All experiments were performed in a 28˚C constant temperature room with 24 hours lights so that webcam imagery could be obtained continuously.

Three respirometry chambers were used simultaneously with one chamber consisting of sand with no hatchlings (hereafter known as 'blank chamber') to measure background microbial carbon dioxide production while the remaining two chambers contained hatchlings. Carbon dioxide production measurements were recorded for 10 minutes at a time in each chamber in sequence via a series of solenoid valves that were controlled through the "event manager" module in Chart 7 software. When swapping from one chamber to another it took 3 minutes for the gas to flush completely through the system so that only the last 7 minutes of $\dot{V}_{CO_2}$ measurement in a 10 minute cycle were used to obtain an average value for that measurement period. Carbon dioxide production of hatchlings was then calculated by subtracting the background microbial $\dot{V}_{CO_2}$ from the raw chamber $\dot{V}_{CO_2}$. Total energy expended by all hatchlings during the digging out process was calculated by first integrating the area under the $\dot{V}_{CO_2}$ versus time curve and converting this to units of energy by assuming a respiratory quotient of 0.72 and a CO_2 calorific equivalent of 1 ml CO_2 = 25.6 J (Withers, 1992). The rate of digging upward (m h^{-1}) was calculated by dividing the distance dug (0.15 m) by the time required to reach the surface (h). The total energetic cost per individual was calculated by dividing the energetic cost for the entire group by the number of individuals within the group. In the cases of hatchlings reaching the surface at different times, each individuals was taken out immediately once on the surface and, the calculation was adjusted using the remaining number of individuals still in the respirometry. Therefore, hatchlings that reached the surface at different times would have different total individual energetic cost as shown in Table 2.1.

Table 2.1. Example from Clutch 8 showing how total individual energetic cost of digging out was calculated when individual adjustment of the total CO_2 production prior converted to energy value in Joule.

Elapsed time (h)	Remaining hatchlings (n)	Total CO_2 produced (ml)	Total CO_2 produced adjusted per individual (ml)	Total CO_2 produced to reach surface per individual (ml)	Energetic cost of reaching surface per individual (kJ)
0.00 - 33.5	10	263.38	26.34	26.34	0.674
33.6 - 51.7	7	159.44	22.78	49.12	1.257
51.8 - 56.8	4	14.89	3.72	52.84	1.353
Clutch average cost per individual				42.57	1.094

Calculating the Net Cost of Transport (NCOT)

The net cost of transport (NCOT) was calculated by first subtracting the resting metabolic rate (RMR) (assumed to be at 0.64 ml O_2 h^{-1}; equivalent to 12.6 J h^{-1} per individual; Booth, 1998b) from the measured metabolic rate during the digging out trials to give the digging metabolic rate and the area under the f digging metabolic rate versus time curve used to calculate the net energy expended on digging ($E_{digging}$). $E_{digging}$ was then divided by the digging distance (0.15 m) to calculate NCOT (kJ m^{-1}).

Calculating residual yolk energy utilization

The total energy within residual yolk and the yolk-free carcass of hatchlings were determined. Hatchlings were divided into two groups, newly hatched (NH) and post digging (PD). NH hatchlings were collected and euthanized immediately after hatching from the egg, and the PD hatchlings were euthanized after they had gone through a digging trial as described in metabolic rate measurement section. Each hatchling was weighed to 0.1 mg before being euthanized by cooling to 3 $^{\circ}$C and then freezing. Hatchlings were dissected while still frozen to separate the residual yolk from the hatchlings' body and these components weighed separately to 0.001 mg. Both samples were dried to constant mass using a freeze drier. Dried samples of residual yolk were homogenized using a mortar and pestle, and the yolk-free carcasses were ground to a homogenous powder using a coffee grinder.

The energy density of dried samples of yolk and yolk-free carcasses was determined using ballistic bomb calorimetry. Triplicate sub-samples (0.1 - 0.2 g) of residual yolk and yolk-free carcasses of individuals were transferred to a metal thimble and fully combusted in 20 atmospheres of oxygen within a ballistic bomb calorimeter (Gallenkamp autobomb, England) to determine their energy density. The calorimeter was calibrated with thermochemical standard benzoic acid (26.442 J g^{-1} British Chemical Standard, Bureau of Analysed Standards Ltd, Newham Hall, Middlesbrough, UK) periodically throughout these analyses. Energy density is reported on a dry mass basis that includes the ash component. The energy used during the nest escaping process was calculated by subtracting the total energy in the residual yolk of PD hatchlings from NH hatchlings.

Statistical analysis

Spearman's correlation was used to explore the relationship between the group size and digging duration, while Pearson's correlation has been used to investigate correlation between duration of the digging out process (independent variable) with energy expenditure and mass loss of hatchlings. ANOVA or ANCOVA with hatchlings mass upon hatching as the covariate were used to compare energy density, and fractional water content (%) between yolk-free carcass and residual yolk, Clutch was included as a random factor in ANOVA and ANCOVA. Statistical significance was assumed if $p \leq 0.05$.

2.3 Results

Observations of digging activity

The digging behaviour of hatchlings could be monitored when individuals were digging near the respirometry chambers' wall. At the beginning of the experiment, newly hatched (6-8 hours after hatching) turtles were placed horizontally plastron down and stacked one on top of another on sand near the bottom of the chamber, and 15 cm of sand placed on top of the topmost hatchlings. When digging began, hatchlings moved their head upwards until their body was turned vertically (~90º from their starting position) and they retained this orientation throughout the digging process. Most of the time, both front and rear feet were used in intermittent asynchronous movements. Hatchlings used the ventral surface of their front feet to scratch for short periods (see supplementary video) to scratch down the sand ceiling. Meanwhile, the rear feet were involved in two distinct movements (i) stomping their feet in a downward motion to compact the sand under them and (ii) to push their body upward by stretching the hind limbs against the floor of the cavity they were in. While they stretched their limbs in this manner, their head was moved from side to side creating space within the sand column. The head was regularly seen to rest in this space. During these resting periods, hatchlings remained still, their limbs and neck motionless. Even though hatchlings were placed in the respirometry chambers at the same time, after they started to dig, hatchlings formed discrete groups containing several individuals while moving upward. The formation of these discrete groups was clearly established by the time they reached half-way to the surface. As a consequence of forming discrete groups, synchronous digging activities only occurred between individuals within the same group. On reaching the surface hatchlings rapidly broke through and moved continuously on top of the sand surface until they were removed from the chamber which in these trials occurred within five minutes of them surfacing.

Digging duration

In general, the nest escaping process consisted of numerous digging bouts that were separated by long pauses. These intermittent digging activities were characterized by 2-3 strokes of front limb digging (mean ± SE: 5.4 ± 0.18 s, *n* = 52) interspersed by a period of rest (35.8 ± 1.35 s, *n* = 52). The nature of this digging behaviour was not consistent among trials with some hatchlings digging almost continuously, while in others the resting period lasted 6 - 15 minutes. Hatchlings took between 12.2 and 162.8 hours to dig up through the 15 cm of sand (Table 2.2). In most trials, the period between first emergence and the last emergence to the surface was less than 12 hours, and in only one trial (Clutch no. 7) was this time interval greater than 24 hours.

Table 2.2. Time taken for the fastest, slowest and average individual to dig upwards through 15 cm of moist sand and average digging rate during these trials.

Clutch no.	Clutch size (n)	Fastest individual (h)	Slowest individual (h)	Difference between fastest and slowest (h)	Avg. Digging Duration (h)	Average Digging Rate (mm h^{-1})
1	10	37.00	49.77	12.8	41.5 ± 1.4	3.62
2	14	39.94	49.94	10.0	47.7 ± 1.0	3.14
3	11	12.20	12.20	0.0	12.2 ± 0.0	12.30
4	6	57.18	74.00	16.8	67.7 ± 3.3	2.22
5	6	49.67	55.67	6.0	50.7 ± 1.0	2.96
6	12	28.75	31.00	2.3	30.6 ± 0.3	4.90
7	4	112.25	162.77	50.5	137.5 ± 14.6	1.10
8	10	33.17	56.83	23.7	48.2 ± 3.3	3.11
9	4	59.33	59.67	0.3	59.5 ± 0.1	2.52
10	10	33.83	34.22	0.4	34.1 ± 0.1	4.40
Average	8.70	46.33	58.61	12.28 ± 3.9	52.97 ± 16.8	4.03 ± 1.3

An average digging duration per clutch was used to investigate the relationship between digging duration and clutch size. Spearman's correlation analysis found a negative correlation between clutch size and digging duration with larger groups having shorter digging durations and this relationship persisted even if the data points for a clutch size of 4 are removed from the analysis (Figure 2.1A). Although clutch sizes as small as four are not laid in wild, mortality of embryos during incubation does occur and this can reduce the number of hatchling within a nest to below the original number of eggs laid.

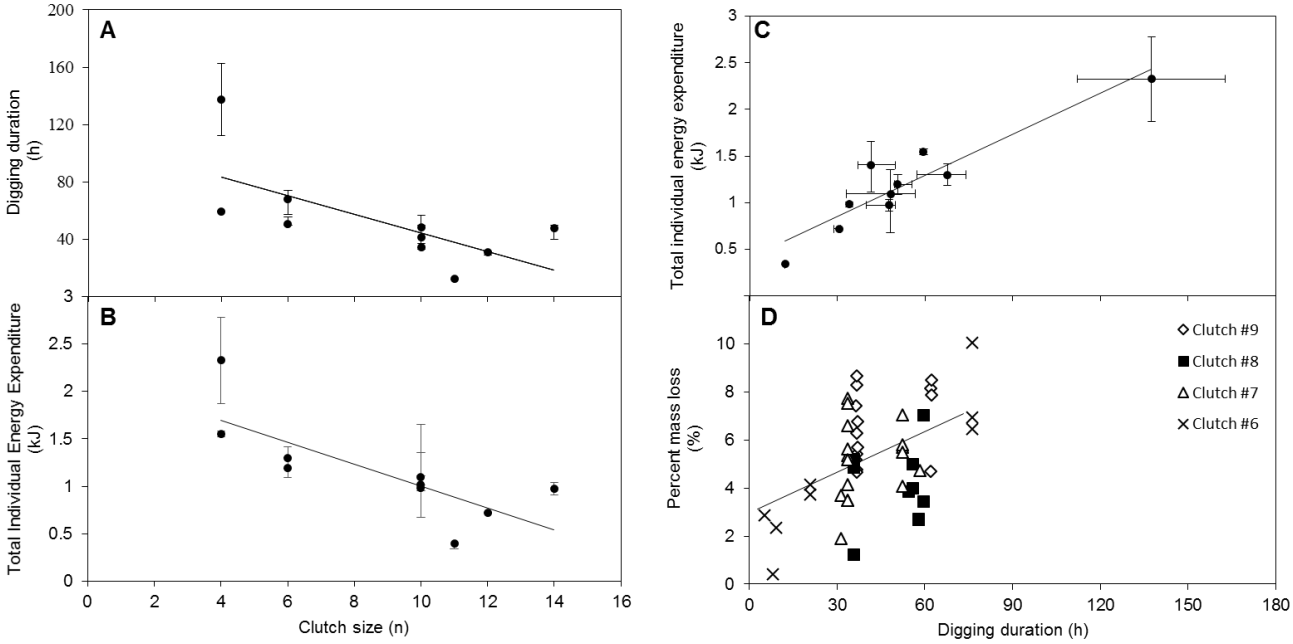

Figure 2.1. (A) Relationship between mean digging duration and clutch size (y = -6.499x + 109.51, r^2 = 0.452, p = 0.005, n = 10). Vertical bars = range of digging times amongst individuals from within a clutch. (B) Relationship of total individual energy expenditure during the digging out process and clutch size (y = -0.115x + 2.16, r^2 = 0.6, p = 0.001, n = 10). Vertical bars = range of total individual energetic expenditure amongst individuals from within a clutch. (C) Relationship of total digging duration (h) and the energetic cost (kJ) of nest escape (Pearson correlation, r = 0.928, n = 10, r^2 = 0.86, p < 0.001). The regression equation: Energetic cost (kJ) = 0.015 (Duration) + 0.41. Vertical bars = range of total individual energetic expenditure amongst individuals from within a clutch. (D) The relationship of hatchlings' digging duration (h) and mass loss (%) from four clutches. Regression line (y = 0.056x + 2.99) represent relationship between digging duration and percentage mass loss after the nest escape process, r = 0.445, r^2 = 0.20, p = 0.001, n = 49).

Because hatchlings formed discrete groups that were separate from each other during the digging out process, and at any moment in time the activity of these groups was asynchronous (i.e. one group could be resting, and another group actively digging) it was not possible to determine a "resting" $\dot{V}_{CO_2}$ and an actively digging $\dot{V}_{CO_2}$ as was originally planned. However, for the purpose of estimating the NCOT, a resting metabolic rate (RMR) value was obtained from Booth (1998b) as mentioned in the method section. For this reason only the average $\dot{V}_{CO_2}$ over the entire digging out trial (Figure 2.2) was calculated and these values converted to energy units.

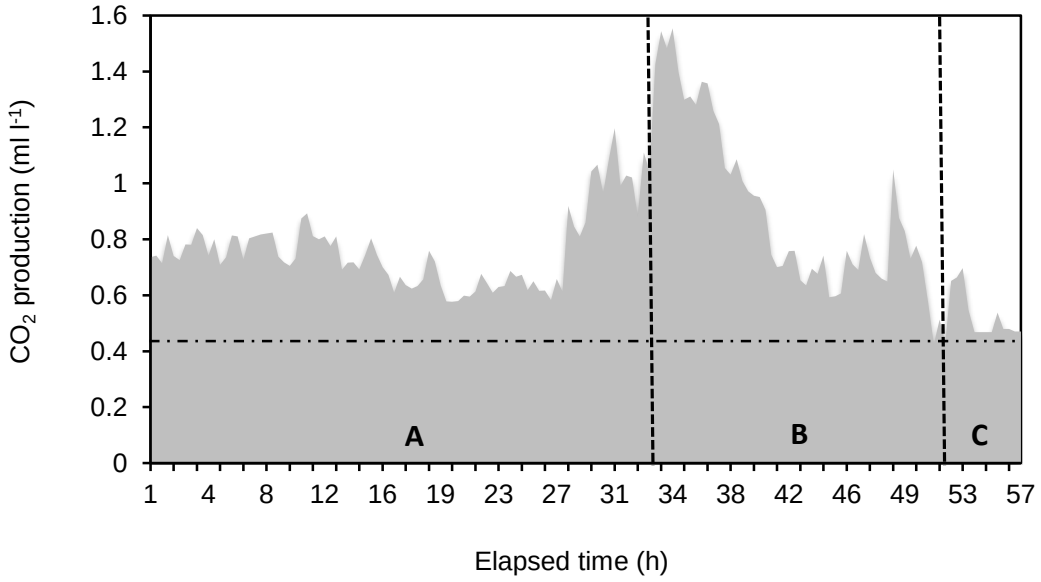

Figure 2.2. An example of an individual CO_2 production from Clutch 8 throughout the entire digging trial. The total energy consumption was calculated by integrating the area under the rate of CO_2 production versus time curve before convert to units of energy (J) as described in the methods section. Letters in the middle of each integrated area represents the total CO_2 produced adjusted per individual within the time frame (A = 26.34 ml, B = 22.78 ml, C = 3.72 ml). Horizontal line in graph at 0.46 ml CO_2 h^{-1} represents the RMR value and has contributed approximately 42.1 % from the total energy consumption, E_{total}.

Energy expenditure and NCOT during nest escape

The total energy expenditure obtained from the respirometry method throughout the digging out process varied between 0.34 and 2.32 kJ per individual and was dependent on clutch size (Figure 2.1B). The duration of the digging out process was positively correlated with energy expenditure (Figure 2.1C). However, there was no significant influence of hatchling mass on the total energy expenditure (ANOVA $F_{1, 9} = 0.063$, $p > 0.05$). RMR was estimated to contribute between 28.5 and 57.7 % of energy expended during the digging out process (Table 2.3). Hence, the higher energetic cost can be attributed to the longer time spent in the nest digging as opposed to differences in body mass. Further, the NCOT calculations ranged from 1.7 to 5.6 kJ m^{-1} per individual and were dependent on the clutch size (Figure 2.3).

Table 2.3. Percentage of energy expenditure for the RMR and the estimation of NCOT by Brisbane river turtle for nest escaping process.

Clutch size	E_{total} [Total individual energy expenditure (kJ)]	$E_{resting}$ [RMR energy expenditure (kJ)]	$E_{digging}$ [Digging energy expenditure (kJ)]	Proportion RMR (%)	Proportion digging (%)	NCOT (kJ m^{-1})
4	2.32	1.62	0.70	69.8	30.2	4.7
4	1.55	0.70	0.85	45.2	54.8	5.6
6	1.3	0.80	0.50	61.3	38.7	3.4
6	1.2	0.60	0.60	49.7	50.3	4.0
10	1.09	0.49	0.60	44.8	55.2	4.7
10	0.98	0.57	0.41	57.9	42.1	3.5
10	1.41	0.40	1.01	28.5	71.5	3.9
11	0.34	0.14	0.20	42.3	57.7	1.7
12	0.72	0.36	0.36	50.1	49.9	2.4
14	0.97	0.56	0.41	58.0	42.0	2.7

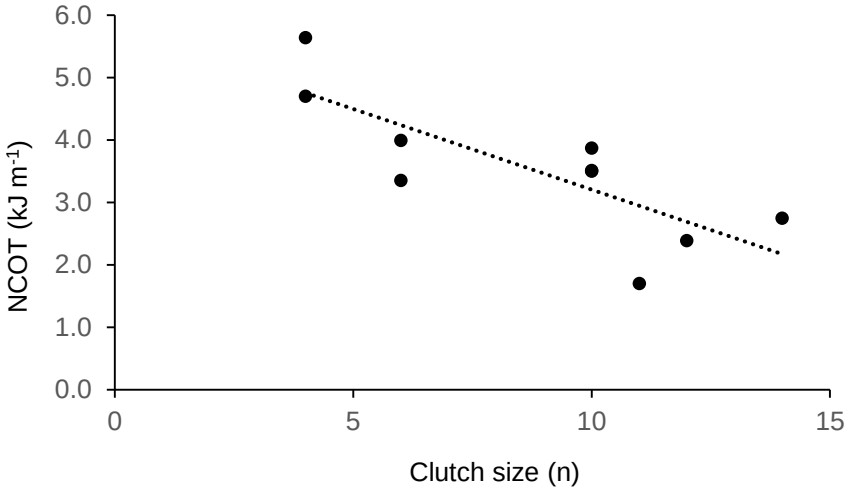

Figure 2.3. Relationship between NCOT and clutch size (y = -0.259x + 5.8, r^2 = 0.63, p = 0.001, n = 10).

