DIPTERAN LARVAE INFESTATION OF LEATHERBACK TURTLE (*DERMOCHELYS CORIACEA*) NESTS ON GANDOCA BEACH, COSTA RICA

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SONIA GAUTREAU

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ABSTRACT

DIPTERAN LARVAE INFESTATION OF LEATHERBACK TURTLE (*DERMOCHELYS CORIACEA*) NESTS ON GANDOCA BEACH, COSTA RICA

Sonia Gautreau
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Co-Advisor: Dr. Ronald J. Brooks
Co-Advisor: Dr. Steve A. Marshall

We examined the ecological characteristics of dipteran larvae infesting Leatherback nests on Gandoca Beach, Costa Rica in 2005-2006. Fly infestation exceeded 75% of nests, but levels were much lower when evaluated as the percent of the clutch infested. Fly larvae seemed to act mostly as scavengers, but a few live hatchlings were attacked indicating that flies also act opportunistically as predators. Several dipteran species were recorded with the sarcophagid *Eumacronychia sternalis* being dominant. Back-calculations from the development timeframe for *E. sternalis* showed that infestation happened shortly after hatchling emergence, suggesting that flies are attracted to the nest because of emanating odours of decomposing material brought to the surface by emerging hatchlings.

Sampling year, bacteria or fungus invasion, and the interaction between nest depth and the number of dead hatchlings best predicted the incidence of larvae within nests. Infestation levels in egg hatcheries were not higher, possibly because of protective baskets. Our results suggest that flies do not seriously threaten Leatherback turtle populations in Gandoca, but they can cause incidental mortality and measures should be taken to protect nests against larval infestation.

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GENERAL INTRODUCTION

1.1. Species Information

The Leatherback sea turtle (*Dermochelys coriacea*) diverged from other sea turtle species during the Cretaceous or Jurassic Period, 100-150 million years ago and is now the only member of the Family Dermochelyidae (Zangerl 1980). Leatherbacks are the biggest of all sea turtle species and may attain a carapace length of two meters and a body mass of 500kg (Zug 1996). Unlike all other sea turtles, Leatherbacks do not have scales as adults, they lack scutes on their carapace and they do not possess claws. The Leatherback's shell is characterized by seven longitudinal ridges and composed of a mosaic of thousands of small dermal bones that underlie a black leathery ectoderm.

1.2. Distribution

Leatherbacks have a larger geographic range than all other reptiles. They are found in tropical and temperate waters of the Atlantic, Pacific, and Indian Oceans, with the northernmost latitude recorded at 70°15N (Gullicksen 1990) and the southernmost at approximately 27°S (Boulon *et al.* 1988). All nesting beaches are located off tropical waters, but studies demonstrate that Leatherback turtles disperse broadly after nesting and frequently journey to northern waters. Adult Leatherbacks are highly migratory and are believed to be the most pelagic of all sea turtles. They feed mostly on jellyfish and other soft-bodied pelagic invertebrates (Grant and Ferrell 1993) and thus migrate long distances to exploit areas of high food concentration (Grant *et al.* 1996). In Canada, Leatherbacks are frequently observed on the continental shelves of the Atlantic (Goff and Lien 1988, James *et al.* 2005, James *et al.* 2006) and the Pacific oceans (DFO 2004).

1.3. Status

The Leatherback turtle is currently listed as critically endangered on the International Union for Conservation of Nature's Red List (Sarti Martinez 2000). Data on males are lacking so estimates of population size are based on the number of nesting females and the accuracy of these estimates is undermined by the possibility of skewed sex ratios. Nevertheless, a declining trend is clear. The global number of nesting female Leatherbacks fell from an estimated 115,000 in 1980 (Pritchard 1982) to 34,500 by 1995 (Spotila *et al.* 1996) when both Pacific and Atlantic stocks are considered. This decline is unevenly distributed with the number of nesting females dropping more severely on Pacific beaches bordering Costa Rica, Mexico and Malaysia (Reina *et al.* 2002). Reductions of up to 90% in numbers of nesting females on these beaches are attributed to illegal egg collection and incidental capture in gillnet and longline fisheries (Spotila *et al.* 2000). Trends in the Atlantic are more difficult to assess because of the variability in nesting between years, so continued monitoring is needed to discern long-term population trends (Troëng *et al.* 2004). In 1987, Berry estimated 4,987 nests per year for the entire Caribbean coast of Costa Rica which is similar to actual trends of 3,686–7,736 estimated for the Caribbean coast of Costa Rica and Northern Panama (Troëng *et al.* 2004). It therefore appears that the numbers of nesting females in Caribbean Costa Rica have remained stable or have experienced a slight decline over the past 15 years. In the Atlantic, there may be less overlap between Leatherback feeding areas and fishing zones, reducing the numbers of incidental catches compared to the Pacific (Troëng *et al.* 2004).

Although trends in Caribbean Costa Rica appear stable, on a world-wide scale Leatherback turtles are declining. Extinction in several populations is considered to be only a matter of time if the current rate of decline continues (Spotila *et al.* 1996). To achieve long-term continuation of all populations of Leatherbacks, rigorous conservation and management practices need to be applied on all stages of their development. Although increased levels of mortality are mostly associated with anthropogenic factors, natural threats such as depredation also merit scientific investigation. Despite several studies on insect infestations of turtle nests, it is still not clear if they have negative impacts on the overall reproductive success of sea turtles.

1.4. Reproduction

Leatherback turtle nesting seasons vary from one geographic location to another. In the Caribbean coast of Costa Rica, females generally nest between March and July (Chacón *et al.* 1996, Chacón 1999). Female Leatherback turtles undertake reproductive migrations to nesting grounds every two to three years, where they generally oviposit five to six clutches at nine to eleven day intervals (Plotkin 2003). Nesting happens at night with peak hours ranging from 8 pm to 4 am. Once a nest site is chosen, the nest cavity is carefully excavated using both posterior flippers. Nest depths range from 50 to 90 cm and are generally constructed near the high tide mark. Females on Gandoca Beach oviposit on average 79 normal eggs and 35 small shelled albumin globes (yolkless eggs) (Chacón 1999). These eggs are simply packets of excess albumin formed in the oviduct and covered with a shell (Bell *et al.* 2003). They are thought to play an important role in gas exchange, temperature regulation and predator diversion (Hall 1990). Most yolkless eggs are laid toward the end of oviposition. Eggs are then buried and the nest is compacted

followed by the female's return to the sea. Incubation time is usually between 55-70 days depending on nest temperature (Chacón *et al.* 1996, Chacón 1999). High temperatures allow for high metabolic rates and shorten the incubation period. Developing embryos are subject to temperature dependant sex determination. Studies have shown that constant incubation temperatures above 29.5°C produce more males whereas constant temperatures below 29.5°C produce more females (Chan and Liew 1995).

1.5. Incubation and the Nest Environment

Sea turtles do not provide any parental care to their offspring. Therefore, the eggshell and associated membranes, together with the albumen and mucus secreted during oviposition are the only protection that the eggs receive. After oviposition, the egg is exposed to environmental changes within the nest and to potential invaders such as bacteria, fungus, viruses or insects. The flexible semi-permeable Leatherback eggshell provides a mechanical/physical barrier against many biotic and abiotic factors (Ackerman 1997). Nest conditions influence embryonic survival and ultimately the success of the clutch. Successful incubation is highly dependant on the female's ability to select a suitable nest-site that will provide an appropriate environment for embryonic development. For example, nests laid below the high-tide line risk being washed away at sea (Mrosovsky 1983) while nests located too close to the supra-littoral vegetation are often invaded by rootlets (Bustard and Greenham 1968). Gas concentrations, temperature and moisture also have profound effects on the development of turtle embryos (Ackerman 1997). Gas exchange in the clutch is limited because oxygen and carbon-dioxide are only able to move through spaces between the eggs (Ackerman 1997). Sea turtle eggs are very sensitive to inundation and desiccation. Developing embryos and

hatchlings may suffocate in the event of excessive rainfall or tidal inundation (Kraemer and Bell 1980) while insufficient amounts of water results in dehydration of the eggs. Rainfall can also harden the upper-sand layer which may have detrimental effects on hatchling emergence.

1.6. Hatching and Emergence of sea turtle hatchlings

When the incubation period is over, the eggs that successfully developed pip and hatch within a few hours or days of each other (Christens 1990). The first few hatchlings to hatch will wait for most of their siblings to break free from their eggs before starting to dig upwards (Christens 1990). After hatching, the young usually take several (three to seven) days to dig to the sand surface (Balazs 1974, Christens 1990). While digging to the surface, hatchlings deplete the oxygen supply in the sand column and need to stop periodically to allow oxygen diffusion to take place (Balazs 1974). The majority of the young will emerge at the same time (Kraemer and Bell 1980), but some stragglers emerge on nights subsequent to the main emergence (Hays *et al.* 1992). Digging out of the nest is facilitated by the coordinated efforts of all the young (Balazs 1974). It is also believed that group emergence is an adaptation that reduces depredation pressure (Mrosovsky 1968). Sea turtles usually emerge at night, except when day temperatures are low (Mrosovsky 1968, Balazs 1974). If hatchlings reach the surface during daylight hours, they generally stop digging and wait until night when a decline in temperature triggers a group emergence (Bustard and Greenham 1968).

The development of an embryo involves complicated physiological processes that are very sensitive to fluctuating temperatures and to changes in the hydric environment.

Abrupt changes in abiotic factors may inhibit embryo formation or deform the embryo (Morris *et al.* 1983). Leatherback turtles have significantly lower hatching success than other species of sea turtles (Whitmore and Dutton 1985, Chan 1989) because of high embryonic mortality rates (Bell *et al.* 2003). Average Leatherback nest productivity in Gandoca varies between years, but hatching success is generally between 50-70% (Chacón and Machado 2005, 2006) which lies within the normal range for the species (Whitmore and Dutton 1985, Leslie *et al.* 1996). Since Leatherbacks have more dead propagules per nest, insect infestation rates of Leatherback nests may be higher compared to other species of sea turtles. Embryos that die early in development will decompose quickly and may carry chemical cues to the surface which could attract gravid flies to the nest. This could potentially put the rest of the clutch at risk of infestation by fly larvae.

1.7. Decomposition and Saprophagous Flies

The actions of cellular enzymes start the decomposition process only minutes after death. The catabolism of tissue into gases, liquids and soft molecules requires work from micro-organisms such as bacteria, fungi and protozoa (Vass 2001). Insects contribute significantly to the degradation of carrion, with flies being the most predominant during all stages of decomposition (Abell *et al.* 1982, VanLaerhoven and Anderson 1999). Carrion-eating flies have adapted life strategies that enable them to exploit an ephemeral food source (Denno and Cothran 1976, Braack 1987). Adult flies disperse rapidly, they quickly detect and colonize new resources, and they maximize reproductive recruitment by producing many young that grow rapidly.

There are several major families of Diptera that are associated with carrion: the blow flies (Calliphoridae), the flesh flies (Sarcophagidae), the muscid flies (Muscidae) and the scuttle flies (Phoridae). Other groups of flies also feed on necrotic remains, but are not as common and usually do not contribute as obviously to tissue degradation. Gravid female flies follow physical and chemical stimuli from the environment to find suitable ovi- or larviposition substrates. Calliphorids, muscids and phorids are all oviparous, and they deposit a large number of eggs that usually hatch rapidly. Sarcophagid flies have the ability to deposit active first instar larvae rather than eggs. Subsequently, the larvae start feeding on the soft tissues and development is very rapid with maggots growing more rapidly when exposed to higher temperatures (VanLaerhoven and Anderson 1999). Once their food requirements have been met, the larvae move away from the food source and pupate in the soil (Braack 1981).

The Calliphoridae are among the first insects to detect decomposing organic material, often within minutes of death and over great distances (Braack 1981, Rodriguez and Bass 1985, Mann *et al.* 1990). Calliphorid flies are generally the most numerous group attending a carcass (Blackith and Blackith 1990, Bourel *et al.* 1999, Archer and Elgar 2003a) and are among the largest maggots of the carrion community, making them effective competitors for space and resources. Interestingly, they have never been recorded infesting turtle clutches, as they may not be able to infest dead turtle eggs and hatchlings inside a nest because of their inability to borrow through sand. Lundt (1964) stated that calliphorids will only lay their eggs on the soil surface in exceptional circumstances.

The Family Sarcophagidae has 2,600 species described world-wide and is divided in three subgroups: Miltogramminae, Paramacronychiinae and Sarcophaginae (Pape 1996). The Sarcophagidae includes many specialists ranging from inhabitants of plants, bat coprophages, crab saprophages, wasp nest inquilines and insect parasitoids (Pape 1996). Several species of sarcophagid are partly predatory (Pickens 1981) and produce myiasis (infestation of live tissue by fly larvae) in selected species of turtles (Dodge 1955), lizards (Dodge 1955, Mullen *et al.* 1984, Trauth and Mullen 1990, Marmels 1994) and frogs (Crump and Pounds 1985). Despite their common name (flesh flies), only a small minority of sarcophagid species breed in vertebrate carcasses and they generally colonize necrotic remains during the initial stages of decomposition (Denno and Cothran 1976). Sarcophagidae are ovoviviparous, the eggs hatch in the uterus of the females before she lays them, which allows the offspring to begin feeding immediately after being deposited directly on the food source. The mobility of the larvae may help them gain access to buried turtle nests if the first instar larvae are capable of burrowing and could also give them an advantage over egg laying flies that would need to wait for the eggs to hatch before starting to feed.

Muscid flies are generally the last of important carrion associated Diptera to colonize decomposing remains. They usually arrive at the site after the calliphorids and the sarcophagids (Arnaldos *et al.* 2001, Byrd and Castner 2001). While some Muscidae species are haematophagous, coprophagous, phytophagous and saproghagous, the vast majority of muscid larvae are at least partially carnivorous in the final larval instar

(Skidmore 1985). Many muscids breed on carrion, but they can also attack wounds of live animals especially when their numbers are abnormally large (Bishopp *et al.* 1917). Lundt (1964) found that two species of muscid, *Muscina prolapsa* and *Ophyra leucostoma*, laid their eggs on the soil surface and that the hatched larvae burrowed to carrion isolated underground (2.5-10 cm). This ability might allow them to infest buried turtle nests.

Phorid flies are small and easily recognized by their humpback appearance. More than half of the Phoridae belong to the single genus *Megaselia*. The adults as well as the larvae of phorids are polyphagous and they can be found on or near many kinds of decomposing plant and animal matter (Peterson 1981). Because of their small size, the larvae tend to be more numerous in carcasses where other bigger species of maggots are absent (Campobasso *et al.* 2004). Females are attracted to carrion to feed on the rich protein fluids that will allow for maturation of their eggs, but the presence of adults on carcasses does not necessarily mean that they are using them as a breeding medium (Disney 2005). Phorid flies have been known to lay eggs almost anywhere even through fine gauze and are often unwanted ubiquitous pests where live insect colonies are maintained (Disney 2005). Adult phorids are capable of burrowing through the soil, up to a depth of one meter in order to lay their eggs on buried corpses (Lundt 1964, Disney 1994) and this adaptation could allow them to access buried sea turtle nests.

1.8. Effects of Dipteran Infestation on Sea Turtle Nests

There are many anthropogenic and natural threats to sea turtle eggs and hatchlings, the two most vulnerable stages in the life cycle of turtles. A natural threat

such as an egg predator or parasite may significantly reduce hatching success and affect hatchling viability. Because of the recent decline in sea turtle populations, it is important to try and maximize the recruitment of young. A possible threat to sea turtle clutches that has not received adequate attention by scientists is that of nest infestation by insects. Several studies have documented the presence of ants (Moulis 1997, Allen *et al.* 2001), beetles (Baran and Türkozan 1994, Broderick and Hancock 1997, Baran *et al.* 2001, McGowan *et al.* 2001a, Donlan *et al.* 2004, Katlmş *et al.* 2006), crickets (Maros *et al.* 2003, Maros *et al.* 2005) and fly larvae (Fowler 1979, Vogt 1981, Lopes 1982, Bjorndal *et al.* 1985, Whitmore and Dutton 1985, López Barbosa 1989, Acuña-Mesén and Hanson 1990, Andrade *et al.* 1992, Iverson and Perry 1994, Vásquez Bustos 1994, Broderick and Hancock 1997, Baran *et al.* 2001, McGowan *et al.* 2001a, McGowan *et al.* 2001b, Özdemir *et al.* 2004, Hall 2005, Phillott 2005, Hall and Parmenter 2006, Katlmş *et al.* 2006) feeding on turtle nest contents and possibly damaging the clutches. However, only a few of these studies have dealt with insect infestation as the main focus of research. The role of these insects in the nest is still unknown and merits further investigation.