Mass loss during the nest escapes process

Hatchlings lost less than 10 % of their initial mass during the nest emergence period (Mean: 5.2 ± 0.3) and there was a positive correlation between digging duration and mass lost during digging (Figure 2.1D).

Residual yolk utilization

Wet mass of residual yolk from NH and PD hatchlings was compared by ANCOVA in which the whole body initial mass of hatchlings was the covariate. Newly hatched hatchlings had larger residual yolks compared to post digging hatchlings (ANCOVA, $F_{1, 36}$ = 7.656, p = 0.009, Figure 2.4).

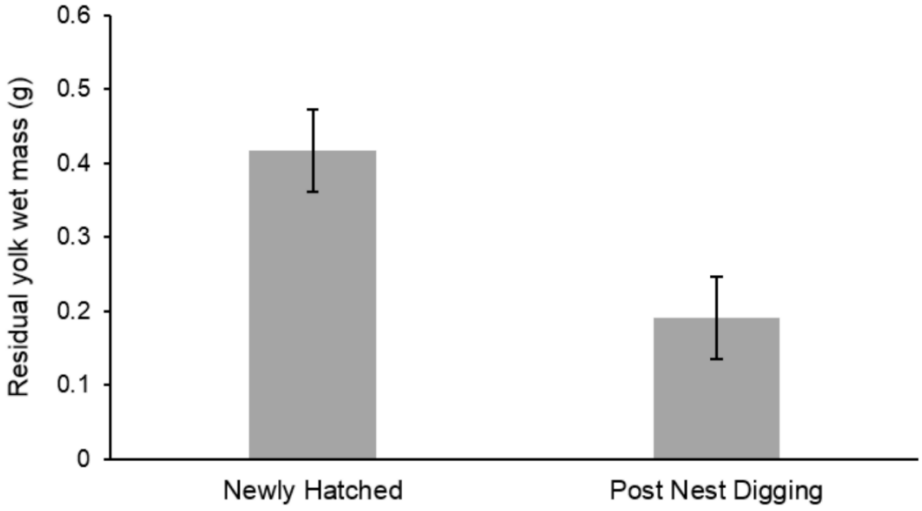

Figure 2.4. Comparison of least square means residual yolk wet mass between the newly emerged and post nest digging hatchling Brisbane river turtles. Error bars represents standard error.

Water fraction within residual yolk (49.0 ± 12.2 %) and yolk-free carcass (72.5 ± 1.7 %) was similar in newly hatched and post digging hatchlings (yolk-free carcass ANOVA, $F_{2,36} = 0.144$, $p = 0.707$; residual yolk ANOVA, $F_{2,36} = 0.349$, $p = 0.559$). However, yolk-free carcass water fraction was independent of body mass, while the water fraction of residual yolk increased with hatchling wet mass (Figure 2.5).

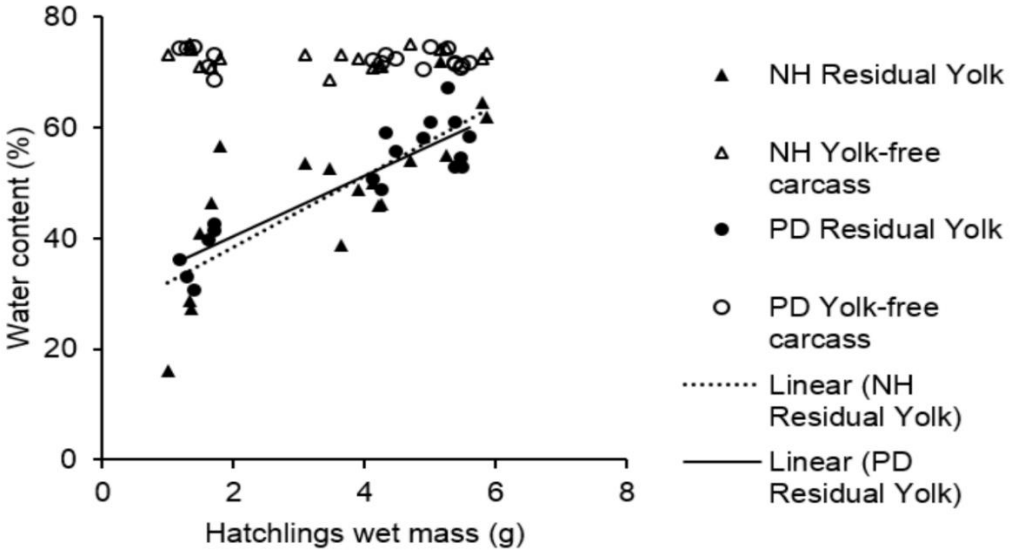

Figure 2.5. Relationships between percent water content of residual yolk and yolk-free carcass and hatchling wet mass. Regression lines represent relationship between residual yolk percent water content and hatchling mass in newly hatched hatchlings (NH) (y = 6.409x + 25.666, $r^2 = 0.584$, $p < 0.001$, $n = 18$) and Post digging hatchlings (PD) (y = 5.489x + 29.284, $r^2 = 0.820$, $p < 0.001$, $n = 18$). Yolk-free water content was not correlated with hatchling mass ($r^2 = 0.007$, $p = 0.62$, $n = 36$).

There were no differences in energy density of yolk-free carcasses (ANOVA, $F_{2, 18} = 1.618$, $p = 0.212$) or residual yolks (ANOVA, $F_{2, 18} = 0.026$, $p = 0.873$) between newly hatched and post digging hatchlings (Table 2.4). However, there was a difference (ANOVA, $F_{2, 72} = 175.817$, $p < 0.05$) in the energy density of residual yolk and yolk-free carcass. Dry mass of yolk-free carcass was similar in newly hatched and post digging hatchlings (ANCOVA, $F_{1, 36} = 3.72$, $p = 0.062$), while residual yolk dry mass was greater in newly hatched compared to post digging hatchlings (ANCOVA, $F_{1, 36} = 7.791$, $p = 0.009$). There was no difference in the calculated total energy in the yolk-free carcass (ANCOVA, $F_{1, 36} = 3.72$, $p = 0.062$), conversely there was a significant difference in residual yolk (ANCOVA, $F_{1, 36} = 7.69$, $p = 0.009$) between NH and PD hatchling groups. Further, the PD group was not classified according to the number of hatchlings involved in digging trials as no significant difference were found (ANCOVA, $F_{2,18} = 1.106$, $p = 0.358$), hence data were pooled and represented by the average value. Therefore, by assuming that hatchlings only relied on their residual yolk to fuel their nest escape, an average of 3.22 kJ (50 % of residual yolk energy) of energy was used during the nest escape process (Table 2.4).

Table 2.4. Total energy contained within hatchlings components based on energy density (calculated on a dry mass basis including ash) and dry mass. Data are mean ± SE (energy density) and least square ANCOVA adjusted means hatchlings mass at 5.21 g.

Group	N	Energy Density (kJ/g)	Dry Mass (g)	Calculated Total Energy (kJ)
Yolk-Free Carcass				
Newly Hatched	18	22.74 ± 0.30	1.183 ± 0.02	26.90
Post digging	18	23.23 ± 0.24	1.239 ± 0.02	28.78
Residual Yolk				
Newly Hatched	18	31.81 ± 0.57	0.203 ± 0.03	6.46
Post digging	18	32.02 ± 1.18	0.101 ± 0.03	3.23
Energy used During digging				3.23

2.4 Discussion

Digging Activity

Hatchlings did not dig continuously, relatively short bouts of digging were separated by relative long breaks of inactivity. According to Seymour (1973), there are two advantages of intermittent digging; (i) this process is more economic in terms of energy usage and (ii) keeping bouts of intense activity which are presumably powered in part by anaerobic metabolism, to short bursts of digging prevents the build-up of a larger oxygen debt, and the inactive period allows sufficient time to pay back the oxygen debt before the next digging bout begins. In this study, both small and large groups of hatchlings used the intermittent digging strategy, but there was a difference in terms of time needed to dig the 15 cm to the surface. The smaller groups spent a longer time resting between digging bouts so they probably accrued a larger oxygen debt during the digging bout compared to larger groups. This larger oxygen debt might result from more intense digging as the digging work is shared across fewer individuals. The net effect is it takes larger groups a shorter time to dig out of a nest not because they dig faster, but because they have shorter rest periods between bouts of digging. Further, both the RMR contribution and the digging contribution to total energy expenditure increased with decreasing clutch size (Table 2.3). The RMR contribution increased because the digging duration time increased as clutch size decreased, and the digging contribution presumably increased because of an increase in digging intensity associated with smaller clutch sizes. Interestingly, the proportion of energy spent on RMR and digging was similar across all clutch sizes and averaged 50.8% for RMR and 49.2% for digging (Table 2.3). Presumably the reason why turtle hatchlings do not dig continuously during nest escape is that the muscles fatigue

due to the accumulation of lactic acid. Compared to mammal and bird species, reptiles have a lower aerobic capacity because of the limitation in respiratory and cardiovascular systems (Schmidt-Nielsen, 1997). In particular, chelonian hatchlings would accumulate lactate in blood during periods of intense activity (Dial, 1987; Baldwin et al., 1989; Hamann et al., 2007; Pereira et al., 2012), but blood lactate was not measured in the present study. My data suggest that the bigger group size most probably required less resting time because they accumulated less lactate in their blood during digging bouts. This hypothesis needs to be experimentally tested in the future.

Webcam recordings showed that the digging movement of one hatchling typically triggered the start of digging in other adjacent hatchlings so that several hatchlings dug simultaneously during a digging bout. The same behaviour has been reported for sea turtle hatchlings during nest escape (Mrosovsky, 1968; Moran et al., 1999). The lead hatchlings that triggered the activity were not necessarily positioned on the top of their group. The group response could also be initiated by hatchlings in the middle or on the edge of their group. However the cue used by a hatchling to start a digging bout is unclear, but may be related to the fall of blood lactate concentration to below a threshold level.

The emergence pattern in terms of the number of hatchlings reaching the surface almost simultaneously varied among trials and was not correlated with clutch size. Hatchlings generally emerged onto the surface together in a few separate small groups, and typically all hatchlings within a clutch emerged within 12 hours of the first individual emerging (Table 2.2). This indicates that hatchlings moved up through the sands column together in the general close proximity of each other.

Typically, chelonian hatchlings tend to emerge from the nest synchronously, and it has been hypothesized that this serves to swamp predators (Bustard, 1972) and thus decrease the probability of an individual being predated (Dehn, 1990). However, the present study reveals there are two other advantages to digging out of the nest in a group synchronously. Larger groups took less time to dig out of the nest (Figure 2.1A), and also expended less energy per individual while escaping the nest (Figure 2.1B). A previous study on *E. macquarii* also found that groups of 10 hatchlings escaped the nest faster compared to single hatchlings (Spencer et al., 2001). Given that in respirometry analysis hatchlings spent between 0.34 and 2.32 kJ per individual (Figure 2.1B) to escape, this constitute approximately 5.3-36 % of their residual yolk reserved energy (as calculated in Table 2.4). From these findings it can be inferred that hatchlings emerging from nests of a larger clutch size have more energy reserves to enter the next stage of their life cycle compared to hatchlings emerging from nests with small clutch size.

Social facilitation

The individual hatchling energy expenditure obtained in this study was calculated as an average using the numbers of hatchlings within in a clutch. Theoretically, in moving forward as a bunch against a resistance such as soil, the leading edge animals would spend more energy than other group members (Fish, 1995). This strategy is used in human bicycle races in which pacemaker riders often sacrifice their energy in order to conserve the energy of the following riders (Trenchard et al., 2015). Similarly, Northern bald ibis (*Geronticus eremita*) were found to share the workload of being the lead bird in a flying formation by swapping positions while flying in echelon formation (Voelkl et al., 2015). In the current experiment, the leading edge individual(s) was not identifiable, but it would be interesting to see if this position was maintained by the same individual throughout nest escape or swapped around between different individuals to share the workload.

Previous studies have claimed the reduction of an individual's total energetic cost in subterranean animals moving through sand when they shared the workload of burrowing between several individuals (Hansell, 1993; Ebensperger and Bozinovic, 2000). One of the most remarkable examples occurs in naked mole rats (*Heterocephalus glaber*) which form a chain of diggers while constructing burrows. *Heterocephalus* often live in colonies of more than 40 individuals huddling behaviour while building tunnels reduces an individual's metabolic cost (Withers and Jarvis, 1980; Lovegrove, 1989). In chelonian, social behaviour begins earlier in the subterranean nest when synchronous hatching occurs among sibling eggs. Remarkably, despite thermal gradients in nests causing differences in embryonic development, embryo use multiple cues; namely vocalizations, physical disturbance, hypoxia and temperature change to induce synchronous hatching (Spencer et al., 2001; reviewed in Doody, 2011). It is clear that this synchronous hatching behaviour could facilitate communal digging and share digging effort among siblings.

E. m. signata typically construct their nest within 2-10 m of the water's edge in either soil or clay medium (Booth, 2010). As egg incubation usually takes 2-3 months (Booth, 1998b), nesting soil would become compacted during this period. As shown by Horrocks and Scott (1991), hawksbill sea turtle (*Eretmochelys imbricata*) hatchlings had a decreased emergence success with an increase in soil compaction. Hence, another plausible explanation for synchronous hatching is that it may facilitate digging through compacted soil. An experimental approach is needed to provide more definitive information about hatchling energetic cost and fitness consequences of female nest site selection with respect to soil type and compactness.

The current study found that it is an advantage having a larger group in the nest as it decreases the energetic cost of digging out of the nest for individuals, suggesting that females should attempt to

maximize their clutch size during a reproductive bout. On the other hand, it is thought that larger offspring are generally fitter than smaller offspring (Smith and Fretwell, 1974). Hence, there may be a trade-off between clutch size and hatchlings size if a female has a fixed amount of resources to allocate to reproduction, with many smaller individuals saving on the energy needed to dig out of a nest, but smaller individuals having lower fitness once they leave the nest. This aspect warrants further investigation because physically larger individuals may be more efficient diggers than smaller individuals, so data on relative digging efficiencies in terms of energy expenditure over a range of hatchling sizes is needed. Freshwater turtles would make good models to study this question because the same populations of breeding females can produce a broad range of egg and clutch sizes (Booth, 1998a).

While having a larger group might be energetically beneficially for chelonian hatchlings during nest escape, asynchronous nest emergence is widespread among chelonian species (Witherington et al., 1990; Hays et al., 1992; Houghton and Hays, 2001). Asynchronous nest emergence is thought to be due to asynchronous hatching caused by thermal gradients within nests, which are more likely to affect shallow nesters (i.e. freshwater species compared to the sea turtles). For example, temperature differences in the Murray river turtle, *Emydura macquarii* nests were found to reach 6 ˚C between top and bottom eggs (Thompson, 1989). Accordingly, Hays et al. (1992) proposed that early hatched hatchlings may exhibit a 'waiting period' in order to take advantage from synchronous digging effort, but this waiting also incurs an energetic cost in the form of maintenance metabolism during the waiting period. An estimate of the energetic cost of the dig alone as soon as you hatch strategy compared to the wait and dig together with your clutch mate strategy can be made. A hatchling digging alone would consume approximately 2.05 kJ, while a hatchling in a group of 14 would consume just 0.59 kJ (Figure 2.1B), a difference of 1.46 kJ. A full-term Brisbane river turtle embryo consumes approximately 0.303 kJ per day (calculated from data in Booth, 1998b), so an embryo could wait 4.8 days until its clutch mates also hatched and spend the same amount of energy in nest escape. Hence, if the wait period is less than 4.8 days, from an energy expenditure point of view, a hatchling is better off waiting for its clutch mates before commencing the nest escape process.

The utilization of residual yolk

Mass was lost from hatchlings when digging out of the nest, and this loss was greater when the digging out period was longer (Figure 2.1D). Bennett et al. (1986) explain that mass loss in chelonian hatchlings can be attributed to the utilization of yolk and to water loss. Thus, water loss could possibly be a major contributor to the mass loss. However, in the current study there was no change in hydration state between the newly hatched and the post-emergence groups, but there was

a decrease in dry yolk matter (0.14 g) between newly hatched and post-digging hatchlings indicating that the observed mass loss was most likely caused by yolk metabolism with mass loss due to respiratory gas exchange and loss of metabolically produced water.

Despite the fact that a significant amount of residual yolk was metabolized while digging, there appears to be no change in the chemical constituents of this yolk as indicated by no significant difference in its energy density of newly hatched (31.8 kJ g^{-1}) and post digging (32.0 kJ g^{-1}) hatchlings (Table 2.3). The difference in energy density between residual yolk and yolk-free carcass can be explained by differences in chemical composition between these two components. Previously, it was postulated that residual yolk has a higher proportion of energy-rich lipid compared to yolk-free carcass, and that the inorganic mineral component which has no combustible energy content is greater in the yolk-free carcass because of the skeleton (Speake and Thompson, 2000; Speake et al., 2003).

In the context of the reproductive energy allocations of this species, Booth (2003) estimated that the amount of energy in a fresh egg is 4.17 kJ g^{-1}. Thus for a typical 8.0 g egg (size range 5.0-10.6 g), egg energy content would be 33.4 kJ. While according to Booth (1998b) the total energy expended during embryo development is 11.52 kJ (~ 35 % of initial energy content) and in current study I found that the digging process consumes 3.23 kJ (approximately 10 % of initial energy content, and approximately 50 % of the energy remaining in the residual yolk at hatching). Thus hatchlings have 55 % of the initial egg energy content for post-nest activities such as predator avoidance, foraging, and early growth.

2.5 Conclusions

The estimated energetic cost of individuals by digging for *E. m. signata* hatchlings are 0.34-2.32 kJ per individual depending upon the number of hatchlings digging together. Although their energetic cost consumed at least 50 % of the remaining energy in the residual yolk, the amount of energy consumed is reduced by synchronous digging of many individuals during nest escape. The present study concludes that the clutch size and the time spent digging within the nest column are important determinants of the energetic cost while digging out of the nest.

CHAPTER 3

Synchronous activity lowers the energetic cost of nest escape for sea turtle hatchlings

3.1 Introduction

Grouping of individuals of the same species resulting in mutual benefits is common in animals. Experimental data from a broad range of taxa show that there are at least four mutual benefits that can result from aggregation behaviour; (i) decreased chance of predation (Colbert et al., 2010; Creel et al., 2014; Unglaub et al., 2013), (ii) increased feeding efficiency (Horst, 1995; Houston, 2008; Hsia and Wood-Gush, 1982; Lazarus, 1979), (iii) increased locomotion efficiency (Ebensperger and Bozinovic, 2000; Fish, 1995; Voelkl et al., 2015), and (iv) decreased energy spent on thermoregulation (Gilbert et al., 2008; Gilbert et al., 2010; Nunez-Villegas et al., 2014; Withers and Jarvis, 1980). However, aggregation behaviour may have more than one function within a single species (Lazarus, 1979). For example, formation flight by migrating birds might increase their flight range (Portugal et al., 2014; Voelkl et al., 2015) but also decrease the chance of predation by way of a dilution effect as the group size increases (Dehn, 1990). Specifically for chelonian species, Chapter 2 has demonstrated the energetic cost of the Brisbane river turtle decreased as the number of individuals digging together out of the nest increased.