Many features of sea turtle nests make them attractive to scavengers and predators alike. On many sea turtle nesting beaches, fly larvae are the dominant insects found in nests. Adult flies locate carrion by chemoreception and gravid female flies presumably smell the decomposing remains inside the nests, deposit live maggots on the sand surface and the larvae subsequently burrow through the sand by following chemical attractants (Vogt 1981, McGowan *et al.* 2001a). However, once the larvae reach the nest contents, it is still unclear whether they function as scavengers (feeding on necrotic nest material) or

as predators (depredating developing embryos and hatchlings). Some saprophagous larvae breed mainly on carrion, but can become predatory when necrotic tissue is unavailable (Zumpt 1965, Hall and Wall 1995). Therefore, larvae that infest turtle nests may attack viable eggs and hatchlings after utilizing the available decomposing nest remains. Most studies conducted on turtle nest infestation by dipterans concluded that they do not pose a serious threat to turtle populations. Most suggested that fly larvae fed on failed eggs and dead hatchlings and they do not appear to be an important source of embryonic mortality (Fowler 1979, McGowan *et al.* 2001a, Hall and Parmenter 2006). However, fly larvae have infrequently been reported to attack viable turtle embryos (Acuña-Mesén and Hanson 1990, Iverson and Perry 1994) and Lopes (1982) found that flies reduced hatching success by over 30%. Because of these contrasting results, further research is needed to confirm whether or not Diptera pose a threat to the overall reproductive success of sea turtles.

The severity of fly infestation can be measured at the level of the rookery or of the individual clutch. Measures of infestation at the rookery level are likely to be higher than levels at the individual clutch because the majority of sea turtle clutches have eggs that fail to develop and the smell of rotting embryos emanating from the nests will attract gravid flies, making it likely that most nests would contain a few larvae. However, the proportion of total nests infested may not be a good measure of the actual magnitude of infestation because no accommodation is made as to whether a few or many maggots are present in any given nest and it does not quantify the damage inflicted on individual clutches. Only two studies have measured infestation at the level of the individual clutch

and both reported high rates of infestation at the rookery level, but the number of infested eggs and hatchlings per nest were typically low (McGowan *et al.* 2001a, Hall and Parmenter 2006). If flies were actively attacking viable embryos, one would expect to see much higher numbers of eggs affected in any given infested nest (Hall and Parmenter 2006).

1.9. Fly Species Recorded from Sea Turtle Nests

Most reports documenting dipteran larvae in sea turtle nests have identified larvae as belonging to the Sarcophagidae (Lopes 1982, López Barbosa 1989, Andrade *et al.* 1992, Broderick and Hancock 1997, McGowan *et al.* 2001a, Hall 2005, Phillott 2005) or the Phoridae (Fowler 1979, Bjorndal *et al.* 1985, Broderick and Hancock 1997, McGowan *et al.* 2001a). Most recently, Hall and Parmenter (2006) in Australia recorded two species of Platystomatidae from Green (C*helonia mydas*) and Loggerhead (*Caretta caretta*) nests. A few studies have identified Muscidae larvae from Loggerhead nests in Turkey (Baran *et al.* 2001, Özdemir *et al.* 2004, Katlmş *et al.* 2006) and in Cyprus (McGowan *et al.* 2001a). Studies in Costa Rica have documented larvae of the Phoridae, specifically *Megaselia scalaris*, from nests of Green (Fowler 1979) and Hawksbill (*Eretmochelys imbricata*) turtles (Bjorndal *et al.* 1985). T. Pape (pers. comm.) has documented *Eumacronychia sternalis* of the Sarcophagidae from Leatherback turtle nests in Costa Rica.

1.10. Mechanisms of Dipteran Infestation of Sea Turtle Nests

Discerning the timing of infestation is important to determine if flies are acting as scavengers or as predators in nests. There are several stages at which turtle clutches could

become infested by fly larvae. During oviposition, the eggs are exposed and cloacal secretion may act as an attractant. Infrequently, flies have been seen landing on eggs while female turtles are depositing their clutch (Broderick and Hancock 1997). However, fly larvae develop quickly, particularly in the prevailing high temperatures found in the tropics, compared to the developmental period of sea turtle embryos. Members of the Phoridae can complete their life cycle in 20-25 days (Disney 1994) and members of the Sarcophagidae in 15-20 days (Kamal 1958). Therefore, infestation at the time of oviposition seems unlikely because larvae are recorded during post-emergence excavation of turtle nests (Andrade *et al.* 1992, Broderick and Hancock 1997, McGowan *et al.* 2001a, McGowan *et al.* 2001b, Hall and Parmenter 2006).

Forensic entomologists use developmental rates of fly larvae to approximate time of death of a corpse and the same technique can be applied to estimate the timing of nest infestation by fly maggots. Two studies have used the development time of flies and back-calculated to estimate when infestation was taking place (McGowan *et al.* 2001a, Hall 2005). In Australia, Platystomatidae species appeared to be specialists of turtle eggs and were infesting nests as much as two to three weeks prior to hatchling emergence (Hall 2005). The Sarcophagid species recorded in Australia and in Cyprus were infesting the clutches a few days prior to or shortly after hatchling emergence (McGowan *et al.* 2001a, Hall 2005). Moving hatchlings in the nest chamber and in the sand column might release chemical attractants and advertise the location of nests. After piping, hatchlings have external undigested yolk which they retract into the body cavity through the umbilical opening in the mid-plastral suture from one to four days after leaving the

eggshell (Kraemer and Bennett 1981). The presence of an external yolk sac coupled with the amount of time hatchlings spend in the nest chamber before emerging may create an opportunity for the larvae to attack. The longer the emergence process takes, the greater the number of infested eggs in a clutch (McGowan *et al.* 2001b). Nests may also be easier to find over time as a result of decaying tissue build up releasing stronger smells.

Some physical and biological nest factors might predispose individual clutches to infestation by flies. To date, few studies have attempted to identify why certain turtle nests remain free of fly larvae and others become infested. Nests containing turtle eggs broken open by ghost crabs (*Ocypode* spp.) or by tree rootlets may influence infestation rates by releasing stronger cues and attracting more flies. Certain sections of the beach may support greater numbers of flies and the likelihood of infestation for the clutches deposited in these areas could be much higher than the nests located in areas of low fly abundance. The selection of a nest-site by female turtles or by someone translocating the clutch affects the likelihood of depredation and could also be of potential significance to fly infestation (Blamires and Guinea 1998). Clutches of Loggerhead turtles laid close to the sea at Alagadi Beach in Cyprus contained significantly more eggs infested with sarcophagid larvae than did nests further from the high water mark (McGowan *et al.* 2001b). In contrast, Vásquez Bustos (1994) found that the location of hatcheries with respect to the high water mark and the density of nests had no effect on larvae infestation rates of Leatherback turtle clutches. Species-specific behaviours of the flies involved could possibly explain the discrepancies between both investigations. Dipterans in Cyprus may be more abundant near the sea to exploit food sources that are washed on

shore or because of their own moisture requirements (McGowan *et al.* 2001b). Nest depth may also be an important factor in determining whether or not an individual nest will be infested by fly larvae. Shallow nests in Cyprus were more prone to dipteran infestation with deep nests containing fewer infested eggs (McGowan *et al.* 2001b). Certain chemical cues associated with decaying tissue may loose potency as they permeate up through the sand column or larvae may have limited burrowing skills and thus cannot reach deeply buried eggs. Research on the digging abilities of larvae and the capacity of female flies to detect decaying tissue is needed to resolve some of these issues.

Nest translocation in a communal egg hatchery and in other areas of the beach is a management practice used by many conservation programs to increase nest success. In some cases, egg handling lowers hatching success (Eckert and Eckert 1990) and this could attract more flies to the remaining developing embryos of the clutch. This was not the case in Cyprus, the clutches that were left *in situ* had more infested eggs than the relocated nests. However, translocated nests were also found to be deeper and depth may be the main factor involved and not relocation per se (McGowan *et al.* 2001b). Communal egg hatcheries may have higher levels of fly infestation because of a high density of nests. On the Pacific coast of Mexico, nests that were translocated to a common hatchery experienced higher levels of dipteran infestation than *in situ* nests (Lopes 1982, Andrade *et al.* 1992) even though in an earlier study near the same location, Vásquez Bustos (1994) demonstrated that the number and density of nests had no effect on infestation rates. The determination of nest factors that are associated with fly

infestation is important for the creation of proper management strategies that will minimize the negative effects of flies on turtle embryos and hatchlings.

1.11. Objectives of Study

This investigation was prompted by the need to determine if flies are a serious threat to Leatherback turtle nests on Gandoca Beach and to elucidate the potential role of the flies in the nest environment. The research objectives of the study were to:

1) Assess whether the flies are acting as scavengers (feeding on necrotic nest remains) or as predators (depredating live embryos and hatchlings).
2) Identify which species of dipterans infest Leatherback turtle clutches.
3) Estimate the timing of infestation.
4) Determine possible nest entry mechanisms used by dipteran larvae.
5) Ascertain which nest factors might affect dipteran infestation rates of individual turtle nests.

We also examined the effects of management practices such as communal egg hatcheries and clutch relocation on fly infestation. Suggestions on how to reduce infestation rates are discussed to try and improve current management policies. Our general hypothesis was that if flies are attracted to decomposing nest contents, then infestation should take place late in the incubation period and the larvae should act mainly as scavengers posing no significant threat to Leatherback turtles.

GENERAL METHODOLOGY

My research was conducted in collaboration with Asociación ANAI, a Costa Rican non-governmental organization that has been working to conserve the Gandoca sea turtle rookery since 1986. All data collection took place during the Leatherback turtle nesting season from the beginning of March to mid-August in 2005 and 2006.

2.1. Study Site

Gandoca Beach lies within the Gandoca/Manzanillo National Wildlife refuge in the south eastern coast of Costa Rica (9°35' N, 82°34' W). The refuge is characterized by humid tropical forest, and the annual average temperature ranges from 25°C to 27°C with a maximum of 31°C in the dry season (December to April) and a minimum of 20°C in the wet season (May to November). This area is considered the most humid of the whole country with a relative humidity between 86% and 88% (Cuevas 2002).

Gandoca Beach, which extends 11 km, lies between Monkey Point and the mouth of the Sixaola river, forming the border with Panama (Figure 2-1). The beach is characterized by strong currents, a medium to steep slope and a crenate to dentate shoreline (Chacón *et al.* 1996). It is highly dynamic and subject to longshore currents, storm waves and high spring tides which regularly change the width and height of the beach. The sand is mainly composed of fine granules of alluvial origin which gives it a grey/black color when humid. The shore is mostly sandy, but due to recent coastal development in the Caribbean lowland, organic waste and land derived sediments are

increasing. These sediments are assumed to be responsible for the degradation of coral reefs and sea grass beds in the area (Chacón *et al.* 1996). For most of the year, the beach is partially covered with assorted debris such as wood, coconut husks and a wide variety of plastic articles, most of which originate from the banana plantations in the Sixaola watershed. Big logs from inland logging frequently wash ashore and often create a barrier for nesting turtles and hatchlings.

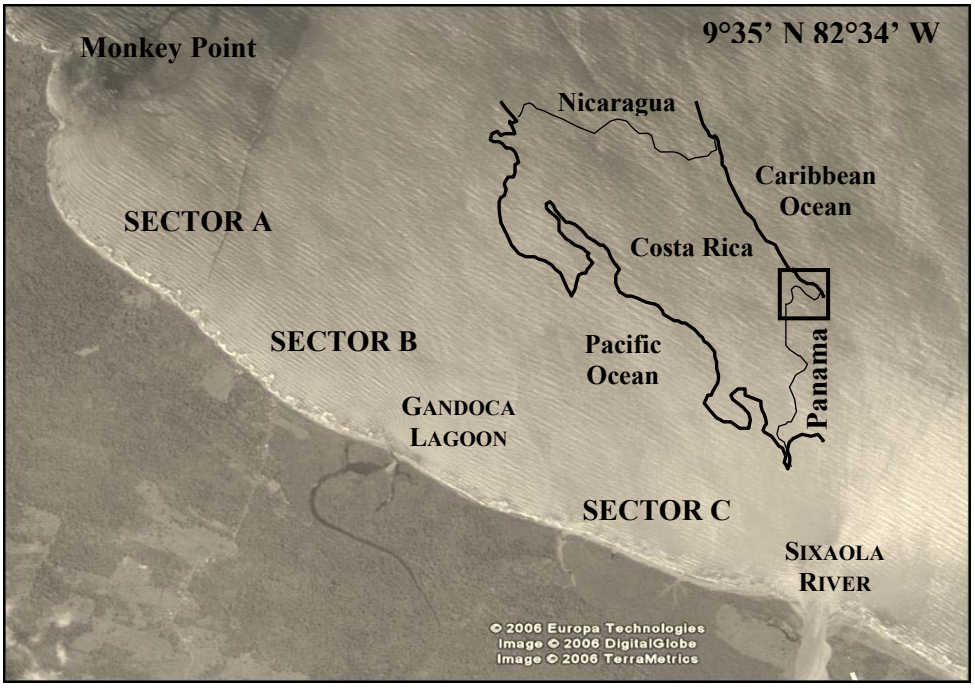


Figure 2-1: Map showing the location of the sectors on Gandoca Beach

Gandoca Beach is one of the most important nesting sites for sea turtles on the Caribbean coast of Costa Rica (Chacón *et al.* 1996, Chacón 1999). All four species of sea turtles known from the Caribbean have been recorded in Gandoca (Green turtle, C*helonia mydas*; Hawksbill turtle, *Eretmochelys imbricata*; Loggerhead turtle, *Caretta caretta*, and Leatherback turtle, *Dermochelys coriacea*). Leatherback turtles are by far the most common sea turtles nesting in Gandoca between 1990-1997, 1,045 females were tagged

and a total of 3,484 nests recorded. The average number of nests per year was 534 with a range of 226–1,135 nests (Chacón 1999).

2.2. Beach Preparation and Nest Translocation

The beach was divided every 50 meters with a wooden marker located near the vegetation border. In total 8.5 km of the beach were marked and divided in three sectors: A, B and C. Sector A extended from Monkey Point to Middle Creek, sector B from Middle Creek to the Gandoca Lagoon and sector C from the Gandoca Lagoon to the Sixaola River (Figure 2-1). Every night from 8pm to 4am, research assistants and volunteers walked the beach in search of gravid sea turtles. As part of Asociación ANAI's ongoing research program, several measurements were taken on female turtles and their nests. All methodologies used during the nesting process are detailed in ANAI's annual reports (Chacón and Machado 2005, 2006).

When it was assumed that the chances of survival of the clutch were low, usually due to the instability of the beach (inundation and/or erosion) or to high human activity, nests were relocated in a safer area of the beach or in one of two egg hatcheries within approximately one hour of the female returning to the sea. The eggs were collected in a plastic bag while the female was ovipositing and the depth of the nests was measured using a wooden stick. Artificial nest chambers were subsequently dug by hand above the high tide mark or in the hatchery while trying to duplicate the nest measurements of the *in situ* nest. Eggs were transferred to the artificial nest, normal/fertile eggs placed on the bottom and yolkless eggs placed on top. Clutch sizes were recorded at this time. Some nests were planted with thermocouples and temperature dataloggers. The temperature of

these nests was recorded every six hours. During oviposition and relocation of eggs, all staff was asked to observe closely if flies were present near the egg chamber to determine if they were depositing eggs or larvae on newly oviposited turtle clutches.

2.3. Communal Egg Hatcheries

Two communal egg hatcheries were built on the beach, one in sector A and another in sector B. Hatcheries were located in different areas for both nesting seasons to reduce the potential of bacteria and/or fungus infection of the sand. Before the construction, the sand was sifted with a 0.25 cm sieve to a depth of 90 cm to remove pieces of wood, rootlets and other debris that could have affected the incubation of the eggs. After the sifting, the hatchery area was surrounded by a fence and sand bags were put on the outside to avoid inundation by the tide. Half of the hatchery was covered with black netting to shade some nests and minimize the possibility of producing skewed sex ratios. Hatchery A covered an area of 47.5 m^2 in 2005 and 90 m^2 in 2006. Hatchery B covered an area of 62.5 m^2 in 2005 and 98.6 m^2 in 2006. Nest density in 2005 was two nests per one m^2 and in 2006 two nests per 1.6 m^2. The inside area of the hatchery was delimited by rope into squares of 50 cm x 50 cm in 2005, and 80 cm x 80 cm in 2006. The emanating rows and columns were each provided with an identification number. One square was left empty between each nest to provide adequate space for individual egg clutches to incubate. The overall size of the hatcheries was bigger in the 2006 nesting season to reduce nest density. For protection of the clutches from predators such as ghost crabs (*Ocypode* spp.) and from flies laying their larvae in the nests, mesh baskets (netting of 0.1 cm x 0.1 cm) with a diameter of approximately 60 cm were placed on top of the nests immediately after the eggs were translocated (Figure 2-2). These baskets also

helped to contain hatchlings when they emerged. A random selection of nests outside the hatchery was also covered with baskets in 2006 to investigate their effectiveness to reduce infestation by flies.

Figure 2-2: Communal egg hatchery showing artificial nests covered by white mesh baskets.