Although sea turtles are typically considered to be solitary species, they also exhibit grouping behaviour (Carr and Hirth, 1961; Hendrickson, 1958) when leaving the underground nest after hatching from a clutch of about 50-150 eggs (Spotila, 2004). Because many eggs within a clutch experience a similar incubation temperature, they hatch almost synchronously with their clutch mates, and at least some species are able to stimulate the hatching of clutch mates if the process has not already begun (Spencer et al., 2001). Synchronous hatching within a clutch results in incidental association among clutch mates and allows simultaneous digging activity of individual hatchlings. Chapter 2 showed that the energetic cost of digging in Brisbane river turtle (*Emydura macquarii signata*) hatchlings decreased as the number of individuals digging together increased. Hence, in the current chapter I explore the effects of clutch size on a species that has larger clutch size, but constructs deeper nests, the green turtle (*Chelonia mydas*).

The term ‘social facilitation’ has been used to describe synchronous digging in sea turtle hatchlings along with the scramble of hatchlings from the nest to the sea (Carr and Hirth, 1961; Hendrickson, 1958), but I only discuss this concept with respect to the nest escape process. Social facilitation may facilitate nest escape because the synchronous digging effort of many individuals might be needed to dig successfully through the column of sand above the nest chamber (Hendrickson 1958, Carr and Hirth 1961). Sea turtle hatchlings do not dig continuously while escaping the nest; periods of intense digging activity are interspersed with periods of rest (Carr and Hirth, 1961; Drake and Spotila, 2002). At the end of a rest period, the spontaneous digging activity of one individual triggers the individuals around it to also start digging resulting in cohort-wide synchronous digging (Bustard, 1967; Carr and Hirth, 1961; Gyuris, 1993). Digging activity is also inhibited by high temperatures. Hence, when hatchlings approach the surface during the day when the sun is shining, the hot sub-surface sand inhibits digging (Bustard, 1967; Gyuris, 1993). After the sand cools in the late afternoon or at night, digging activity can resume and this is the reason why most sea turtle hatchling emergence events occur at night or during cool cloudy days (Bustard, 1967; Gyuris, 1993). The group digging behaviour is hypothesised to result in sea turtle hatchlings emerging from the nest in large cohorts, which can result in a predator swamping phenomenon that may decrease the overall predation rate of hatchlings as they leave the beach and swim out to sea (Bustard, 1972).

Early reports of upward digging activity in sea turtles hatchlings were based primarily on excavation of nests during the hatch to surface period (Hendrickson, 1958), and observations made through a glass pane inserted into nests (Carr and Ogren, 1959). Bustard (1972) suggested that hatchlings may actively dig upwards through the nest column at any time during day or night. Recent studies (Baldwin et al., 1989; Dial, 1987; Hamann et al., 2007) have used hatchlings’ blood lactate concentration as an index of digging intensity, but were unable to relate these values to the overall energetics of the nest escape process. Another study addressed the energetics of nest escape in olive ridley, *Lepidochelys olivacea*, hatchlings using the doubly labelled water (DLW) technique (Clusella Trullas et al., 2006). These authors estimated that the energy used by olive ridley hatchlings digging out from 25 cm below the surface in a cohort of 20 individuals was 39.52 kJ per individual. However, this approach required the taking of multiple blood samples, which disturbed the natural digging behaviour of turtles.

The fixed amount of energy available in the freshly laid egg is the only energy available for embryonic development, nest escape, beach escape, and the initial swim frenzy of sea turtle hatchlings. Hence, any energy saved during these progressive stages will ultimately be available for the energetically demanding initial swim frenzy before hatchlings reach oceanic waters where feeding behaviour begins. In sea turtles, it is thought that a substantial proportion of the energy

reserve in the residual yolk is used during hatchling dispersal (i.e. nest escape, crawling from nest to the beach and early swimming effort) (Clusella Trullas et al., 2006; Kraemer and Bennett, 1981), and the social facilitation hypothesis suggests that this energy will vary depending on cohort size. This hypothesis may be tested experimentally by measuring energy expenditure during the digging out process across different cohort sizes. In the current study, I investigated two aspects of the social facilitation hypothesis: (i) larger cohort size decreases the time taken to dig out of the nest, and (ii) the energetic cost per individual decreases as the cohort size increases. I also evaluate the energy used during nest escape so it can be placed in the context of the total energy available in the egg, and the proportion of that energy used during embryonic development, nest escape, and the off-shore swim.

3.2 Materials and methods

Animals

The whole study procedure was approved by The University of Queensland Animal Ethics Committee (AEC approval number: SBS/133/13/URG). Getting green turtle hatchlings to dig in a natural way within respiratory chambers was problematic, and several iterations of different protocols were trialled before a protocol that worked was discovered. In these 'developmental' trials, four clutches of the green turtle (*Chelonia mydas*) eggs were collected (Department of Environment and Heritage Protection, Queensland, Permit no: WITK12887013) during oviposition from females at Heron Island (Queensland, Australia). These clutches were chilled to 7-10˚C for eight hours to retard embryonic development (Harry and Limpus, 1989) before being transported by boat to the mainland and then by car to the laboratory facilities (approximately 10 hours traveling time) at The University of Queensland, St. Lucia campus. Upon arrival in the laboratory, eggs were incubated in moist heat sterilized beach sand at a constant temperature of either 27˚C or 30˚C until they were about to hatch. Hatchlings were euthanized at the end of these trials by cooling to 3˚C then freezing. Because hatchlings were to be euthanized at the end of these trials, I was only permitted to collect four clutches. Some eggs and hatchlings from these trials were also used to determine their energy content so that the energy expended during nest escape could be put into context of the overall egg to hatchling energy budget.

For the major trial of this study, green turtle eggs were collected at Chagar Hutang (Redang Island, Malaysia) Marine Turtle Research Station of Universiti Malaysia Terengganu (UMT). Six clutches that had been incubating *in situ* for between 53-57 days were transferred periodically (two clutches at a time) to the Institute of Oceanography and Environment (INOS, UMT) laboratory facilities by placing clutches into an icebox (no cooling used) for the three-hour journey by boat and car. Thus

all clutches were expected to begin hatching within 24-72 hours of being collected. A semi-permanent mark in permanent pen was placed on top of each egg as it was removed from the nest to maintain egg orientation during transportation. Hatchling metabolic expenditure during nest escape was measured once, and after surfacing hatchlings were kept together in darkness on top of moist beach sand (~ less than 20 minutes) prior to being released in a dark section of a nearby beach to complete their crawl to the sea.

Measuring energy expenditure

Open flow respirometry (which assumes that ultimately all energy is derived from aerobic metabolism) was used to measure energy use during digging. Using the clutches collected from Heron Island, Queensland I developed by trial and error a protocol that finally resulted in the successful measurement of metabolic rate during digging upward through a sand column. This perfected protocol appeared to show similar nest digging out behaviour to hatchlings in natural nests and was then used in the trials conducted in Malaysia.

Preliminary System used in University of Queensland trials: Respirometry chambers were constructed from transparent cylindrical Perspex placed vertically (7.5 cm in radius and 80.0 cm in height). Chambers were sealed at both ends with the top cover made of clear acrylic so that hatchlings could be seen when surfacing. Newly hatched hatchlings were randomly selected and buried under a column of beach sand in respirometry chambers. As the total number of hatchlings used differed among chambers, the depth of sand from the uppermost hatchlings to the surface was standardized to 40 cm (the typical depth from the top of an egg chamber to surface sand of green turtle nests) to ensure that hatchlings dug through the same volume of sand to reach the surface. Air entered through a tube at the base of the chamber and exited via a tube in the lid of the chamber. All preliminary trial experiments were performed in a 28˚C constant temperature room with 24 hours lights so that webcam images could be recorded continuously.

Final System used in UMT trials: Respirometry chambers were constructed from light-proof PVC cylindrical pipe placed vertically (10.5 cm in radius and 80 cm in height). Chambers were sealed at both ends with the top cover made of clear acrylic so that hatchlings could be seen when surfacing. Air entered through a tube at the base of the chamber and exited via a tube in the lid of the chamber. A five-centimetre layer of vermiculite was placed at the bottom of the chamber to avoid fluid retention at egg level during egg hatching. Pipping eggs (those in which the hatchling had pipped the shell with their egg tooth prior to hatching) were randomly selected and buried in groups of varying size (10-60 eggs) under a column of beach sand in respirometry chambers. As the total number of pipped eggs differed among chambers, the depth of sand from the uppermost eggs to the

surface was standardized to 40 cm. Sand from Chagar Hutang beach, Redang Island was used in these experiments and analysed following Folk (1974) by dry sieving method to determine the mean grain size (graphic mean). The sieving indicated the sand was a medium sand grade (phi (ϕ) value of mean grain size: 1.05 – 1.24, (Folk and Ward 1957)). In order to determine the time of hatching within the opaque respirometry chambers, a thin strip (~2 mm) of aluminium foil was laid on the uppermost eggs before the sand was added and connected in-line with a 1000 ohm resistor, 1.5V alkaline battery and a voltmeter (analogue/digital converter, ADInstrumens, PowerLab 4/30) placed across the resistor. At hatching, the hatchlings broke the aluminium foil causing the electrical potential to fall to zero. Digging duration time was assumed to be the difference between the time when the electric potential fell to zero and when hatchlings appeared on the sand surface as recorded by a webcam setup that viewed the sand surface through the chamber's transparent lid. These experiments were performed in UMT laboratory facilities at 28 ˚C with 24 hours lights so that the emergence event could be monitored continuously.

An open-flow respirometry system was used to measure the rate of oxygen consumption ($\dot{V}_{O_2}$) throughout these experiments (Figure 3.1). Outside air was pumped (Cole-Parmer, Air Cadet) sequentially through a soda lime (scrubbing CO_2), drierite (absorbing water vapour) and a mass flow controller (OMEGA, FMA5400/5500) that regulated the air flow at 1000 ml min^{-1}. The outflow air from the top end of the chamber was sub-sampled at 100 ml min^{-1} through scrubbing columns of soda lime and drierite prior to entering an oxygen analyser (Sable System, PA-1B). The voltage output of the oxygen analyser was sampled every 30 s through an analogue/digital converter (ADInstrumens, PowerLab 4/30) connected to a computer running Lab Chart 7 software ADInstruments). Calibration of the oxygen analyser was performed every three-hours with CO_2-free dry air. Oxygen consumption was calculated using equation 11.1 of Lighton (2008):

$$\dot{V}O_2 = FR_i\,(F_i\,O_2 - F''_e\,O_2) \,/\, (1 - F_e\,O_2)$$

where FR_i is the incurrent mass flow, $F_i\,O_2$ denote the fractional O_2 concentration of dry CO_2-free incurrent air and assumed to be at 0.2095, $F''_e\,O_2$ denote the fractional O_2 concentration of the dry, CO_2-free excurrent gas.

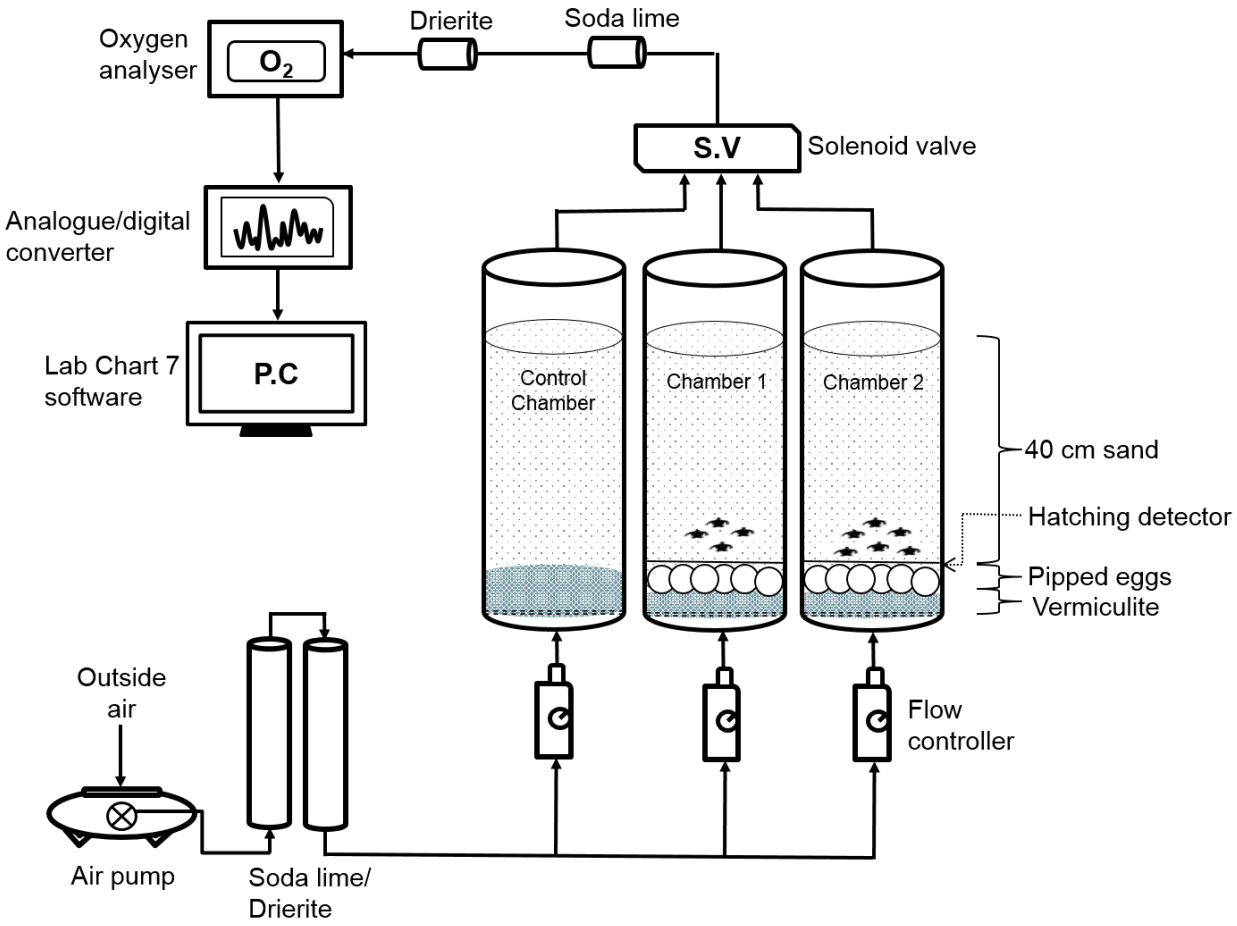

Figure 3.1. Schematic diagram of the open-flow respirometry system. Flow path: Air pump (Cole-Parmer, Air Cadet) – soda lime – drierite - mass flow controller (OMEGA, FMA5400/5500) –respirometry chambers – solenoid valve (S.V.)- soda lime – drierite – oxygen analyser (Sable System, PA-1B) - analogue/digital converter (ADInstrumens, PowerLab 4/30) – computer.

Calculation of energy expenditure from oxygen consumption

Three respirometry chambers were used simultaneously with one chamber consisting of vermiculite and 40 cm of sand with no hatchlings (hereafter known as 'control chamber') to measure background microbial $\dot{V}O_2$, while the remaining two chambers contained hatchlings. The $\dot{V}O_2$ was recorded for 10 minutes at a time in chambers containing hatchlings in sequence via a series of solenoid valves that were controlled through the "event manager" module in Chart 7 software. Oxygen consumption of the control chamber was recorded periodically for 10 minutes once every three hours. When swapping from one chamber to another, it took three minutes for the gas to completely flush through the system so that only the last seven minutes of $\dot{V}O_2$ measurement in a 10 minutes cycle were used. The $\dot{V}O_2$ of hatchlings was then calculated by subtracting the background microbial $\dot{V}O_2$ which was relatively constant and small (0.02-0.03% $\dot{V}O_2$of hatchlings) from the raw hatchling chamber $\dot{V}O_2$.

The total energy consumed by hatchlings (Et_{otal}) during the digging out process was calculated by first integrating the area under the $\dot{V}O_2$ versus time curve and converting this to units of energy by assuming an RQ of 0.72 and every litre of oxygen consumed corresponded to the expenditure of 19.7 kJ of energy (Schmidt-Nielsen, 1997). To calculate the net cost of transport (NCOT), the resting metabolic rate (RMR) of hatchlings was first subtracted from the empirically measured total metabolic rate to give a 'cost of digging' (COD) metabolic rate. RMR was assumed to equal the measured metabolic rate of pipped hatchlings before they hatched and integrated with time to calculate the resting energy ($E_{resting}$). COD was then integrated with digging time to calculate the total energetic cost of digging ($E_{digging}$), and this divided by the digging distance (0.4 m) to reveal NCOT (kJ m^{-1}).

To compare energy expended per individual among trials, E_{total}, $E_{resting}$, $E_{digging}$ and NCOT for the whole group was divided by the number of individuals within the group. Once on the surface, hatchlings were removed from the chamber and weighed. When hatchlings emerged onto the surface asynchronously, each individual was removed from the chamber immediately on surfacing and the calculation of individual oxygen consumption adjusted using the number of individual remaining in the chamber (Table 3.1). The $\dot{V}O_2$ trials were terminated 96 hours after the last cohort appeared on the surface because our observations indicated it was unlikely that anymore hatchlings would make it to the surface after this time. In some trials some hatchlings failed to make it to the surface, but these hatchlings were alive when the experiment was terminated. None of these hatchlings had progressed upward from the egg level, so it was assumed they did not contribute to

the digging effort made by their clutch mates. Hence, the $\dot{V}O_2$ of these remaining hatchlings after their emerged siblings had been removed from the respirometry chamber was treated in the same way as background microbial $\dot{V}O_2$ when calculating the $\dot{V}O_2$ of their siblings that successfully dug up through the sand column.

Table 3.1. Example from Trial 2B demonstrating how total individual energetic cost of digging through 40 cm of sand was calculated when adjusting for varying numbers of individuals remaining in the respirometry chamber.

Elapsed time (h)	Hatchlings remaining in chamber (n)	Total O_2 consumed (ml)	Total O_2 consumed adjusted per individual (ml)	Total O_2 consumed to reach surface per individual (ml)	Energetic cost of reaching surface per individual (kJ)
0.00 - 72.3	50	8415	168.3	168.3	3.316
72.4 - 87.7	15	503	33.5	201.8	3.975
87.8 - 135.8	14	1349	96.4	298.2	5.874
135.9 - 231.8	9				
Clutch average cost per individual				184.95	3.640

Trial 2B had three separate emergence cohorts with 41 hatchlings successfully reaching the surface (cohort size that reached the surface together were 35, 1 and 5 hatchlings respectively) while nine hatchlings remained at the base of the chamber and never began the journey upwards.