2.4. Nest Identification

Nests were identified using various methodologies. Several nests were marked by triangulation directly after the female finished ovipositing. The distance of the clutch to two distinct beach markers (e.i. palm tree, wooden marker) was measured, as well as the distance between the two markers. In areas of high nest density, nests were planted with numerated metal tags to assure correct identification of the clutch. This allowed a comparison of relocated nests versus *in situ* nests and provided the exact oviposition date. All nests brought to the hatchery were individually marked.

Volunteers patrolled the beach several times daily when hatchlings were expected to emerge to locate *in situ* and relocated nests. Hatchery nests were reviewed by volunteers every half hour for emerging turtles. Hatchling emergence was determined on the basis of the following: (1) hatchlings observed emerging from the nest (2) hatchling tracks observed (3) slight depression observed in the sand-surface indicating the

reduction of sand volume at hatchling emergence. The top of the sand column (approximately 30 cm below the surface) above the emerged nests were checked for straggling hatchlings. This was done to reduce mortality caused by high sand temperatures at the sand surface and to avoid fly depredation of the hatchlings. All hatchlings recovered from *in situ*, relocated or hatchery nests were left to crawl at sea as soon as possible. Whenever the sand temperature was too hot for immediate release of the hatchlings, they were kept in boxes filled with humid sand and left in a shaded area near the hatchery. The hatchlings were then released late in the afternoon. All emerged nests were marked for subsequent nest excavation.

2.5. Post-Emergence Nest Excavations

Nests were excavated by hand within five days of hatchling emergence. When the first eggshell fragments were uncovered, the depth to the top of the egg chamber was recorded. The nest contents were then removed and the depth to the bottom of the egg chamber was measured. All eggshells were counted to assess nest success. When eggshell fragments were bigger than 50% of one egg they were counted as one emerged hatchling, the remaining pieces were puzzled together to the approximate size of one egg. Nest contents were examined and separated using the categories listed in (Table 2-1). The number of days between hatchling emergence and excavation was calculated. When the oviposition date was known, the number of incubation days was determined.

Table 2-1: Characterisation of excavated nest contents using morphological descriptors.

Type of nest content	Attributes allowing the categorisation of the nest content
Eggshell	A shell fragment greater than 50% of one egg.
Undeveloped	An egg that did not show gross signs of embryonic development
Embryo	An egg that contained a dead embryo
Pipped	A pipped egg with a fully developed hatchling that died prior to emergence from the eggshell
Depredated	Bacteria/Fungus: Eggs found with signs of bacteria or fungus invasion
	Crab hole: Eggs found with characteristic snip-marks made by crabs
	Larvae hole: Eggs found with small circular holes made by fly larvae
Live hatchling	Any live hatchling found inside the nest chamber or in the sand column
Dead hatchling	Any dead hatchling found inside the nest chamber or in the sand column
Yolkless egg	A shelled albumin globe
Infested	Any egg or hatchling found infested with fly larvae

CHAPTER I

VARIATION IN LEATHERBACK TURTLE NEST SUCCESS ON GANDOCA BEACH DUE TO INFESTATION BY DIPTERAN LARVAE

3.1. Introduction

Developing turtle embryos are sensitive to variations in the physical conditions of the beach sand, such as temperature, humidity, salinity and levels of respiratory gases (Ackerman 1997). Therefore, the success of a clutch is highly dependent on the ability of the female turtle to excavate an appropriate egg chamber on a favourable location of the beach. Many characteristics of sea turtle nests make them attractive to predators and scavengers alike and depredation is often an important source of mortality for turtle eggs during incubation. Carnivorous mammals, birds, crabs, fish and insects feed on sea turtle clutches. Other sources of embryonic mortality include developmental abnormalities, infertility, tidal inundation, microbial infection, plant root invasion and extreme weather conditions (Ackerman 1997, Phillott *et al.* 2001).

Leatherback turtle (*Dermochelys coriacea*) clutches generally have significantly lower nest success rates than other species of sea turtles (Whitmore and Dutton 1985, Chan 1989). Reported hatch success for Leatherbacks is quite variable, but is generally around 50% (Whitmore and Dutton 1985, Chan 1989, Eckert and Eckert 1990, Chacón *et al.* 1996, Chacón 1999). Egg failure in this species is mostly attributed to high embryonic mortality rather than egg infertility (Bell *et al.* 2003). Since Leatherback nests contain many dead embryos and hatchlings, the smell emanating from necrotic tissue built-up

would presumably attract many carrion-associated flies to the nests. The damage caused by maggots infesting sea turtle clutches may lower nest success, but the research on infestations has yet to determine whether or not they pose a serious threat to sea turtle populations. So far, the majority of studies have only focused on the overall infestation rate at the rookery level and have not measured the impact on individual nests (Fowler 1979, Lopes 1982, Bjorndal *et al.* 1985, Andrade *et al.* 1992, Vásquez Bustos 1994, Baran *et al.* 2001, Özdemir *et al.* 2004). However, calculating the proportion of nests infested in a rookery may over-represent the infestation as it makes no accommodation as to whether the maggots were consuming one or many eggs within a given nest. The few studies that have looked at infestation levels beyond the rookery levels have found that the actual number of infested eggs and hatchlings per clutch were quite low compared to the infestation rates found on the beach as a whole (Broderick and Hancock 1997, McGowan et al. 2001a, Hall and Parmenter 2006). Moreover, infestation levels may be high at the rookery level, but flies may be scavenging nest remains and not actively depredating developing embryos.

Sea turtle nests contain a variety of materials that could be used as a food resource and fly larvae may have feeding preferences for specific nest contents. If larvae are not able to breach the eggshell, they would be restricted to feeding on pipped or broken eggs, as the shell would act as a physical barrier protecting viable eggs from maggots. Previous studies suggested that the larvae prefer to feed on necrotic nest material (Fowler 1979, McGowan *et al.* 2001a, Hall and Parmenter 2006) but some saprophagous maggots can occasionally act as agents of myiasis in wounded and dying animals (Zumpt 1965, Hall

and Wall 1995), and this may put some eggs and hatchlings at risk of being attacked. Discriminating exactly what the larvae are consuming inside turtle nests could provide insight into whether the flies are occupying a scavenger or a predator niche (or both) and help to assess when infestation is taking place. We investigated the relationship between nest success and larval infestation rates at both the rookery and individual nest levels, and examined what larvae used as a food resource within Leatherback turtle nests. We hypothesized that higher rates of larval infestation in leatherback nests will be associated with lower nest success since nests with greater amounts of decaying material would attract more flies to the clutch.

3.2. Materials and Methods

Effects of infestation on nest success

To measure the impact of fly infestation on Leatherback turtle nest productivity, infestations were examined both at the rookery level and at the individual clutch level. Nests were located and excavated following the procedures in Section: 2.5. Nest contents were categorized based on morphological characteristics (Table 2-1). No attempts were made to count the number of larvae per nest because of the difficulties involved. Larvae were often present in great numbers, they were often too minute to see and many would have been lost during nest excavations. The number of infested eggs or hatchlings per clutch was considered to be more relevant to nest productivity than the actual number of larvae in each nest. Nests that failed completely to hatch, usually because of tidal inundation, sand contamination, nest depredation or female infertility were removed from all calculations and analyses.

To determine infestation rates at the rookery level, the percentage of infested nests on the beach was calculated. Next, hatch and emergence success values were calculated for each nest using the following equations:

$$\text{Clutch Size} = \text{eggshells} + \text{undeveloped eggs} + \text{embryos} + \text{pipped eggs}$$

$$\text{Hatch Success (\%)} = (\text{eggshells} \div \text{clutch size}) \times 100$$

$$\text{Emergence Success (\%)} = ((\text{eggshells} - (\text{live hatchlings} + \text{dead hatchlings})) \div \text{clutch size}) \times 100$$

Hatch success of a turtle clutch is calculated as the percentage of hatchlings that successfully come out of their eggshells. Emergence success is the percentage of hatchlings that successfully emerge out of the nest. Values for emergence success are lower because hatchlings encountered inside the egg chamber are not counted.

The effect of dipteran infestation and clutch translocation (*in situ*, relocated, hatchery A and hatchery B) on hatch and emergence success values for both nesting seasons was analyzed using two-factor ANOVAs with replication. The dependant variables were expressed as proportions and were transformed using $x' = \arcsin \sqrt{(x)}$ in order to comply with the requirements of the ANOVA tests (Zar 1999). Results were considered significant at the 0.05 level.

Infestation was then examined at the level of individual nests by calculating the percentage of each individual clutch that was infested and the percentage of nest remnants (includes all hatchlings and eggs that were available in the nest during nest excavation) that were infested. These calculations give an idea of larval prevalence in the nests. The percentage of each individual clutch and of total nest "remnants" that was infested was calculated as follows:

Total Infested = infested undeveloped egg + infested embryo + infested pipped egg + infested dead hatchling + infested live hatchling

Number of Nest Remnants = undeveloped egg + embryo + pipped egg + dead hatchling + live hatchling

% of Nest Remnants Infested = (Total Infested ÷ Number of Available Nest Remnants) x 100

% Clutch Infested = (Total Infested ÷ Clutch Size) x 100

Next, the possibility that variability in hatch and emergence success values could be predicted by the number of eggs and hatchlings per nest infested with larvae was tested using a Linear Regression. Before analysis, the data were transformed using $x' = \arcsin \sqrt{(x)}$ (Zar 1999).

Larval feeding preferences

The feeding preferences of the larvae within nests were assessed by calculating the proportion of individual nest "remnants" (including eggshells) that were used as a food resource. The proportion of each type of nest "remnant" that was infested by larvae was converted to a percentage value by dividing the number of infested "remnants" by the total for that type of "remnant" in the nest, then multiplied by 100.

All statistical analyses were performed using the Statistical Package for the Social Sciences, version 12 (SPSS, Inc) and Microsoft Excel (Microsoft Corporation).

3.3. Results

Effects of infestation on nest success

The prevalence of nest infestation for Leatherback nests on Gandoca Beach was high in both nesting seasons. Dipteran larvae were found in a total of 166 and 135 nests in 2005 and 2006, respectively. This represented 80.2% and 75.0% of the assessed nests, respectively (Table 3-1). Hatch (HS) and emergence success (ES) for clutches were lower in 2005 than in 2006. Average hatch success was 66.9% in 2005 and 70.1% in 2006. Average emergence success was 61.7% in 2005 and 64.8% in 2006 (Table 3-1).

Coefficient ± SE

(P-value)

The two-factor ANOVA analyses showed that the effect of dipteran infestation (whether or not the nest was infested by fly larvae) and nest translocation (*in situ*, relocated, hatchery A and hatchery B) on hatch and emergence successes was significant in 2005 (HS: $F_{(7,182)}=4.61$, $p<0.001$, $R^2=0.12$, ES: $F_{(7,182)}=4.19$, $p<0.001$, $R^2=0.11$), but not in 2006 (HS: $F_{(7,164)}=1.43$, $p=0.197$, $R^2=0.02$, ES: $F_{(7,164)}=1.37$, $p=0.220$, $R^2=0.02$) (Figure 3-1 and Figure 3-2), suggesting that the variability in nest success can not be explained by these two variables alone. The interaction between whether or not a nest was infested by fly larvae and nest translocation was significant in 2005 (HS: $F_{(3,182)}=3.03$, $p=0.031$, ES: $F_{(3,182)}=3.28$, $p=0.022$). Therefore, the individual effects of fly infestation and nest translocation on nest success can not be elucidated.

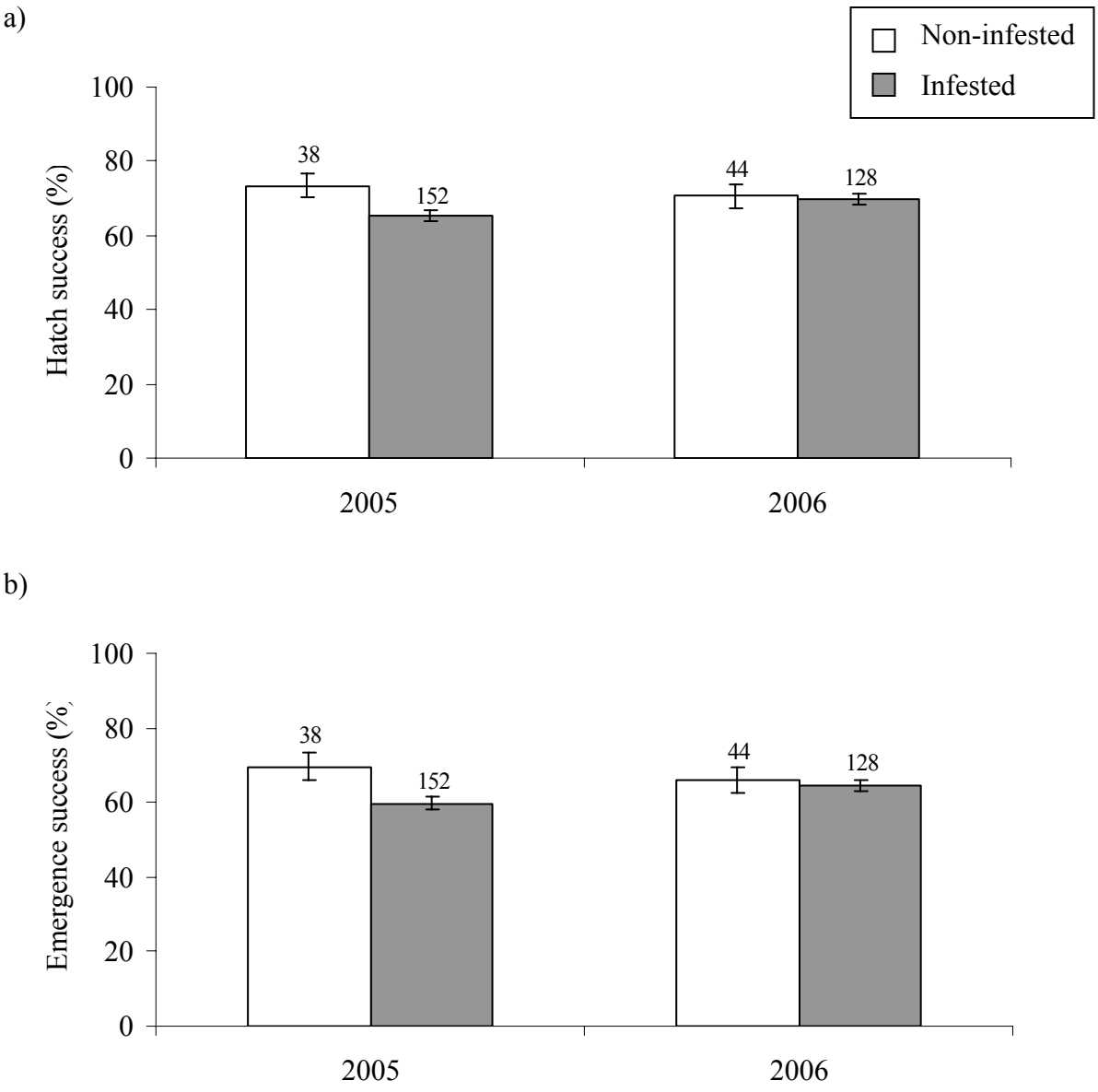
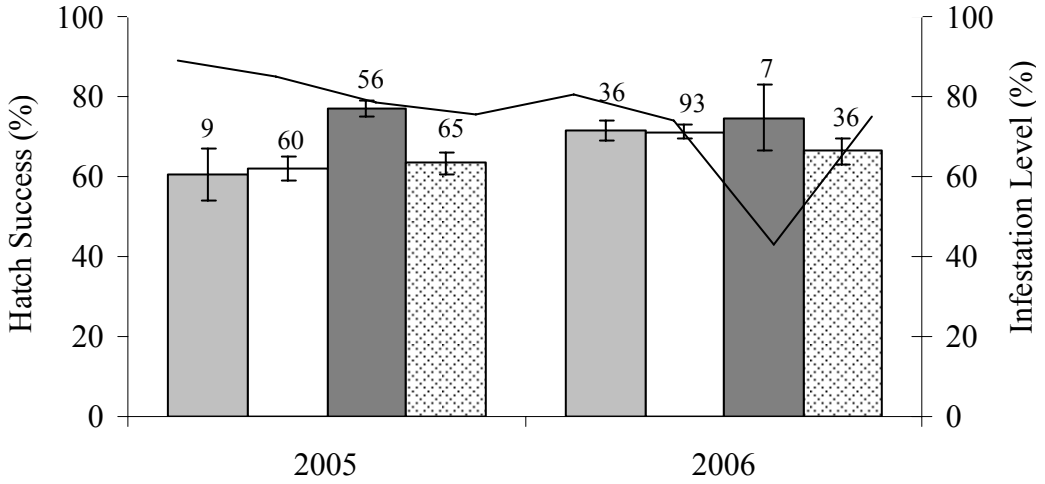


Figure 3-1: Mean hatch success (a) and emergence success (b) values (% ± SE) for infested and non-infested Leatherback nests on Gandoca Beach for the 2005-2006 nesting seasons. Sample sizes (n) are shown above columns.