Energy content of eggs and hatchlings

Energy content analysis was performed on eggs and hatchlings from the Heron Island green turtle clutches. Twenty freshly laid eggs (five eggs from each of four clutches) were separated into albumen and yolk, and 26 recently hatched hatchlings (six or seven hatchlings from each of four clutches) were euthanized and dissected to separate the residual yolk from the hatchlings' body. Each component was weighed to 0.1 mg prior to being dried to constant weight in a freeze drier. The dried samples of egg components and residual yolk of hatchlings were homogenized using a mortar and pestle, and yolk-free carcasses were ground using a coffee grinder to a homogenous powder.

The energy density of dried samples was determined using ballistic bomb calorimetry. A sub-sample (0.1 - 0.2 g) of each component was transferred to a metal thimble and fully combusted in 20 atmospheres of oxygen inside a ballistic bomb calorimeter (Gallenkamp Autobomb, England). Energy density value was determined in triplicate for each component. Periodically, the calorimeter was calibrated with thermochemical standard benzoic acid (26.442 J g^{-1} Bureau of Analysed

Standards Ltd, Middlesbrough, UK). Energy density is reported on a dry mass basis that includes the ash component.

Data Analysis

Spearman's correlation was used to examine the association between clutch size and digging duration, and clutch size and total energy expenditure. Individuals from a particular nest were not used in more than one trial, so that each trial was independent of each other. Pearson correlation was used to examine the relationship between digging duration and total energetic cost. Statistical significance was assumed if $P \leq 0.05$.

3.3 Results

Emergence Pattern

In the initial trials with hatchlings from the Heron Island population, all hatchlings failed to show natural digging and emergence behaviour. Some hatchlings made it to the surface, but they emerged as individuals and not in a group. As a consequence, the experimental design and protocol went through several different iterations until a protocol that resulted in 80% of eggs producing hatchlings reaching the surface was developed. This protocol was used in all trials conducted at UMT in Malaysia. Overall, hatchlings took between 3.7 and 7.8 days to emerge from beneath 40 cm of sand, and emerged onto the surface in one to four cohorts (Table 3.2). In seven out of eleven trials, I noticed that hatchlings remained slightly buried with their heads partially protruding from the sand on the surface for 2 to 4.5 hours before completely moving out onto the sand's surface. In all trials, the first cohort to emerge onto the sand surface contained the largest number of hatchlings. Trials that consisted of a larger number of individuals took the shortest time to dig upward through the 40 cm of sand (Figure 3.2A).

Table 3.2. Time taken to emergence and cohort characteristics of green turtle hatchling digging up through 40 cm of sand in respirometry chambers.

Clutch No.	Group (A = Small group vs. B = Big group)	Number of eggs placed in chamber	Proportion of hatchlings reaching sand surface (%)	Mean digging duration ± SEM (days)	Time between emergence of first and last cohorts (days)	Number of separate cohorts that emerged	Proportion of hatchlings in the first cohort (%)
1	A	10	90	7.83 ± 0.7	5	3	77.8
	B	20	100	6.74 ± 0.3	5.7	4	85
2	A	30	93.3	3.78 ± 0.0	0.6	2	96.6
	B	50	82	3.95 ± 0.1	2.2	3	85.4
3	A	10	90	6.00 ± 0.0	0	1	100
	B	47	80.9	4.00 ± 0.0	0	1	100
4	A	12	100	8.10 ± 0.0	0	1	100
	B	60	100	4.00 ± 0.0	0	1	100
5	A	15	100	5.70 ± 0.3	0	1	100
	B	60	91.7	3.65 ± 0.2	3.9	2	83.3
6		25	84	5.03 ± 0.3	4.1	3	85.7

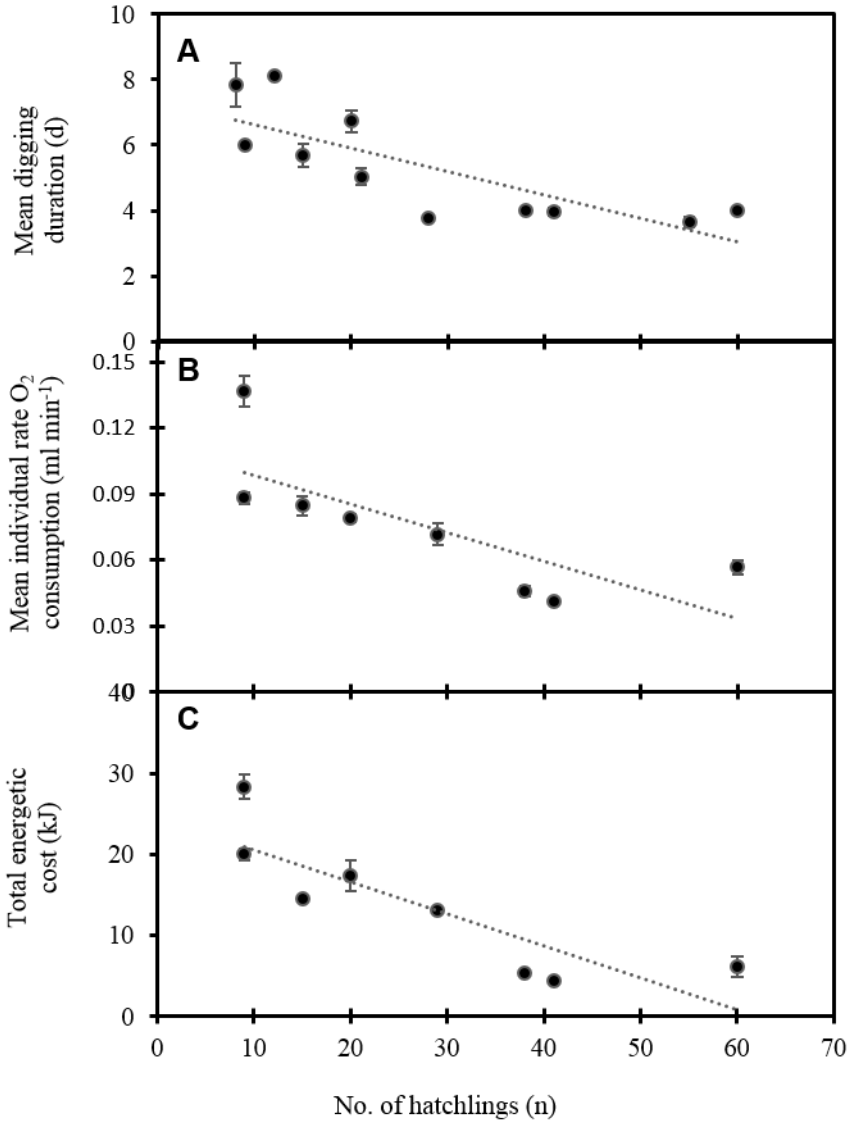

Figure 3.2. Variation in (A) digging duration (y = -0.072x + 7.36, r^2 = 0.64, P < 0.001, N = 11), (B) mean individual oxygen consumption throughout the digging period (y = -0.001x + 0.11, r^2 = 0.58, P = 0.008, N = 8), and (C) total individual energetic cost, E_{total} (y = -0.4x + 24.5, r^2 = 0.73, P = 0.002, N = 8) with clutch size. Vertical bars represents standard error of mean.

The energetic cost and NCOT of digging upward

Out of the eleven trials conducted, I recorded $\dot{V}O_2$ throughout the entire upward digging process from eight clutches. Oxygen consumption data from three trials was lost due to file corruption when power to equipment briefly failed. The pattern of $\dot{V}O_2$ was similar in all trials, with large fluctuations throughout the entire time and occasional sharp peaks that lasted 10 to15 h (Figure 3.3).

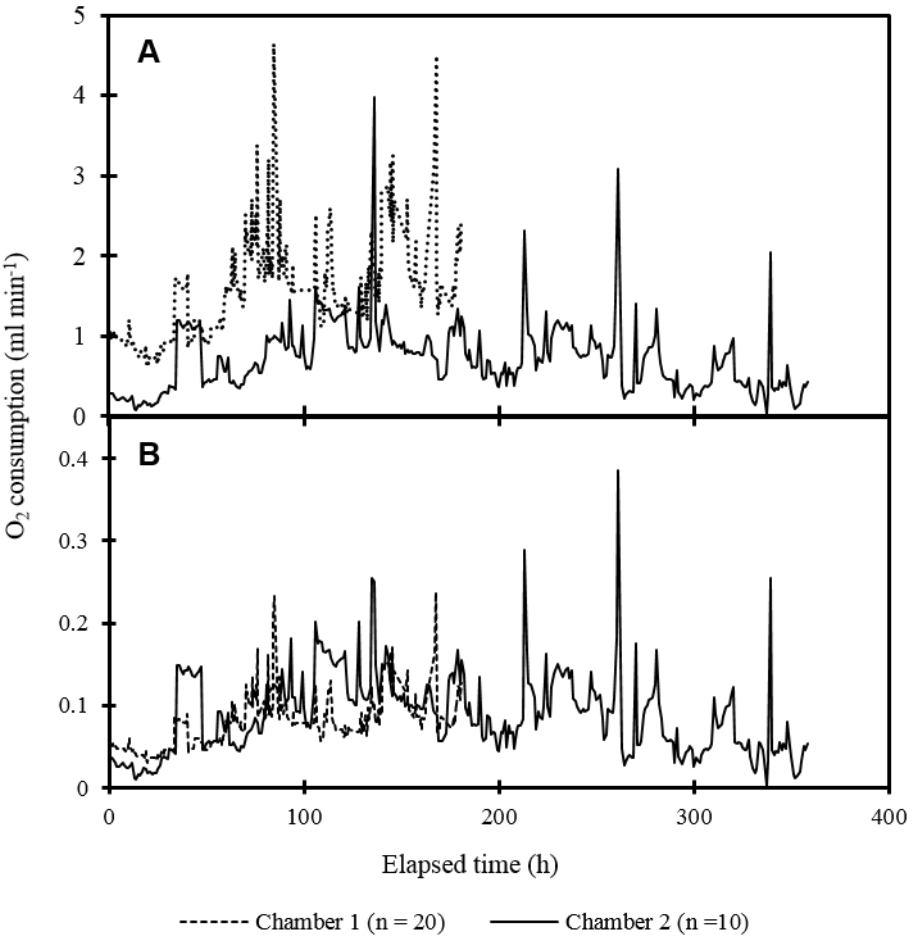

Figure 3.3. An example of real-time $\dot{V}O_2$ data (A) total oxygen consumption of all hatchlings combined, and (B) oxygen consumption per individual. Note the differences in time taken to reach the surface between chambers one and two. The resting metabolic rate (RMR) was assumed to be at 0.04 ml min^{-1} per individual. Chamber one contained 20 individuals whereas chamber two contained 10 individuals.

The per individual $\dot{V}_{O_2}$ average over the entire digging out period varied between 0.04 and 0.14 ml min^{-1}, and decreased as cohort size increased (Figure 3.2B). The per individual total energy expended during the digging period, calculated by integrating respirometry data, varied between 4.4 and 28.3 kJ and decreased as cohort size increased (Figure 3.2C). Hatchling individual energetic cost was correlated with digging duration (Figure 3.4). The NCOTs ranged from 0.4 to 21.5 kJ m^{-1} (Table 3.3) and dependent on clutch size (Figure 3.5). The RMR was estimated to contribute less than 5% across the entire range of clutch sizes.

Table 3.3. The estimation of NCOT by green turtle for nest escaping process.

Clutch size	E_{total} [Total individual energy expenditure (kJ)]	$E_{resting}$ [RMR energy expenditure (kJ)]	$E_{digging}$ [Digging energy expenditure (kJ)]	Proportion RMR (%)	Proportion digging (%)	NCOT (kJ m^{-1})
9	20.01	0.37	19.64	1.85	98.15	11.13
9	28.3	0.28	28.02	1.00	99.00	21.49
15	14.53	0.27	14.26	1.85	98.15	8.06
20	17.37	0.32	17.05	1.83	98.17	9.72
29	13.04	0.18	12.86	1.37	98.63	8.75
38	5.28	0.19	5.09	3.58	96.42	0.74
41	4.44	0.19	4.25	4.21	95.79	0.39
60	6.09	0.19	5.90	3.11	96.89	1.55

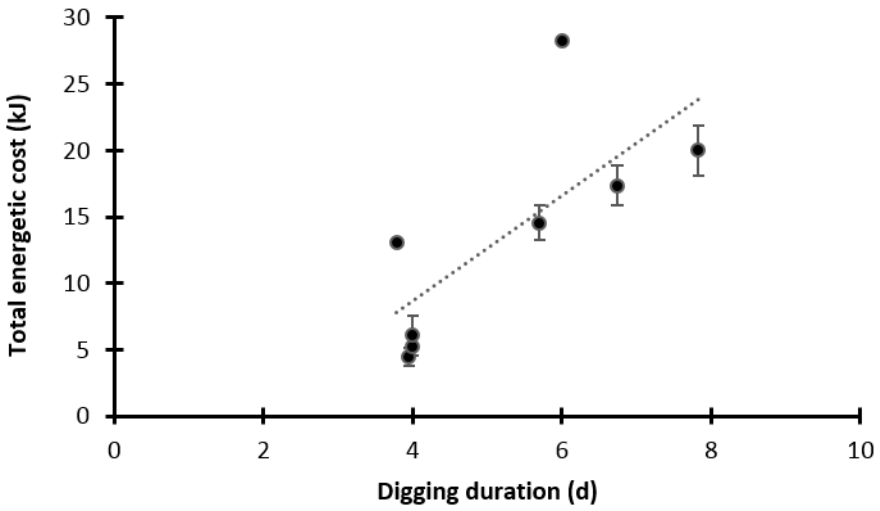

Figure 3.4. Relationship between the energetic cost of digging through 40 cm of sand per individual hatchling and digging duration (y = 3.98x -7.3, r^2 = 0.55, P = 0.028, N = 8). Vertical bars represents standard error of mean.

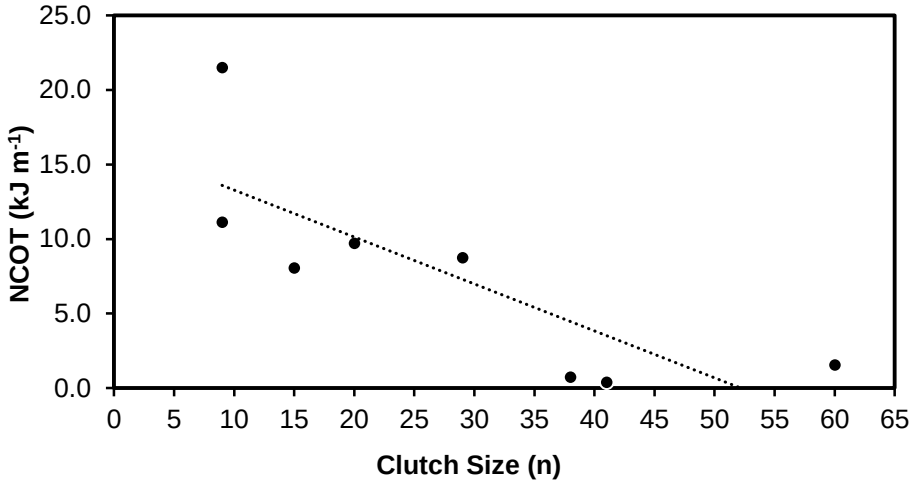

Figure 3.5. Relationship between NCOT and clutch size (y = -0.315x + 16.4, r^2 = 0.64, P = 0.007, N = 8).

Energy content

The yolk samples had a much greater energy density and total energy content than albumen (Table 3.4). Although residual yolk had a greater energy density than the yolk-free carcass, the greater mass of the yolk-free carcass resulted in there being more energy in the yolk-free carcass than in the residual yolk (Table 3.4). I calculated that eggs contributed 172.4 kJ of their energy to the embryonic development and hatching process. Based on the energy content of the residual yolk at hatching, 41.7 kJ was available for nest escape and dispersal from the nest.

Table 3.4. Estimation of the total energy contained within freshly laid eggs and newly hatched hatchling components based on energy density (calculated on a dry mass basis including ash) and mean dry mass of green turtles.

	N	Mean Dry Mass ± SEM (g)	Mean Energy Density ± SEM (kJ g dry mass^{-1})	Calculated Total Energy (kJ)
Fresh laid Eggs				
Albumen	20	0.37 ± 0.02	13.1 ± 0.5	4.8
Yolk	20	11.61 ± 0.40	30.8 ± 0.4	357.8
Entire egg contents				**362.6**
Newly Hatched Hatchlings				
Yolk-free carcass	26	6.07 ± 0.1	24.5 ± 0.2	148.5
Residual Yolk	26	1.09 ± 0.1	38.3 ± 0.3	41.7
Entire hatchling				**190.2**
Energy used during embryonic development				**172.4**

The least square ANCOVA adjusted means for freshly laid eggs and hatchlings mass are at 47.8 g and 25.1 g respectively (wet mass).

3.4 Discussion

System Troubleshooting and Improvement

This is the first study to measure metabolic rate continuously of sea turtle hatchlings during the nest escape process, and I encountered a number of unanticipated problems in my first trials. In my first attempt, active newly hatched hatchlings were placed under 40 cm of sieved beach sand in a transparent cylindrical Perspex chamber, with the aim of continuously monitoring digging activity visually while also recording $\dot{V}O_2$. However, hatchlings remained more or less motionless under the sand column for more than 48 h before this first trial was abandoned. A possible explanation for this unexpected lack of digging behaviour is that hatchlings require some working air space into which to start their digging activity (Carr and Hirth, 1961). In natural nests, there are usually some air spaces between the spherical eggs in the egg chamber (Carr and Hirth, 1961; Kraemer and Richardson, 1979), and additional space would be created during the hatching process as fluids drain away from the nest when hatchling escape their eggs. Hence, burying hatchlings under a solid column of sand with no air spaces may have inhibited the start of digging behaviour. To overcome this problem, in the next iteration of these experiments, I used eggs from a clutch that had just started to pip. During the egg transfer process I meticulously maintained the orientation of the egg so that the top of the egg always remained upwards by marking the top surface before movement began because it is known that chelonian embryos at the late stage of incubation orientated themselves into a hatching position prior to the hatching event (Miller, 1985). Therefore, changing an egg's orientation could prolong the hatching process as embryos reposition themselves within the egg if eggs were rotated while being placed in the chamber. I also introduced a layer of vermiculite below the eggs to allow a space for the fluids released during hatching to drain into.