a)



b)

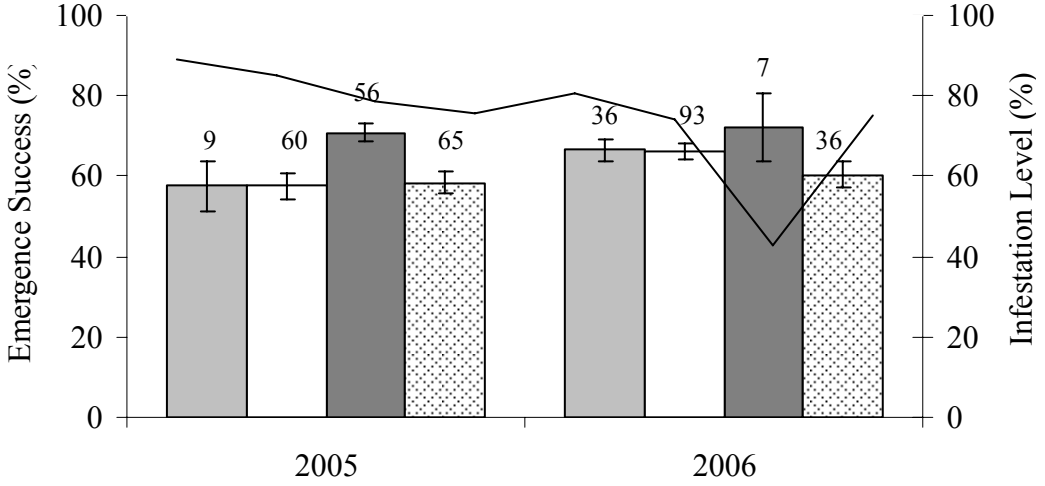


Figure 3-2: Mean hatch (a) and emergence (b) success values (% ± SE) compared to infestation levels for *in situ*, relocated, hatchery A and hatchery B Leatherback nests on Gandoca Beach for the 2005-2006 nesting seasons. Infestation level is presented as the number of nests infested by fly larvae. Sample sizes (n) are shown above columns.

Hatch and emergence success were found to be significantly related on the number of infested eggs and hatchlings per clutch both in 2005 and 2006. This relationship was negative; as the number of infested eggs and hatchlings increased in a clutch, hatch and emergence successes decreased. In 2005, the number of infested eggs and hatchlings predicted 8% of the variation in hatch success ($F_{(1,205)}$=19.13, $p<0.001$) and 14% of the variation in emergence success ($F_{(1,205)}$=34.92, $p<0.001$). In 2006, the number of infested eggs and hatchlings predicted 4% of the variation in hatch success ($F_{(1,178)}$=8.74, $p<0.004$) and 10% of the variation in emergence success ($F_{(1,178)}$=19.94, $p<0.001$).

Calculations were conducted to assess the prevalence of larvae within individual nests. Clutches in 2005 were more highly infested with an average of 13.4% of each affected clutch infested compared to 8.9% in 2006. Proportions of infested nest remnants (excluding eggshells) were 35.1% and 26.1% in 2005 and 2006, respectively (Table 3-2).

Table 3-1: Percent of the clutch and nest "remnant" of Leatherback turtle nests infested with dipteran larvae on Gandoca Beach in the 2005-2006 nesting seasons.

Year	% Infested Clutch Mean ± SE, (Range)	% Infested Nest Remnant Mean ± SE, (Range)	n
2005	13.4 ± 0.9 (1.1-58.1)	35.1 ± 1.8 (1.5-85.7)	166
2006	8.9 ± 0.8 (1.0-51.8)	26.1 ± 1.7 (1.6-78.6)	135

Larval feeding preferences

Larvae demonstrated feeding preferences for particular nest remnants. Of all the available nest contents (including eggshells) at nest excavation, larvae clearly preferred

pipped eggs and dead hatchlings as a food resource. Dipteran larvae utilized from 72.1% to 81.6% of the available pipped eggs and dead hatchlings present in assessed Leatherback nests (Table 3-3). Undeveloped eggs were the least utilized by fly larvae. Of all the undeveloped eggs available in the nests, the larvae only used 6.8% in 2005 and 5.1% in 2006 (Table 3-3). Although quite rare, larvae were found feeding on live hatchlings recovered in nests during excavations. The usual points of entry into the hatchlings were through the external yolk sac and umbilicus, the cloaca or through openings in the head (eyes, nose and mouth). However, two hatchlings were found with a small circular hole through the posterior flipper and several hatchlings had the anterior part of the carapace (just above the posterior flipper) damaged by fly larvae. Of all the available live hatchlings found in nests, the larvae used 10.0% and 10.1% in 2005 and 2006, respectively (Table 3-3). In total at the time of nest excavations, larvae were found feeding on 21 live hatchlings in 2005 and 23 live hatchlings in 2006. The total number of hatchlings recovered during post-emergence nest excavations was 298 and 330 in 2005 and 2006, respectively.

The mean number of infested undeveloped eggs, dead embryos, pipped eggs, dead hatchlings, live hatchlings and eggshells was also calculated for each infested nest. The mean number of infested eggshells per nest had the highest values, 14.0 in 2005 and 8.3 in 2006 (Table 3-4). The mean number of infested live hatchlings per nest yielded the lowest values for both years (0.1 and 0.2 for 2005 and 2006, respectively) (Table 3-4).

Table 3-2: Percent of available undeveloped eggs, embryos, pipped eggs, dead hatchlings, live hatchlings and eggshells utilised by dipteran larvae in Leatherback turtle nests on Gandoca Beach in the 2005-2006 nesting seasons. Values are presented as Mean ± SE (n, range).

Year	% Undeveloped Eggs Infested	% of Embryos Infested	% of Pipped Hatchling Infested	% of Dead Hatchlings Infested	% of Live Hatchlings Infested	% of Eggshells Infested
2005	6.8 ± 0.9 (n=202, 0-66.7)	14.7 ± 1.6 (n=185, 0-88.9)	81.6 ± 3.2 (n=131, 0-100)	80.3 ± 3.5 (n=114, 0-100)	10.0 ± 2.7 (n=94, 0-100)	23.7 ± 1.9 (n=207, 0-100)
2006	5.1 ± 0.8 (n=179, 0-54.5)	9.6 ± 1.8 (n=161, 0-100)	74.9 ± 3.9 (n=110, 0-100)	72.1 ± 4.2 (n=104, 0-100)	10.1 ± 3.0 (n=79, 0-100)	11.8 ± 1.7 (n=180, 0-100)

Table 3-3: Mean number of infested undeveloped eggs, embryos, pipped eggs, dead hatchlings, live hatchlings and eggshells in infested Leatherback turtle nests on Gandoca Beach in the 2005-2006 nesting seasons. Values are presented as Mean ± SE (range).

Year	Mean Number of Infested Eggs	Mean Number of Infested Embryos	Mean Number of Infested Pipped Eggs	Mean Number of Infested Dead Hatchlings	Mean Number of Infested Live Hatchlings	Mean Number of Infested Eggshells	n (Infested Nests Only)
2005	1.0 ± 0.1 (0-14)	1.8 ± 0.3 (0-25)	5.7 ± 0.6 (0-42)	2.5 ± 0.3 (0-35)	0.1 ± 0.0 (0-3)	14.0 ± 1.1 (0-72)	166
2006	0.9 ± 0.1 (0-12)	0.8 ± 0.1 (0-10)	3.4 ± 0.4 (0-29)	2.1 ± 0.3 (0-24)	0.2 ± 0.0 (0-3)	8.3 ± 1.1 (0-70)	135

Decomposing eggs from post-emergence nest excavations were often found with larval entrance holes (Figure 3-3). These holes were the only opening into the egg and the eggs contained maggots. The diameter of the holes ranged from 0.7 mm to 3.1 mm. Fly larvae were observed entering decomposing eggs (Figure 3-4) and were the only insects found feeding on nest remains on Gandoca Beach. No observations of larvae breaching through viable eggs were made.

Figure 3-3: Larval entrance holes (size ~2-3 mm) in decomposing Leatherback turtle eggs from an excavated nest on Gandoca Beach.