This experimental protocol resulted in hatchlings starting to dig upwards after escaping the eggs, as would occur in natural nests, providing experimental evidence that hatchling sea turtles do indeed need an air space in order to begin their nest escape digging behaviour. However, I found that rather than concentrating their digging effort in a vertical direction, as would be expected in a natural nest, many hatchlings directed their digging effort sideways and became stuck against the chamber's wall. It would appear that rather than rely solely on gravitational cues to directly their digging effort upwards as is suspected to occur in natural nests which are completely dark, hatchlings were attracted to light entering through the transparent chamber walls. It is well known that hatchling sea turtles use light cues to direct their movement towards the sea once they exit the nest in nature (e.g. Mrosovsky and Kingsmill 1985, Kawamura et al. 2009), and it appears that they can be attracted to light even while buried underground, but in nature such cues are absent during the nest escape

process. Thus, in the final experimental protocol, I used light-proof PVC pipe as respiratory chambers. In this way, hatchlings remained in the dark and light naïve until they reached the surface of the sand column. To determine the time when hatchlings reached the surface I used a transparent chamber lid and a 24-hour webcam. To determine the time then hatchlings emerged from eggs and started to dig upwards, a thin strip of aluminium foil (~2 mm width) was placed on top of the uppermost eggs and connected in series with a 1000 ohm resistor and 1.5 volt D-cell battery. The electrical potential was monitored continuously on one channel of the PowerLab system, and the beginning of the digging out process detected then the electrical potential fell to zero as the aluminium strip was broken. This hatching detector was assumed not to interfere with the upward movement of hatchlings.

Sea turtle hatchlings do not dig continuously during their nest escape; periods of activity digging are punctuated by periods of rest (Carr and Hirth, 1961; Drake and Spotila, 2002). Originally, I hoped to continuously monitor the digging progress of hatchling as they moved upward, but discovered that light interfered with this process. Other work from my laboratory also shows that hatchlings are distracted by infrared light, so I did not attempt to use the transparent chambers and infrared video. I did find that by placing an ear directly against the opaque chamber wall, scratching noises presumably generated by digging activity, could be heard intermittently. Unfortunately, I was unable to source a surface mounted microphone system during my experiments, but this would be a useful addition to future experiments. An important observation was that external sound such as talking in close vicinity to the chamber could trigger an activity bout. For this reason, care was taken to minimise external noise during these experiments. Noise may be a natural cue used to trigger synchronous digging activity amongst clutch mates as it is known that chelonian hatchlings can detect and respond to sound stimuli (Ferrara and Vogt, 2014; Ferrara et al., 2012; Ferrara et al., 2014; Harms et al., 2014).

Immediately before the emergence event, I noticed that in most trials hatchlings spent some time resting just below the sand surface before finally emerging onto the surface. This behaviour is similar to that described by Dial (1987) for sea turtle hatchlings emerging from natural nests. There are three hypotheses about the cues used by sea turtle hatchlings to trigger the final nest emergence process: (i) the threshold temperature hypothesis (Drake and Spotila, 2002; Hendrickson, 1958; Matsuzawa et al., 2002), (ii) the rapid temperature change in surface sand hypothesis (Witherington et al., 1990), and (iii) the negative thermal gradient hypothesis, when surface sand became cooler than the sub-surface sand (Gyuris, 1993). As the current trials were conducted at a constant temperature, none of the temperature change hypotheses can explain my observations. However it is possible that the delay I observed was caused by a build-up of lactic acid in the blood, and

hatchlings waited until blood lactate levels dropped to a lower level before completing the emergence process as suggested by Dial (1987).

Metabolic Rate and Hatchling Activity

The spikes in $\dot{V}O_2$ that occurred throughout the digging out process were presumably caused by bouts of synchronous digging. These spikes were separated by periods of decreased oxygen consumption. During periods of active digging, lactic acid accumulates in hatchling sea turtles (Dial, 1987; Hamann et al., 2007) and causes the hatchlings to stop digging once lactate reaches an upper threshold (Edwards and Gleeson, 2001). Oxygen consumption would probably remain high for a period after digging in order to pay back an oxygen debt, a period of time when lactate is oxidised to pyruvate following cessation of activity via aerobic metabolism (Randall et al., 1997). I intended to continuously observe digging behaviour so that timing of digging and resting could be correlated with $\dot{V}O_2$ but this was not possible because hatchlings would not engage in their normal digging behaviour in the transparent chambers.

Oxygen consumption during "rest periods" typically varied between 0.03 and 0.05 ml min^{-1}, and during peak $\dot{V}O_2$ typically varied between 0.2 and 0.4 ml min^{-1} (Figure 3.3). These values compare to 0.06 ml min^{-1} for full-term green turtle embryos just prior to pipping (Booth and Astill, 2001) and 0.55 ml min^{-1} for green turtle hatchlings maximally swimming immediately after entering the water during their frenzy swim (Booth, 2009). This indicates that maximum aerobic digging effort is less than maximum aerobic swimming effort, and is consistent with the suggestion that anaerobic effort during digging is also lower than anaerobic swimming effort as reflected by lower blood lactate concentrations in digging compared to swimming hatchling green turtles (Hamann et al. 2007). Clusella Trullas et al. (2006) also found that digging had a lower metabolic requirement than swimming.

Digging Duration and Energetic Cost

The digging duration and energetic cost were dependent on cohort sizes, with larger cohorts requiring shorter digging out times (Figure 3.2A) and lower per individual energetic cost (Figure 3.2C). The lower energetic cost of individuals digging out in large groups resulted from a combination of a lower absolute mean metabolic rate per individual and a shorter digging out time. The relative importance of both these factors to individual energy expenditure is similar, with an increase from a group size of 10 to 60 resulting in ~ 50% decrease in both digging duration and mean metabolic rate. Hence, in nature, it is likely that hatchlings receive a significantly benefit by belonging to a large clutch because of a reduced per individual energetic cost of nest escape. The

role that an individual sea turtle hatchling plays during the nest escape process varies depending on its position within the digging cohort: some scratch down the sand roof and others compact the sand underneath the group (Carr and Hirth, 1961). These roles probably have different energetic costs. In theory, moving in a cohort against the resistance (i.e. sand) requires the leading edge individual(s) to incur higher energetic costs than others (Fish, 1995; Trenchard et al., 2015). However, this added energetic cost to lead individuals can be shared by rotating positions while moving, which shares the workload of being the lead across many individuals (Voelkl et al., 2015). Hence in future studies it would be good to be able to identify individuals to see if they share the workload by rotating positions and roles, or if certain individuals do more of the digging work than others.

The greater number of actively digging hatchlings in a cohort there are, the greater the metabolic heat production will be, and as a consequence the nest temperature may also be higher causing an increase in hatchling body temperature. Ectotherms locomotor performance generally increases with an increase in body temperature; for example, swimming performance of newly emerged sea turtle hatchlings is greater in warm water (Booth and Evans, 2011). Hence, sea turtle hatchlings digging in a larger cohort may also indirectly receive a benefit through the effect of increased body temperature on locomotion performance. The total energetic cost per hatchling of digging vertically through 40 cm of sand varied between 4.4 kJ and 28.3 kJ and decreased as cohort size increased (Figure 3.2C). Because energy is limited for sea turtle hatchlings as they escape the nest, the energy saved by social facilitation would be allocated to the remaining dispersal activities such as crawling across the beach and the swimming frenzy. The grouping of individuals during locomotion to save on individual energetic costs is well established (reviewed in Portugal et al., 2014). For example, Lissaman and Shollenberger (1970) estimated that a group of 25 birds flying in “V” formation would have 71% more flight range than a bird flying by itself, and a power saving of up to 14% has been estimated for flocks of pink-footed geese (Cutts and Speakman, 1994). In the current study, I found that variation in group size could cause a 4-fold change in an individual’s energetic cost of nest escape.

The energetic cost of nest escape can be put into an ecological context when considering it in the context of available energy in the freshly laid egg, energetic costs of embryonic development, terrestrial movement from nest to water, and the off-shore swim (Table 3.5). Although the energetic cost of nest escape is small compared to the overall energy in a fresh egg, it is can be a very large proportion of the energy remaining in the residual yolk at hatching, and is similar or larger than the energy used during the first day of swimming. Hence, hatch success which determines the number of hatchlings in a cohort can have a significant influence on the per-individual cost of nest escape, and as a consequence a large influence on the amount of energy remaining in the residual yolk after

nest escape. Hatchlings entering the sea with larger energy reserves are presumably at an advantage as they can survive longer before find food and therefore escaping the nest in larger cohorts probably results in hatchlings of greater fitness. This finding may have implications for active conservation management of sea turtles in some regions of the world like Malaysia where a common strategy is to split natural clutches into smaller clutches (Mortimer, 1999; Mortimer et al., 1994) when relocating them into hatcheries. Sometimes clutches are also partially harvested before being placed into a hatchery, a practice which also reduces the cohort size (Koch et al., 2007). Reducing the number of eggs in a clutch may ultimately result in the production of hatchlings with reduced energy reserves when they enter the sea.

Table 3.5. A summary of energy budget of the green turtle (*C. mydas*) hatchlings from freshly laid egg through to end of the first day of the frenzy swim.

Component	Energy (kJ)	Fraction of energy in fresh egg (%)	Fraction of energy in residual yolk (%)	Source
Fresh egg	360	100		Current study
Yolk-free hatchling	150	42		Current study
Residual yolk	40	11	100	Current study
Used during embryonic development	170	47		Current study
Used during nest escape *	5 to 25	1.4 to 7	13 to 63	Current study
Used during crawling **	0.54	0.15	0.36	Williams (2012)
Used during first 24 h of swim	6	2	15	Booth (2009)

* Varies depending upon clutch size.

** Rough estimation from *C.mydas* crawling trial on treadmill hard ground surface for 200m under controlled environment ($\dot{V}O_2$ = ~ 0.7ml O_2 g^{-1} h^{-1}, crawling duration = 1.55 h, adjusted to hatchlings mass at 25.1 g). Note that the entire estimation has been compared with hatchlings originating from different populations (eggs, embryonic development and the first day of swimming from southern Great Barrier Reef population, while nest escape from the mainland Malaysia population) as discussed in the methodology section.

3.5 Conclusions

Over 50 years ago, Carr and Hirth (1961) suggested that 'social facilitation' within the nest plays an important role in the nest escape process of newly hatched sea turtles. However, empirical evidence for the existence of mutual benefits to individual hatchlings during nest escaping has been limited. My study provides new insight into sea turtle hatchling synchronous activity during nest escaping, and how it influences the energetic cost of nest escape. I found that an increase in group size from 10 to 60 hatchlings resulted in ~ 50% decrease in the time taken to escape the nest and mean metabolic rate during this time resulting in reduced energy expenditure during nest escape. Because a finite amount of energy is available to hatchlings upon hatching, the energy saved by synchronous digging can be allocated to other activities such as the frenzy off-shore swim.

CHAPTER 4

Soil type influences the energetics of nest escape in Brisbane river turtle hatchlings

4.1 Introduction

Freshwater turtles typically crawl to an upland terrestrial nesting site to deposit their eggs. Unlike sea turtle nests that are always constructed in beach sands, freshwater turtle nests can be found in varying soil types ranging from fine sand to heavy clay (Ratterman and Ackerman, 1989; Kuchling, 1999; Hughes and Brooks, 2006; Booth, 2010). However, some species of freshwater turtle are only found nesting in the sandy substrate (Micheli-Campbell, 2012), so sand might be a more suitable substrate than other soil types. Spencer (2002) demonstrated that incubation success and probability of nest predation of field incubated freshwater turtle eggs is influenced by maternal nest site selection. This variation in hatchling emergence success has been attributed to the influence of the nest substrate on embryo development during incubation, but the nest escape process, once the eggs have hatched may also contribute to emergence success.

Embryonic development and hatching success can be affected by nest substrate because the nest substrate influences the way in which heat, water and respiratory gasses are exchanged between the nest and the developing embryo inside the egg (Ackerman, 1997). For example sand particle size has been reported to influence turtle hatching success (Schwartz, 1982; Miller, 1985; Mortimer, 1990; Foley et al., 2006). In the post-hatching phase, digging during nest escape may also be influenced by soil composition and compaction (Crain et al., 1995). Apart from variation in natural soil types, anthropogenic pollution such as cement dust on a nesting beach at Ras Baridi, Saudi Arabia has been documented to reduce hatchlings emergence of sea turtles (Pilcher, 1999).

Once eggs hatch, nest emergence success may be influenced by how difficult it is for hatchlings to escape the nest. Soil type most probably influences the effort required to dig out of the nest and therefore the energy expenditure required for nest escape. For example, it is likely that digging through a sand substrate is easier than through a heavy clay substrate due to difference in compaction of these substrates. Hatchlings have a finite energy reserve in their residual yolk at hatching, and this yolk must fuel nest escape, the crawl from the nest to the water, and hatchling growth and maintenance until food can be found once they reach the water. Hence, the less energy

spent escaping the nest, the more energy is available for post-nest activities. Clearly, experimental investigations are needed to determine if differences in nest substrate also result in differences in digging intensity and the time spent digging out of the nest which would result in differences in the energy required to escape the nest.

Although freshwater turtles may nest in a variety of soil types, it appears that most species hatch more or less synchronously (Thompson, 1989; Spencer et al., 2001), a strategy widely believed to have evolved to swamp predators (Dehn, 1990). Another possible advantage of synchronous hatching and group formation is that 'social facilitation' can be used to share the workload of digging out of the nest. In previous chapters, I found that an increase in group size resulted in a decrease in time taken to escape the nest and reduced energy expenditure during nest escape of Brisbane river turtle (Chapter 2) and green turtle hatchlings (Chapter 3). Particularly for the Brisbane river turtle hatchlings, digging in a group of 14 used 71% less energy on a per individual basis than a hatchling digging alone through moist fine sand.

In this chapter, I used open flow respirometry to investigate the influence of soil type on the energetics of digging during nest escape in Brisbane river turtle. I tested three hypotheses: (i) that the nesting substrate affects the time required to escape the nest, (ii) that the energy required to dig through soil varies with soil type, and (iii) that the energetic cost of nest escape per individual increases as clutch size decreases. In the previous two chapters, I used the total energetic cost of digging (the sum of energy while resting and digging, E_{total}) to represent the energy budget of nest escaping in hatchlings so that the data can be compared with the amount of reserved energy available in hatchlings' yolk. However, in the current chapter, I use the metabolic cost of digging ($E_{digging}$) and the net cost of transport (NCOT) to compare the energetic cost of digging through different substrates.

4.2 Materials and Methods

Study animals

This study was approved by The University of Queensland Animal Ethics Committee (AEC approval number: SBS/133/13/URG). The Brisbane river turtle (*Emydura macquarii signata*) is commonly found in waterways of Southeast Queensland and northern New South Wales and is restricted to the eastern coastal plain due to the Great Dividing Range (Cann, 1998). Females nest after or during rain events between September and January at all times of the day, but more frequently at night, and typically construct their nests within 2-10 m of the water's edge (Booth,

2010). A clutch consists of between 10 and 30 white hard-shelled eggs that take between 44 and 76 days to incubate (McCosker, 2004).

Egg collection and incubation

Five gravid Brisbane river turtles were intercepted upon emerging to their upland nesting area from a population inhabiting artificial lakes on the St. Lucia campus of The University of Queensland, Australia (27^{o} 29' 54" S, 153^{o} 00' 58" E) during October 2014. Gravid females were transferred to a laboratory facility and induced to lay eggs by intramuscular inject of synthetic oxytocin (activity = 10 iu ml^{-1}) at a dose of 2 ml kg^{-1}. After the injection, females were placed individually into a plastic Nally bin (80 cm x 30 cm x 40 cm) containing 10 cm of water. Eggs were immediately removed from water upon oviposition. The females were then returned to their original habitat after oviposition was completed. Eggs were buried in moist sand (~ -150 kPa) incubated at a constant temperature of 28 ˚C but three clutches were initially incubated at 24 ˚C for two weeks to space out hatching time among clutches. Booth (1998b) demonstrated that incubation temperature does not affect phenotypes and energetics of hatchlings, hence the different incubation temperature procedure in the current study most likely does not significantly affect their energy content at hatching.

Measurement of energy expenditure during digging

Each newly hatched hatchling was weight on an electronic balance prior to introduction into a cylindrical respirometry chamber (2.85 cm in radius and 30 cm in height) that was positioned vertically to simulate a hatchling's dig upward during a typical nest escape. A clutch of newly hatched hatchlings (6-8 hours after hatching) was split into two groups and positioned horizontally, plastron down, and stacked one on top of another on sand near the bottom of the chamber before being buried in either river sand or soil obtained from the UQ lake site. A fixed volume of water was added to standardize a water potential of approximately -150 kPa in the both soil types. The number of hatchlings involved in each digging trial was manipulated between two and six in order to investigate the influence of clutch size on digging time and energy expenditure. As the number of hatchlings involved differed between trials, the height from the top most hatchling to the substrate surface was standardized to 15 cm. Open-flow respirometry was used to quantify carbon dioxide production ($\dot{V}_{CO_2}$) and energy expenditure as described in Chapter 2. Briefly, air was pumped into the respirometry chamber and then directed to a carbon dioxide analyser so that carbon dioxide production could be calculated. Two respirometry chambers of different soil types were used simultaneously so hatchlings from the same clutch that had been split equally into two groups of

between 2-6 hatchlings could be measured. The $\dot{V}_{CO_2}$ of these two chambers was recorded for 10 min at a time in each chamber via a solenoid valves system (Bürkert type 6012, Bürkert Weke GmbH & Co. Christian-Bürkert-Staβe 13-17, Germany) which switched from one chamber to the other by using 'event manager' module in Chart 7 software throughout the trial period. $\dot{V}_{CO_2}$ was monitored in chambers that contained hatchlings three times per hour throughout the entire digging trial. When transferring from one chamber to another, it took three minutes for the gas to completely flush through the system, hence I only used the last seven minutes of $\dot{V}_{CO_2}$ measurement in a 10 min cycle and use the average value during this time as the datum for integration of area under the $\dot{V}_{CO_2}$ versus digging time curve.

I calculated the average individual metabolic rate and the individual metabolic cost throughout the hatchlings' digging out process. Upon completion, turtles were removed and the background microbial $\dot{V}_{CO_2}$ measured. The net $\dot{V}_{CO_2}$ of all turtles was calculated by subtracting the background microbial $\dot{V}_{CO_2}$ from the raw data. Total energy expended by all hatchlings during the digging out process was calculated by first integrating the area under the corrected $\dot{V}_{CO_2}$ versus time curve. This value was then converted to energy units using a carbon dioxide calorific equivalent of 1 mL CO_2 = 25.6 J (Withers, 1992) to give the total energetic cost (E_{total}). The energetic cost of digging ($E_{digging}$) was calculated by subtracting the resting metabolic cost ($E_{resting}$) (assuming individual hatchlings have a resting metabolic rate of 0.64 mL O_2 h^{-1} equivalent to 12.6 J h^{-1}; Booth 1998b) from the total energetic cost ($E_{\text{digging}} = E_{total} - E_{resting}$). Per-individual metabolism was calculated by dividing the metabolic cost of digging (E_{digging}) by the number of hatchlings in each digging trial, and compared across different group sizes. NCOT was calculated by dividing E_{digging} by the digging distance (0.15 m). Summary of calculation can be found in Table 4.1 in the result section. Digging trials were performed in a 28˚C constant temperature room with 24 hours lights so that webcam imagery could be obtained to assess hatchlings digging behaviour and emergence time.

Soil particle size characteristics

Samples of the UQ lake soil and river sand used in digging trials were characterized in terms of the frequency distribution of particle diameters. Samples of river sand and UQ lakes soil were air-dried at room temperature (~24-28˚C) for five days. Triplicate 100 g samples of each air-dried sample were sieved for 15 minutes using mechanical sieves of different sizes (2000 µm, 1400 µm, 1000 µm, 710 µm, 500 µm, 355 µm, 250 µm, 180 µm, 125 µm, 90 µm and 63 µm) (Folk, 1974).

Separated sand in each sieve was then weighed and classified according to the phi (ϕ) value of mean grain size and sorting according to the criteria in Folk and Ward (1957). A grain size distribution has then constructed from the mass percentage of separated particle sizes to illustrate the spread (sorting value) of the soil samples. Soil's sorting value reflects its compactness (Kenny and Sotheran, 2013), with poorly sorted soil being more compact than well-sorted sand (Tucker, 1996).

Statistical analysis

Spearman's correlation (r_s) was used to determine whether digging duration, mean $\dot{V}_{CO_2}$ production and total energetic cost were correlated with number of hatchlings within a trial. I used ANCOVA with the hatchling number in trial as the covariate to compare energetic cost, digging duration and metabolic rate during nest escape between two soil types. All statistical analyses were performed using IBM SPSS Statistics 21 software, and statistical significance was assumed if $P < 0.05$. Data are presented as mean ± SE.

4.3 Results

Sediment characteristics

Relative to river sand, the UQ lake soil had a higher proportion of larger particle sizes and a wider distribution of particle sizes (Figure 4.1). Using the criteria in Folk and Ward (1957), river sand was classified as fine sand, and UQ lake soil classified as course sand with mean particle size at 2.53 ± 0.01 ϕ and 0.72 ± 0.12 ϕ respectively. Further, in terms of sorting classification, river sand was classified as well sorted and UQ lake soil classified as poorly sorted.

Digging duration

Hatchlings mass varied between 3.89 and 5.65 g and all appeared healthy and active when they were buried in the respirometry. They dug intermittently (2-3 strokes in 5 s) followed by a pause, but the pause was highly variable in length. Hatchlings typically dug synchronously within a group, and then rested as a group, but the length of the rest period varied between trials, with some groups occasionally resting for up to 15 minutes before starting a new digging bout. The exception was the group of five hatchlings digging through river sand that dug almost continuously throughout the trial and consequently emerged in a shorter time than might have been expected (Figure 4.2). Upon approaching the soil surface, hatchlings did not pause below the substrate surface; they simply dug continuously until they broke out onto the surface. Digging through river sand took a shorter time than digging through UQ lake soil, and in river sand groups of 5 and 6 took a shorter time to dig out compared to smaller group sizes (Figure 4.2A, Table 4.1).

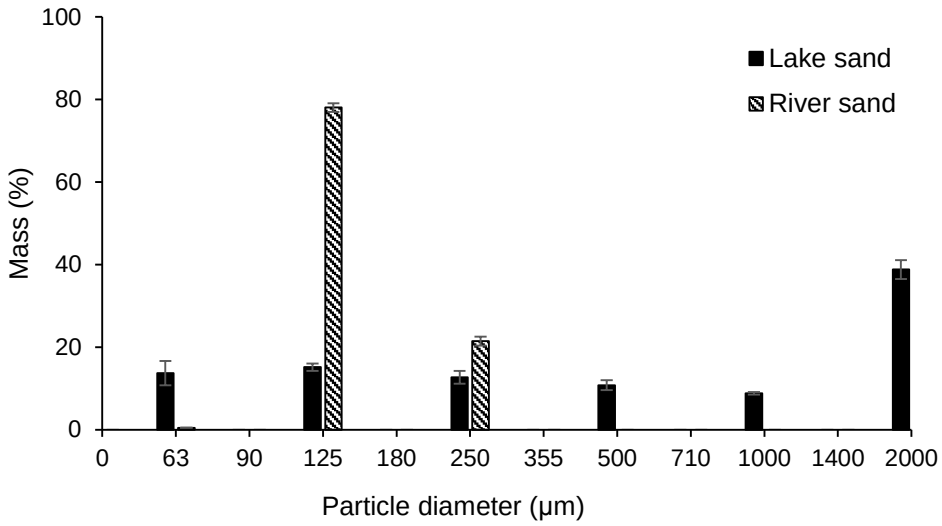

Figure 4.1. Proportion of particle size from lake and river sand used in digging trials. Error bars represent standard error of mean value.

Table 4.1. Least square means digging duration, digging metabolic rate, total energetic cost, resting metabolic cost, metabolic cost of digging and NCOT of individual *E. m. signata* hatchlings digging through fine or coarse sand. Values are mean (adjusted by clutch size of 4 hatchlings using ANCOVA) ± SE.

Variable	River sand (n = 5)	UQ lake soil (n = 5)	P-value
Digging duration (h)	74.74 ± 5.9	103.25 ± 5.9	0.011
Mean $\dot{V}_{CO_2}$ production (ml h^{-1})	1.04 ± 0.5	1.17 ± 0.5	0.106
Total energetic cost, E_{total} (kJ)	2.08 ± 0.1	3.14 ± 0.1	0.001
Resting metabolic cost, $E_{resting}$ (kJ)	0.88 ± 0.04	1.22 ± 0.01	0.043
Metabolic cost of digging, $E_{digging}$ (kJ)	1.20 ± 0.1	1.9 ± 0.2	0.266
Net cost of transport (NCOT)	7.98 ± 0.7	12.84 ± 1.0	0.005

Cost of digging

The mean digging $\dot{V}_{CO_2}$ and $E_{digging}$ of *E. m. signata* hatchlings in fine sand was not significantly different from that in coarse sand, but digging duration was longer, and E_{total}, $E_{resting}$ and NCOT were greater in UQ lake soil compared to river sand (Table 4.1). Digging duration did not correlate with group size (Figure 4.2A), but E_{total} (Figure 4.2B), $E_{resting}$ (Figure 4.2C), $E_{digging}$ (Figure 4.2D) and NCOT (Figure 4.2E) all decreased as group size increased in both soil types.

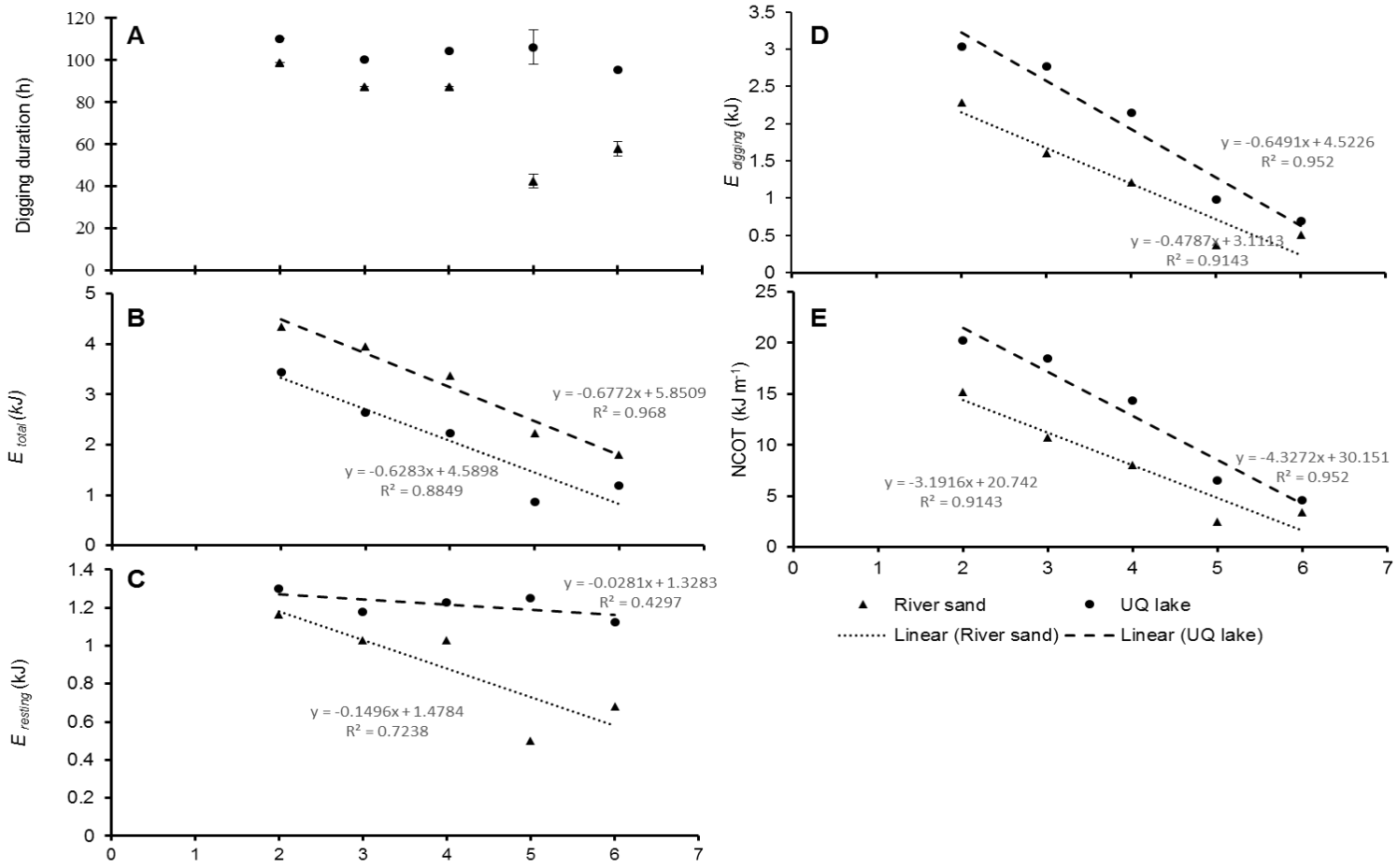

Figure 4.2. (A) Scatter plot of digging duration against number of hatchlings in each trial, there was no significant correlation between these variables (River sand: $R^2 = 0.720$; P = 0.54; UQ lake soil: $R^2 = 0.43$, P = 0.285). ANCOVA with number of hatchlings as the covariate indicated a difference in digging times between River sand and UQ lake soil ($F_{1,7} = 12.75$, P = 0.011). The relationship between E_{total} (B), $E_{resting}$ (C), $E_{digging}$ (D) and NCOT (E) with the number of hatchlings. Vertical error bars represent standard error of mean value.

4.4 Discussion

Soil characteristics influence the digging duration and overall individual metabolic cost of nest escape in *E. m. signata* hatchlings (Figure 4.2A). My particle size analyses indicated that river sand had a smaller mean particle size and was better sorted than UQ lake soil (Figure 4.1). Hence, the UQ lake soil was probably more compact than the river sand, and this made UQ lake soil more difficult to dig through as reflected by the longer digging times and greater energetic cost of nest escape and greater NCOT for hatchling digging through UQ lake soil. In sea turtles, nest escape success decreases as sand compaction increases (Horrock and Scott, 1991; Peters et al., 1994; Crain et al., 1995; Pilcher, 1999), supporting the hypothesis that it is more difficult to dig through compacted soil.

A limitation of this study was that I could only monitor hatchling digging behaviour when individuals were digging close to the chamber's wall. The initial plan was to calculate the mean duration of a digging bout, and of a resting period to see if there were differences between soil types and group sizes. However, it was not possible to make this comparison because (i) individuals digging through the middle of the sand column could not be observed through the chamber wall and (ii) the soil from UQ lakes was dark brown in colour which caused reflection of light back out across the respirometry chamber wall resulting in a poor quality video recording.

For a given group size, there was no difference in the mean metabolic rate of hatchlings digging through river sand and UQ lake soil. This may be because the metabolic scope of the hatchlings was similar in all hatchlings, and hatchlings metabolic rate is independent of the substrate they are digging through. However, because river sand was easier to dig through, the time taken to dig out of the nest was shorter, and as a consequence of a shorter digging duration, the energetic cost of nest escape was less in river sand compared to UQ lake soil. In other words, the greater cost of nest escape and NCOT in UQ lake soil was due to the longer time taken to dig out of the nest rather than to a difference in the intensity of digging effort while digging. Across all hatchling group sizes, hatchlings escaping the nest in river sand spent 33.8 % less energy compared to hatchlings digging through UQ lake soil (Table 4.1). The total energy in residual egg yolk at hatching averages 6.46 kJ (Chapter 2; Rusli and Booth, 2016), so that after nest emergence hatchlings digging through UQ lake soil would have considerable less energy left in their residual yolk (3.32 kJ) compared to hatchlings digging through river sand (4.38 kJ). Hence, from a nest escape point of view, hatchlings emerging from nests constructed in river sand have an advantage over hatchlings emerging from nests constructed in UQ lake soil because they would have greater energy reserves,

potentially giving them a longer survival time if food is difficult to find in the post-hatch environment, or more energy that could be channelled into post-hatch growth.

Besides influencing the nest escape process, soil type is also known to influence the embryonic development and the phenotype of hatchling oviparous reptiles (reviewed in Shine, 2004). For example, hatchlings incubated in nests constructed in clay emerge considerably earlier than nests constructed in sand suggesting that less energy is consumed during embryonic development resulting in larger residual yolks (Kuchling, 1999). However, clay soils are generally more compact than sand soils (Kenny and Sotheran, 2013), so it is likely that hatchlings expend more energy escaping the nest in clay soils than in sandy soils. Hence, there may be a trade-off in energy expenditure between the different phases of nesting biology, with clay soils resulting in less energy expenditure during embryonic development but more energy expenditure during nest escape compared to sandy soils. The significance of such trade-offs and its potential effects on the soil type chosen by a female to nest in are yet to be explored.

Freshwater turtles typically emergence from their nest *en masse* (Doody et al., 2001; Spencer, 2001; Nagle et al., 2004) and this behaviour is associated with the synchronizing of activities of hatchlings in the nest chamber (Spencer et al., 2001; reviewed in Doody, 2011). The current study found that the per-individual energetic cost of nest escape and NCOT decreases as the number of hatchlings digging together increases in both soil types tested (Figure 4.2C). This finding is consistent with the 'social facilitation' hypothesis (Carr and Hirth, 1961) which states that it is advantageous to hatch as a group rather than as individuals. My data indicate that the bigger the clutch, the greater the advantage during the nest escape process, so I would anticipate selection for larger clutch size. However, when there is a limited supply of material for manufacturing eggs as is the case in freshwater turtles, the production of more eggs will result in smaller eggs, and ultimately smaller hatchlings. Smaller hatchlings may be less efficient at digging than larger hatchlings, so further work is needed to explore the possible trade-offs between egg size and clutch size with respect to the nest escape process.

4.5 Conclusions

To conclude, soil type affects digging performance and energetics of nest escaping in Brisbane river turtle hatchlings. Hatchlings in river sand escaped faster and spent less energy than hatchlings in UQ lake soil. Moreover, larger group size extended a clear energetic advantage over small group size while digging out of the nest in both soil types tested.

CHAPTER 5

Tonic immobility in newly emerged sea turtle hatchlings

5.1 Introduction

Animals have a wide variety of defensive behaviours that are adaptive in certain situations. Thanatopsis or tonic immobility (TI) is a distinctive behaviour defined as a temporary loss of muscle and/or neurological function (partial paralysis) in response to a threat. The human equivalent is known as "hypnosis" which dates back to the Old Testament (Ratner, 1967). This phenomenon may last for a few seconds to over several hours (Gallup, 1974). Although animals in a TI state seem to be unresponsive to external stimulus, evidence indicates some animals can continue to process information about the environments that surround them (Sigman and Prestrude, 1981). Recent evidence suggests that TI is not associated with any suspension of consciousness (Marx et al., 2008). Indeed, Mauk et al. (1981) noted that the lizard *Anolis carolinensis* in TI can exhibit hyperalgesia (increased sensitivity to pain).

Tonic immobility has been observed in a variety of animals including fish (Tobler 2005, Wells et al. 2005), amphibians (Toledo et al., 2010), reptiles (Gehlbach, 1970; Edson and Gallup, 1972; Hennig et al., 1979; Santos et al., 2010) birds (Sargeant and Eberhardt, 1975), and mammals (Fraser, 1960; Francq, 1969; Carli, 1974), and TI in invertebrates also appears to be common (as reviewed in Coutinho et al., 2013). However, as far as I know, TI has not been documented in sea turtle hatchlings.

Tonic immobility can be induced in vertebrates by turning them upside down suddenly so that their dorsal side faces the ground (Edson and Gallup, 1972; Carli, 1974; Hennig et al., 1979; Hohtola, 1981). However, turning newly emerged turtle hatchlings onto their backs does not induce TI, but triggers a self-righting behaviour in which the neck is used to turn the animal right side upward (Booth et al., 2013). This behaviour is probably an adaption to accidental tipping over when hatchlings rapidly crawl from the nest to the water's edge during the frantic nest emergence and off-short swim that occurs during the first 24 hours after nest escape (Carr and Ogren, 1959). Frenetic activity can be problematic to researchers when handling and restraining hatchlings. For example measuring mass, and body dimensions, and counting carapace scutes are regular procedures at many sea turtle nesting beaches and these procedures are most efficiently done when hatchlings are immobile. Hence, the inducing of TI could be a valuable tool when conducting routine research and

management procedures on sea turtle hatchlings. Here I describe a technique that can be used to induce TI in green turtle *Chelonia mydas* hatchlings.

5.2 Methods

This study was approved by The University of Queensland Animal Ethics Committee (AEC approval number: SBS/133/13/URG). Newly emerged green turtle hatchlings were collected from Chagar Hutang Turtle Sanctuary, Redang Island, Malaysia in July – August 2014. Enclosures made of plastic mesh were placed on the surface of selected *in-situ* nests to trap newly emerged hatchlings. Enclosures were checked every half hour throughout the expected emergence night to ensure hatchlings were not on the surface for longer than 30 minutes. Once on the surface, hatchlings were transferred to a hut (approximately 20 m from the nesting beach) to measure their TI potential. I defined TI by the cessation of any movement (suspended animation) once induced and the "TI period" as the period of time from cessation of movement until the restart of movement.

Tonic immobility was induced by placing hatchlings upside down (dorsal side on a flat surface), then gently pressing the thumb and first finger on both eyes (to eliminate visual stimulus) while the middle finger was placed on the plastron to stabilize the animal (Figure 5.1). Duration of TI was recorded with a stopwatch. At least 10 hatchlings were tested from each clutch sampled. Immediately after tests, hatchlings were released back onto the beach where they completed their crawl to the sea.

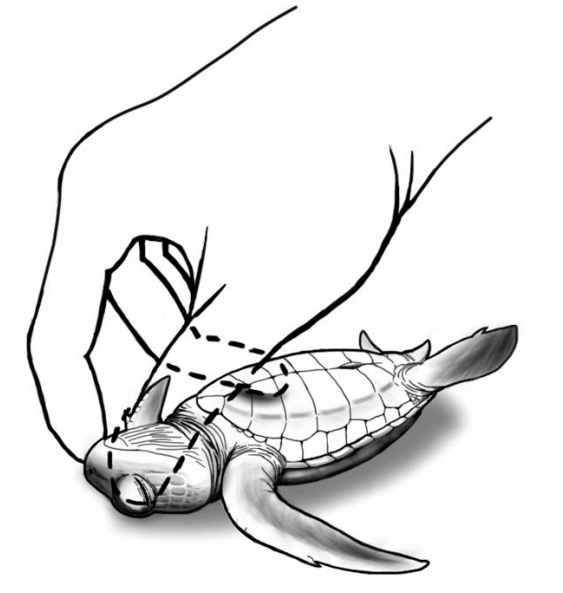

Figure 5.1. Diagrammatic representation of inducing tonic immobility (TI) in a green a turtle (*Chelonia mydas*) hatchling. Operator's fingers are positioned as shown to induce TI. Image by N. Wu.

Results are presented as mean ± SE of each clutch for the duration of tonic immobility. Effect of different clutches was tested with One-way ANOVA and statistical significance assumed if $P < 0.05$. I also investigate if the period of time between nest emergence, and the TI trial was correlated with TI duration using Pearson correlation.

5.3 Results and Discussion

Ten clutches were sampled, and TI duration differed between clutches ($F_{10, 210} = 6.009$, $P = 0.005$). In six out of ten clutches, the TI duration was short, less than 16 sec, while in one clutch TI duration was approximately 40 sec (Table 5.1). Clutch coefficient of variation (CV) of TI varied between 48 and 78%. There was no correlation between the waiting period of hatchlings to TI trial and TI duration (Figure 5.2).

Table 5.1. Tonic immobility duration (sec) from 10 clutches of green turtle hatchling clutches sampled from Chagar Hutang Turtle Sanctuary, Redang Island, Malaysia. n = number of individuals sampled per clutch. Clutches were ranked to the mean value of TI. SE = standard error and CV = coefficient of variation.

Clutch no.	*n*	Mean	SE	CV (%)
9	19	9.04	2.3	72
3	19	9.37	9.2	78
10	18	10.32	1.5	64
6	22	13.99	2.8	73
8	22	14.14	2.8	76
2	33	15.30	1.9	70
5	16	21.95	5.7	75
4	10	26.77	11.1	59
7	26	28.48	6.7	78
1	24	40.24	7.1	48

The TI duration varied among clutches and this variation could be due to a maternal effect. All hatchlings came from the first cohort of hatchlings to emerge from the nest and were assumed to have a similar body temperature. The CV's for TI duration are remarkably large ranging between 48 and 78%, compared to typical biological data such as morphological variables or growth which normally ranges between 10 - 15% (Balaam, 1972). Earlier studies also found much higher variables in locomotor performance of animals such as sprint velocity on swimming of Atlantic cod (35.4%; Reidy et al., 2000), crawling of green turtle hatchlings (31.6%; Ischer et al., 2009), and sprint speed of tree lizards (24.8%; Robson and Miles, 2000). Essentially, the immense inter-individual variability in physiological traits is believed to associated with the natural selection if

found to be repeatable (Bennett, 1987). However, in the current study I did not measure the repeatability of TI duration within an individual.

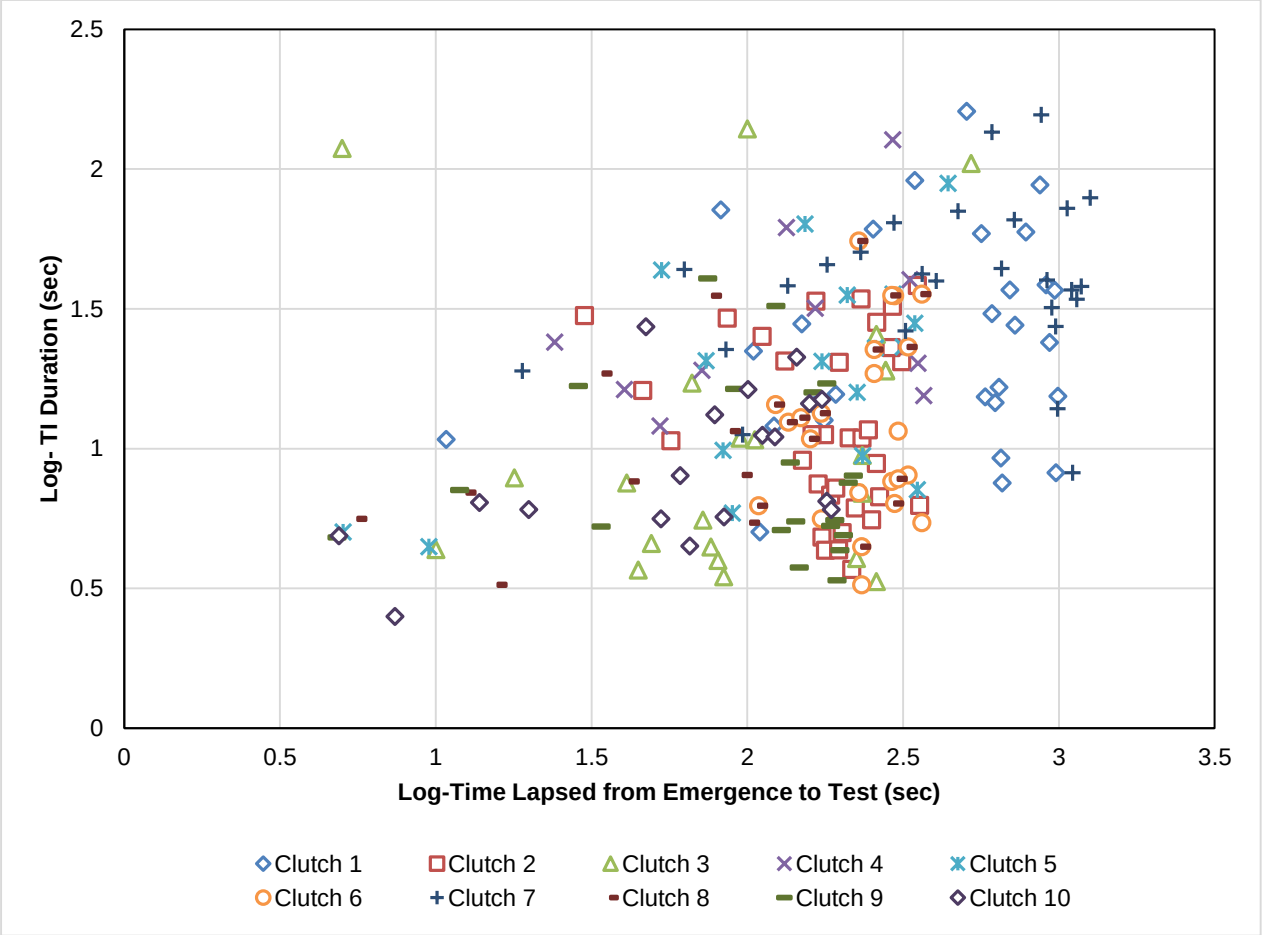

Figure 5.2. TI duration for each hatchling within the clutches to the retention time before test taken. Variables have been log transformed as actual data were centred at bottom-left in graph. There was no significant correlation between TI duration and time elapsed between nest emergence and TI trial. Regression values for each clutch were Clutch 1: r = 0.054, p = 0.785, n = 24; Clutch 2: r = 0.007, p = 0.969, n = 33; Clutch 3: r = 0.201, p = 0.371, n = 19; Clutch 4: r = 0.287, p = 0.422, n = 10; Clutch 5: r = 0.445, p = 0.073, n = 16; Clutch 6: r = 0.210, p = 0.336, n = 22; Clutch 7: r = 0.147, p = 0.473, n = 26; Clutch 8: r = 0.190, p = 0.342, n = 22; Clutch 9: r = 0.234, p = 0.321, n = 19; Clutch 10: r = 0.290, p = 0.243, n = 18.

During the waiting period between nest emergence and measurement of TI, hatchlings were kept in a bucket and they continuously crawled around inside the bucket for up to 30 minutes. This is an energetically demanding process and I propose there might be a relationship between depletion of energy reserves and TI duration. However, there was no relationship between TI duration and length of time between nest emergence and measurement of TI, suggesting that energy reserves are not related to TI duration. Alternatively, the amount of energy used during the holding period would not have been enough to significantly deplete energy reserves and thus influence TI duration.

There is no clear evidence for an adaptive advantage of TI behaviour, but two likely hypotheses for its evolution have been proposed. The first is 'playing possum' in response to the presence of a predator to decrease the probability of being detected by the predator (Gallup Jr et al., 1980) and the second is immobility associated with reproductive activities (Whitman et al., 1986). The majority of TI research has focused on the predator avoidance hypothesis. TI may have evolved as an alternative to the "fight-or-flight" response to increase the chance of survival in the face of a predator (Alboni et al., 2008), and five predator-induced TI hypothesis have been suggested by Miyatake et al. (2009). For example, TI might reduce the attention of predators, as some predators rely on visual movement cues to detect prey or trigger an attack (Heinen, 1995; Gregory, 2008), or escape when a predator's prey is left unguarded (Gallup Jr, 1974), or making it physically difficult to swallow a prey (Honma et al., 2006). Experimental studies suggest that this behaviour can increase the survival rate (Hoagland, 1928; Sargeant and Eberhardt, 1975; Thompson et al., 1981) and is passed on to future generations (Miyatake et al., 2004; Nakayama and Miyatake, 2009b). Interestingly, there seems to be a genetic trade-off for TI in red flour beetles (*Tribolium castaneum*) in which males with a long TI duration experienced increased survival against predation, but have lower mating success even in predator-free environments (Nakayama and Miyatake, 2009a).

In terms of anti-predator defence, hatchling sea turtles can actively flee from predators, freeze or take no perceivable action. Observations of hatchling turtles swimming away from their natal beach in Florida show that when threatened or attacked by an aquatic predator, green turtle hatchlings continue swimming, but loggerhead turtle hatchlings (*Caretta caretta*) often become immobile, assuming a "tuck" position (Wyneken et al., 1994). Experimentally, when hatchlings were given a simulated predation experience loggerhead turtles and hawksbills turtles (*Eretmochelys imbricata*) became immobile (325.4 sec and 89.5 sec respectively) but the green turtles continued to actively swim within 1.8 sec (Mellgren et al., 2003). Hence, different sea turtle species hatchlings respond differently to encounters with predators, and thus the technique of inducing TI described for green turtle hatchlings may differ or be ineffective on the other sea turtle species.

There are different methods to induce TI, most involve some sort of restraint such as stroking an animal's ventral surface, forcing it to fixate its gaze on a chalk line, or placing a hood over its head. In particular, many animals become calm and inactive when in an upside down position. However, newly emerged sea turtle hatchlings struggle to self-right when turned upside down. Thus, the first step of flipping them onto their back alone does not induce TI. We had to also gently press their plastron at the same time with one finger. This stimulus may imitate the pressure placed on individuals by other hatchling turtles while they are still within their nest. The third step was closing their eyes with two fingers as shown in Figure 5.1. This technique was instigated because sea turtle

hatchlings placed in total darkness soon stop moving presumably because the visual cues they use in sea-finding behaviour are absent in complete dark. Therefore, closing the eyes also shuts off these visual cues making body movements less likely. Hence three steps appear to be necessary to induce TI in green turtle hatchlings: i) flip them onto their back, ii) gently press their plastron, and iii) close their eyes.

The ability to induce TI in animals can provide practical applications for researchers attempting to obtain quantitative variables. Specifically for sea turtles hatchlings, inducing a state of TI may allow researchers to measure the animals' body mass accurately, and in other studies where a still animal is necessary such as x-ray studies and photographic studies where fine details are necessary (e.g. scute pattern or flipper morphology). All of these methods require the animal to be still for at least a short period. It is advisable to perform TI on one hatchling at a time when using this method to take measurements because TI duration is not consistent among hatchlings and typically of short duration. As soon as TI is broken, hatchlings attempt to self-right and immediately start moving again. From my observations, TI can be re-induced if necessary but I did not measure TI duration when induced for a second time on the same individual. TI does not appear to harm the sea turtle hatchlings, they assume their normal frenzied behaviour as soon as TI is broken, and in my study rapidly crawled without distraction to the sea when placed on the beach. In conclusion, inducing TI could be a valuable tool in reducing the risk of injury to the struggling hatchlings when undertaking body measurements.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE STUDIES

6.1 Research Context

To date, the description of the nest escape behaviour of turtle hatchlings from their underground nest has been limited to the observations made through a glass pane (Carr and Ogren, 1959). These classic observations made on leatherback and green turtle nests proposed that turtle hatchlings have to work together with clutch mates to successfully escape from their underground nest, a finding supported by a follow-up study on green turtles (Carr and Hirth, 1961). Since these pioneering studies, researchers have used the term 'social facilitation' to describe the way hatchlings work together to escape the nest. However, no investigations have explored how 'social facilitation' might influence the energy spent by individual hatchlings escaping the nest. Previous studies have investigated the amount of energy used during embryonic development (Ackerman, 1981; Booth and Astill, 2001), crawling to the beach (Williams, 2012) and early swimming effort (Booth, 2009), but the lack of information on energy expenditure during nest escape left a significant gap in the knowledge of energy expenditure in the early life stage of turtles. The information about nest escape in freshwater turtles was even scarcer. Therefore, the central theme of my study was to further understand the behaviour and energetics of nest escape in chelonian hatchlings.

I used two methods to quantify energy expenditure during nest escape. The first used open flow respirometry (which assumes that ultimately all energy is derived from aerobic metabolism), and the second used differences in energy content of hatchlings before and after the digging out process. Results from these two methods were then compared and discussed in the ecological context of embryonic development and the immediate post-nest life phase of chelonians.

6.2 Research Development

6.2.1 The Brisbane river turtle

Brisbane river turtles are typical of freshwater turtles worldwide, constructing relatively shallow nests (10-20 cm) which contain 12-30 eggs (Booth, 2010). To investigate the hypothesis that hatchlings truly shared their workload while digging out of the nest, my research focused on how per individual energy expenditure varied as group size varied

during the digging out process. My initial plan was also to investigate if there were differences in metabolic rate during resting and active digging phases during the nest escape process. However, it was not possible to make this differentiation because hatchlings formed into discretely different digging groups that separated them from each other (especially in larger groups) and, the activities of these separate groups were asynchronous.

The energetic cost of digging upward through 15 cm of soil varied between 0.34 and 2.32 kJ per individual with the energetic cost increasing as group size decreased (Chapter 2). This energy expenditure amounted to 5.3 – 36 % of the energy remaining in the residual yolk of a just-hatched hatchling. These results support the hypothesis that there is a fitness advantage to hatching and digging out of the nest as a group, and are in agreement with observation of other animals that pose similar aggregation behaviour as an energy saving strategy while moving together (Hansell, 1993; Fish, 1995; Ebensperger and Bozinovic, 2000). I also discovered that soil type can influence the energetics of digging vertically, not through the difference in the absolute metabolic rate during digging, but through the time it took to dig through the soil (Chapter 4). This aspect of my research needs to be expanded in the future because freshwater turtles are known to nest in a wide variety of soil types from coarse sands to heavy clays (Vestjens, 1969; Ratterman and Ackerman 1989, Hughes and Brooks, 2006; Kuchling, 1999; Booth, 2010), and these soils can also vary in their compactness which is likely to influence greatly the ease of nest escape and its energetic cost.

6.2.2 The green turtle

The energetics of nest escape was also investigated in green turtles, a species which has deep nests (60-100cm) and large clutch size (80-150 eggs). Getting green turtle hatchlings to dig in a natural way within respiratory chambers was problematic, and several iterations of different protocols were trialled before a protocol that worked was discovered. Preliminary trials were conducted on four egg clutches collected from Heron Island, Queensland and I was able to develop a protocol that worked well and this was used in the main trials used on clutches of eggs in the Malaysian population of green turtles. This protocol used about to hatch eggs which were placed on a bed of vermiculite (to absorb liquids released from eggs at hatching) and covered in sand to a standard depth of 40 cm. The respiratory cylinder was light-tight because hatchlings dug towards the side walls rather than vertically if cylinder walls were clear. A ‘hatching detector’ consisting of a line strip of aluminium foil was used to detect the time hatchlings escaped their eggs and began their journey upward.

Hatchlings in respirometry chambers showed similar nest digging out behaviour to hatchlings in natural nests, and typically emerged onto the sand surface in groups, with the first group to emerge being the largest group. Even though the lids of respirometry chambers were made of transparent Perspex and lights were on 24 h per day to monitor hatchlings emergence, hatchlings still exhibited their natural behaviour of resting just under the surface for several hours before completing their final movement of emergence (Dial, 1987). This waiting period might be a time when lactate accumulation within the body was being reduced in anticipation of the rapid crawl seawards immediately after nest emergence (Dial, 1987).

As the digging experiments took placed in dark chambers, I could not monitor hatchlings digging activity and progress up through the sand column visually. However, I discovered that if I placed my ear onto the chamber wall, I could detect active digging as intermittent scratching noises. I also discovered that external sound such as talking in close vicinity to the chamber would trigger hatchlings to start digging activity inside the chamber. Recent studies have found that chelonian hatchlings can detect and respond to sound stimuli (Ferrara et al., 2012; Ferrara et al., 2014; Ferrara and Vogt, 2014; Harms et al., 2014). Hence the question of whether or not a noise could be one of the cues useful by hatchlings to trigger synchronous digging activity among clutch mates needs to be explored in future studies. The use of microphones placed on the inside wall of respirometry chambers could facilitate such studies and also provide information on the lengths of digging and resting bouts during the nest escape process.

6.3 Investigating group movement during nest escape and future studies

The incidental association of turtle hatchlings when hatched together among clutch mates could be classified as one of the social behaviour (Parish et al., 1997). For a better understanding, we might break this group movement study into three questions:

(i) Does grouping facilitate nest escape?

(ii) Are there differences between individuals in digging effort and energy expenditure?

(iii) Can a model be developed to predict an individual's energy expenditure during nest escape?

First, my results show that grouping does facilitate nest escape as indicated by larger groups taking shorter times to escape the nest (Figure 2.1A and 3.2A). My experimental manipulations of clutch size during the simulated nest escape process clearly show that hatchlings escaping in small groups

not only experience longer nest escape times but they also incur greater individual energetic cost in both Brisbane river and green turtle hatchlings. This is the first time such a relationship has been demonstrated and supports the social facilitation hypothesis that states synchronous hatching and nest escape increases the fitness of hatchlings. Consequently, one might expect selection pressure to increase clutch size, particular in the Brisbane river turtle which has a much smaller clutch size than the green turtle. However, there is a well-known trade-off between clutch size and egg size in freshwater turtles (e.g. Iverson et al., 1993; Brooks et al., 1991; Rowe, 1994; Janzen and Warner, 2009), so the production of a larger clutch would result in smaller eggs and consequently smaller hatchlings. Hence it would be interesting to explore the influence of hatchling size on the energetics of nest escape. In these experiments groups of the same number of hatchlings, with hatchlings within each group being of the same size, but of different size between groups would have their energetic cost of nest escape measured. A priori, one might expect larger hatchlings to be more efficient diggers than smaller hatchlings, but this would need to be tested. If larger hatchlings are indeed more efficient diggers than smaller hatchlings, then the advantage of having many smaller hatchlings might be countered by having fewer larger but more efficient hatchlings from an energetic expenditure point of view.

Second, in my calculations of per individual energy expenditure, I assumed that each individual contributed evenly to the digging effort and therefore that within a group the energy expenditure of each individual was the same. This is probably not the case as the digging effort of individuals is likely to differ amongst individuals as it is known that the digging behaviour of individuals is different between the top, centre and bottom positions of the digging cohort (Carr and Ogren, 1959). I hypothesised that hatchlings at the centre and bottom of the digging formation would expend less energy than those on the edges of the formation, especially those located at the front/top which actually scrape away at the sand. It may be that the ‘heavier work load’ of individuals at the digging front is shared among individuals by the mean of changing position regularly (Parish et al., 1997). Hence, it would be interesting to discover if the labour of digging is shared equally amongst clutch mates, or if there are a few individuals that are ‘up-front’ almost continuously and do the majority of digging work. These experiments would be technically difficult to perform because they would have to be conducted in complete darkness, but with infrared camera technology rapidly advancing such experiments may be possible in the future.

In sea turtles which have large clutches, differences in oxygen availability within different regions of the nest may also influence the ability of individuals to contribute to the digging effort. For example, differences in oxygen partial pressure of 5-15 torr have been observed between the centre and periphery of full-term green turtle nests (Ackerman, 1977), and as such, may inhibit the ability

of centrally located hatchlings to elevate their aerobic metabolism compared to hatchlings located in the periphery. Hence future experiments might also examine digging effort and behaviour at different oxygen concentrations.

Thirdly, there are many variables that will influence the energy expenditure of an individual hatchling during nest escape. The key variables in determining nest escaping energetics of turtle hatchlings can be divided into three categories; (i) maternal factors (nest depth, yolk component, clutch size and egg size), (ii) incubation environment (gas partial pressure, humidity, and temperature), and (iii) individual variation (hatching success, position in nest and during nest escape) (Figure 6.1). All these variables have been mentioned earlier in various section in my thesis, however many of them are yet to be investigated. For example, the influence of nest depth and substrate moisture content on the energetics of digging during nest escape. Clearly the deeper the nest, the further the hatchlings have to dig, and therefore the greater the energy expenditure will be. But will the relationship between nest depth and digging out energy expenditure be a linear function or somewhat more complicated? Dry sand has a tendency to collapse and flow downwards with gravity making air spaces difficult to create, while moist sand has the opposite effect, tending to stick together and allowing large airspaces to be created. How these different conditions influence digging activity, digging efficiency and energy expenditure are completely unknown. Experiments using the same protocols I developed during the current study could answer these questions, the same sand could be used but its depth and water content manipulated across a series of digging trials. Likewise, the oxygen concentration during a digging trial could be manipulated and the time taken and the energy expended during nest escape explored.

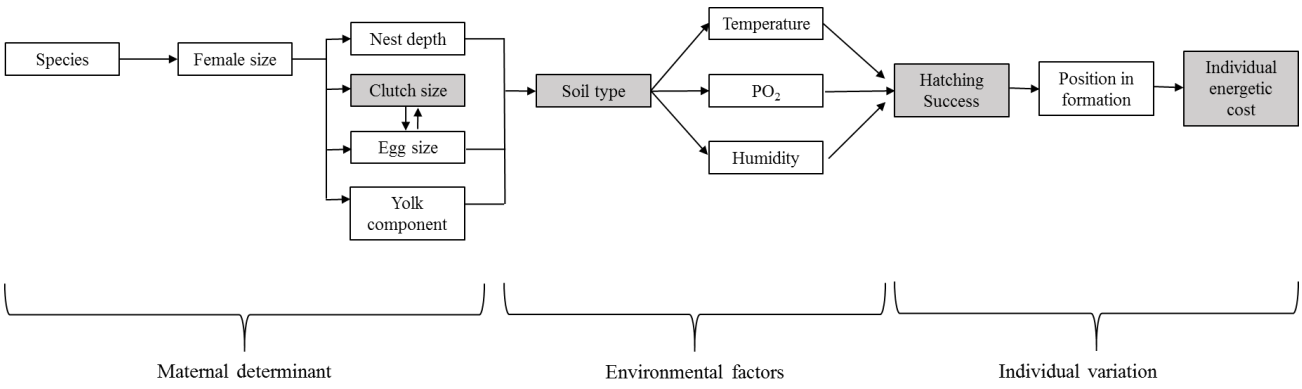

Figure 6.1: Predicted variables that may influence the energy expenditure of an individual hatchling during nest escape. Variables in grey boxes denote some of the variables that had been covered in my thesis.

In another investigation the role of sand surface temperature in triggering hatchling emergence onto the sand surface could also be experimentally explored. It has long been known that the vast

majority of natural sea turtle nest emergences occur at night, typically earlier in the night, and that when daylight emergences occur they are usually associated with rainy or cloudy weather. Such observations have led to the idea that sand surface temperature is the cue used by hatchling sea turtles to trigger the timing of their final escape from the nest (e.g. Witherington et al., 1990; Gyuris, 1993; Matsuzawa et al., 2002). In my experiments the temperature of the sand column was held at a constant 28°C throughout its entire length, but the use of incandescent light bulbs to heat the surface sand for periods of time, could be used to manipulate sand surface temperature, and this method used to explore the role sand surface temperature plays in nest escape timing.

It is also important to note that, currently there are only two studies that have investigated the energetics of terrestrial movement from the nest to water in sea turtle hatchlings. The first study had conducted by the application of DLW method on olive ridley turtle (*Lepidochelys olivacea*) hatchlings (Clusella Trullas et al., 2006), while the second used open flow respirometry with green turtle (*Chelonia mydas*) hatchling (Williams, 2012). Hence further studies in this area are needed to build a more complete picture of the energetics of incubation, nest escape, and movement from the nest to water. This is particularly true for freshwater turtles where no data is currently available. Having said this, the data presented in my thesis indicates that the energy during embryonic development is greater that the energy used for nest escape, and that the energy used traveling from the nest to water is much smaller than the energy used to escape the nest, at least in sea turtles.

To place my results in the context of other digging animals a comparison of the net cost of transport (NCOT) can be made. NCOT varied with the number of hatchlings digging together in both Brisbane river and green turtle hatchlings (Figure 6.2). Interestingly the slope of the relationship between NCOT and group size was similar in both species, but for a given group size NCOT was greater in green turtles compared to Brisbane river turtles (ANCOVA, NCOT dependent variable, species as fixed factor, group size as covariate, $p < 0.001$, $F_{1,18} = 31.6$) most probably because of a difference in their body masses. Indeed if body mass is also included in the ANCOVA model, the species difference disappears (ANCOVA, NCOT dependent variable, species as fixed factor, group size and hatchling mass as covariates, $p = 0.450$, $F_{1,18} = 0.61$) confirming that at least statistically, the species difference can be explained by differences in body mass. The relationship between group size and NCOT described in Figure 6.2 was extrapolated to a group size of one and this value plotted along with data from other digging animals (Figure 6.3). For their body mass, hatchling turtles have a higher NCOT compared to other digging animals, and in particular the other two reptiles for which there are data, two species of semi-fossorial skinks from the genus *Liopholis* (Figure 6.3). This is probably due to differences in body shape, the skinks having long thin bodies with reduced limbs (general attributes of many fossorial animal species), compared to the short-

squat bodies of hatchling turtles which are not well adapted to digging. Hence, the energetics of nest escape of hatchlings needs to be explored in other underground nesting reptiles, the most obvious ones being lizards, snakes and crocodilians which have different body forms. Do the elongate bodies of these non-chelonian hatchlings make it easier to dig out of a nest? Of particular interest would be the energetics of nest escape in species that construct very deep nest such as the recently reported yellow spotted goanna, *Varanus panoptes* (Doody et al., 2015), and the many species that nest communally – is communal nesting a strategy to reduce the energetic cost of nest escape?

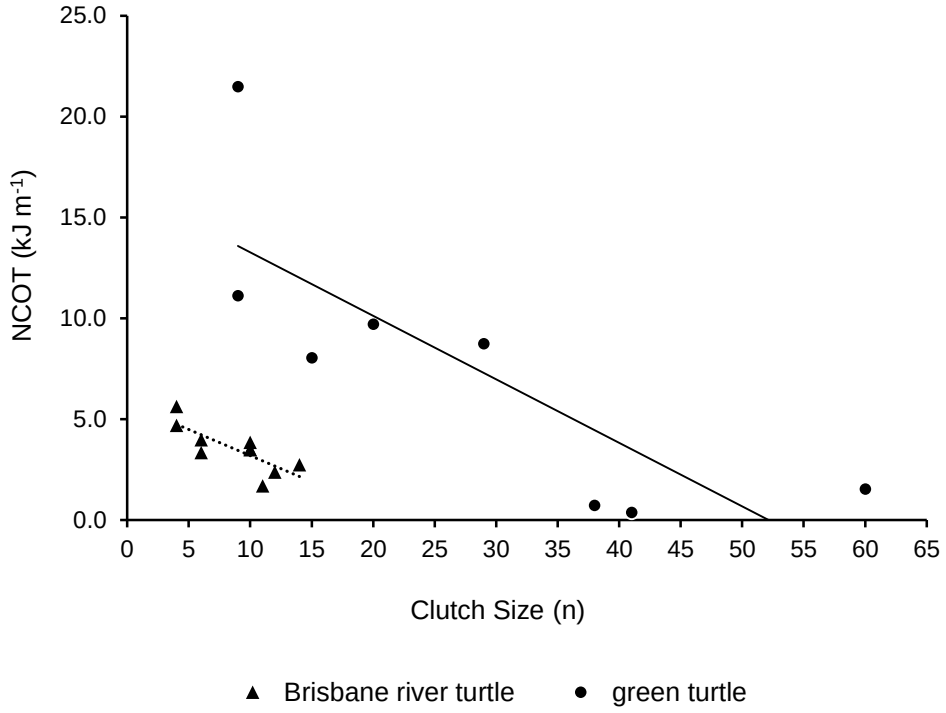

Figure 6.2: Relationship between NCOT and clutch sizes (Green turtle: y = -0.315x + 16.4, r^2 = 0.64, P = 0.007, N = 8; Brisbane river turtle: y = -0.259x + 5.8, r^2 = 0.63, P = 0.001, N = 10).

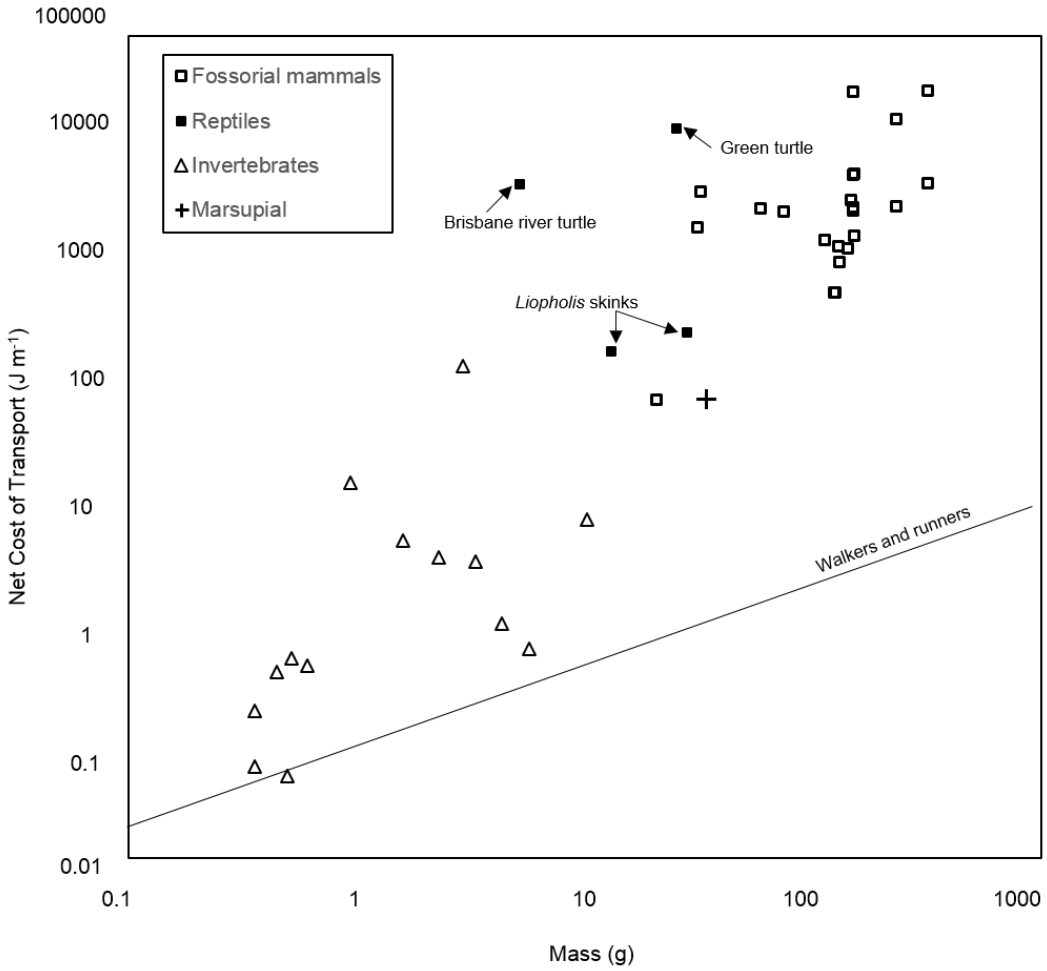

Figure 6.3. Interspecific relationship between the body mass and average net cost of transport for a selection of burrowing animals. Species included are from published data presented in Appendix I. Regression line represents the NCOT for walkers and runners extracted from Full et al. (1990) (NCOT = 0.092 $M_a^{0.69}$).

Finally, the nest escaping process is an energetically demanding process, resulting in the green turtle expending 19,000 times more and the Brisbane river turtle 14,000 more energy moving the same distance than the predicted cost of terrestrial locomotion depicted in Figure 6.3. Digging out to escape the nest consumes a large amount of the residual yolk in chelonian hatchlings. However, this study has provided the first estimates of energy sharing in synchronous digging as one of the chelonian hatchling's behavioural adaptations for energy conservation.

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APPENDIX

Appendix I. The Net Cost of Transport (NCOT) on selection of burrowing animals.

Species	Mass (g)	NCOT (J m^{-1})	Reference
Invertebrates			
Emerita portoricensis	0.5	0.05	Ansell and Trueman (1973)
Donax incarnatus	0.52	0.5	Ansell and Trueman (1973)
Sipunculus nudus	3.33	3.33	Trueman and Foster-Smith (1976)
Nephtys cirrosa	0.45	0.38	Trevor (1978)
Nereis diversicolor	0.61	0.43	Trevor (1978)
Arenicola marina	10.2	7.65	Trevor (1978)
Bullia digitalis	4.34	1	Brown (1979)
Polyphysia crassa	1.6	5.08	Hunter and Elder (1989)
Priapulus caudatus	2.3	3.61	Hunter and Elder (1989)
Tylos granulatus	5.7	0.6	Brown and Trueman (1996)
Urodacus yaschenkoi	2.93	153.61	White (2001)
Gryllotalpa monanka	0.94	15.73	White et al. (2008)
Cirriformia moorei	0.36	0.06	Dorgan et al. (2011)
Cirriformia moorei	0.36	0.18	Dorgan et al. (2011)
Reptiles			
Chelonia mydas	25.1	16100	Current study
Emydura m. signata	5.21	5500	Current study
Liopholis striata	27.9	296.03	Wu (2015)
Liopholis inornata	13	204.68	Wu (2015)
Fossorial Mammals			
Thomomys bottae	150	3250	Vleck (1979)
Thomomys bottae	150	33100	Vleck (1979)
Thomomys bottae	150	6430	Vleck (1979)
Thomomys bottae	150	3420	Vleck (1979)
Georychus capensis	113	1814.39	Du Toit et al. (1985)
Cryptomys damarensis	152.1	1967.50	Lovegrove (1989)
Cryptomys damarensis	152.1	6583.52	Lovegrove (1989)
Heterocephalus glaber	31.5	2319.82	Lovegrove (1989)
Heterocephalus glaber	32.3	4701.65	Lovegrove (1989)
Thomomys talpoides	75	3160	Lovegrove (1989)
Scapanus townsendii	148	3920	Lovegrove (1989)
Scapanus orarius	59	3380	Lovegrove (1989)
Eremitalpa namibensis	20.62	78.96	Seymour et al. (1998)
Ctenomys talarum	125	643.29	Luna and Antinuchi (2006)
Ctenomys talarum	130	1604.62	Luna and Antinuchi (2006)
Ctenomys talarum	131.6	1162.87	Luna and Antinuchi (2007)
Ctenomys talarum	126.4	647	Luna and Antinuchi (2007)
Ctenomys talarum	142.4	1532	Luna and Antinuchi (2007)

Fukomys mechowii	320	33800	Zelová et al. (2010)
Fukomys mechowii	320	5500	Zelová et al. (2010)
Heliophobius argenteocinereus	232	19300	Zelová et al. (2010)
Heliophobius argenteocinereus	232	3500	Zelová et al. (2010)
Marsupial			
Notoryctes caurines	34	81	Withers et al. (2000)