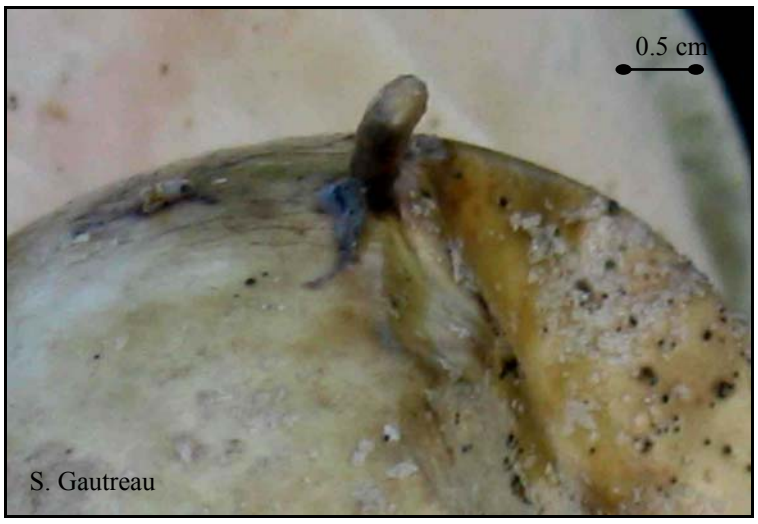


Figure 3-4: Maggot entering a decomposing Leatherback turtle egg from an excavated nest on Gandoca Beach.

3.4. Discussion

Effects of infestation on nest success

The prevalence of dipteran larvae on Gandoca Beach when evaluated as the proportion of nests infested was high for both nesting seasons (Table 3-1). These results are comparable to levels reported by Lopes (1982) in Michoacán, Mexico of 90% and by Hall and Parmenter (2006) in Central Queensland, Australia of 55.6% to 84.6%. McGowan et al. (2001a) reported lower infestation levels in Cyprus, Greece with only 3.3% to 20.7 % of infested nests. Sea turtle clutches are likely to be a major food resource for species of flies able to reach the buried nests. Most sea turtle clutches have some embryos that die during development and their decomposition attracts saprophagous dipterans to the nest chamber. As a result, most nests will contain at least a few maggots and the proportion of infested clutches in a rookery will usually be high. However, measuring the total number of infested nests on a nesting beach does not provide any information on how many eggs and hatchlings per clutch are affected. When infestation level is evaluated as the actual rate of propagule (egg/hatchling) infested per nest, the rate is lower. Only two studies have calculated infestation rates at the individual clutch level and no records exist for Leatherback turtle clutches. In Cyprus, of the total estimated number of eggs laid in a season, 0.5% and 0.8% of Loggerhead turtle (*Caretta caretta*) eggs and 0.01% and 0.2% of Green turtle (*Chelonia mydas*) eggs in 1996 and 1997, respectively were infested by fly larvae (McGowan *et al.* 2001a). Rates of clutch infestation in Central Queensland for the Green turtle were 5.7% in the 2002/03 nesting season and 1.5% for the Loggerhead turtle in the 2002/03 and 2003/04 nesting seasons (Hall and Parmenter 2006). It was concluded for both studies that the larvae were only

infesting eggs that had failed to hatch, and that if flies were functioning as active predators of viable embryos, the number of affected propagules in any given infested nest would be much higher (McGowan *et al.* 2001a, Hall and Parmenter 2006). The percentages of clutch and nest remnant (undeveloped egg, embryo, pipped egg, dead hatchling and live hatchling) infested for nests on Gandoca Beach were higher than the aforementioned studies (Table 2-1), but they are still lower than what would be expected if flies were actively attacking viable embryos. In the majority of infested nests, we found thousands of maggots, but only on a handful of eggs and hatchlings suggesting that the larvae were feeding on embryos and hatchlings that were already dead.

The higher levels of nest infestation on Gandoca Beach suggest that some characteristic of the beach or of Leatherback nests can support high fly numbers. Egg mortality in Leatherbacks is significantly higher compared to other species of sea turtles (Bell *et al.* 2003) and this could lead to high levels of dipteran infestation as more necrotic tissue becomes available. Physical and biological characteristics of the beach and its surroundings could also affect infestation levels. The tropical habitat of the area may be able to sustain large populations of carrion-eating flies since necrotic material would presumably be more readily available in areas of rich biodiversity. Gandoca Beach is a high-energy beach with high spring tides that renew the sand periodically (Chacón *et al.* 1996). As a result, the beach carries much debris that could attract female flies. Some Sarcophagidae breed on dead sea animals (Reinhard 1965, Méndez and Pape 2002) and may exploit sea turtle nests whenever they are available.

In 2005, dipteran infestation and nest translocation successfully predicted some of the variation in nest success (Figure 3-1 and Figure 3-2). However, because of the significant interaction between nest infestation and nest translocation, our results are inconclusive as to whether or not infested nests had lower nest success than non-infested nests. Hatch and emergence success is affected by many interacting biological and physical factors and isolating one particular cause of mortality for individual eggs is difficult. We found that measures of nest success were significantly dependent on the number of infested eggs and hatchlings per clutch for both nesting seasons. Although larval incidence within nests explained less than 15% of the variation in nest successes observed, this provides further evidence that infestation and measures of nest success are related. It may be that maggots lowered the success of the clutches, but it is also possible that infestations of dipteran larvae were dependent on increased mortality caused by other factors such as tidal inundation, microbial infection, and extreme weather conditions etc. If the larvae were acting as occasional predators, their effects on nest success appear to be minimal. To demonstrate that the fly larvae were directly responsible for the death of embryos and hatchlings, direct observations of the clutch during incubation would be necessary. This would require intrusive methodology and may not be compatible with proper conservation practice. Hall and Parmenter (2006) in Australia found that nest success was significantly lower for infested nests than for non-infested nests, but these results were not consistent for all years of their study. They concluded that infestations are associated with decreased success of turtle nests, but that fly larvae may not have been the actual cause of death of the turtle embryos. If larvae were active predators of

viable embryos and hatchlings, nest success for infested nests would have been significantly lower in all seasons of their study (Hall and Parmenter 2006).

Larval feeding preferences

Analysis of the proportions of available nest contents that were used by dipteran larvae revealed that, overall, maggots preferred pipped eggs and dead hatchlings (Table 3-3), and only fed on a few undeveloped eggs and embryos (Table 3-3). The moist environment of these eggs may not be as conductive to larval feeding, and larvae would need to locate an existing passage into the egg or breach the eggshell to reach the food resource. Therefore, the maggots seemed to utilise the more readily accessible pipped and dead hatchlings rather than embryos which were enclosed inside eggs. It has been reported that fly larvae use natural pores in the eggshell surface of freshwater turtle eggs to gain access to developing embryos (Acuña-Mesén and Hanson 1990), but this could not be the mechanism used for entrance into sea turtle eggs because they do not possess true pores (Acuña-Mesén 1989, Chan 1989). Hatching young moving inside the nest chamber, tree rootlets or ghost crabs (*Ocypode* spp.) could inflict damage on eggs that might create access points for the flies. In this study, the larvae recorded from Leatherback turtle clutches were capable of breaching eggs that failed to develop. We found many decaying eggs with small circular holes (Figure 3-3) and larvae were observed chewing directly into eggs (Figure 3-4). Eggs that have been rotting for some time will have gas built up and the eggshells would be softer than viable eggs, making it easier for maggots to puncture the egg surface.

Muscoid larvae possess two sclerotised mandibular hooks that protrude through the mouth and can be used to perforate the cuticle of prey (Ferrar 1979). The mouth hooks of saprophagous larvae are generally unspecialized compared to carnivorous larvae because they do not need to catch or kill prey (Roberts 1971). Larvae also break down tissues using digestive enzymes found in their saliva. Although no observation was made of viable eggs being breached by larvae, maggots could potentially use their specialized mandibles to perforate intact eggs. Other studies have found larval entrance holes in freshwater turtle eggs (Iverson and Perry 1994), lizard eggs (Mullen *et al.* 1984, Trauth and Mullen 1990) and sea turtle eggs (Whitmore and Dutton 1985, Hall 2005). Mullen et al. (1984) concluded that fly larvae could perforate the shell of *Sceloporus undulates* and that they were lizard egg predators.

Many nests contained pipped eggs with the skeletons of well developed young inside. The maggots may only have infested these hatchlings after they died, but it is possible that some may have been killed by larvae after hatching. Generally, myiasis causing flies penetrate pre-existing wounds or body orifices and tear the softer portions of exposed flesh before attacking healthy tissues (Hall and Wall 1995). Therefore, pipped hatchlings may be attractive to maggots because of the external yolk sac they possess after emerging from the egg. Consumed pipped embryos were also noted by Hall and Parmenter (2006), but it could not be concluded whether or not the larvae were the actual cause of death. In a study of a species of freshwater turtle (*Graptemys pseudogeographica*), Vogt (1981) recorded infestation of eggs containing live hatchlings and suggested that most infestation happened at piping.

Phorid flies have been known to cause the death of Common slider turtle hatchlings (*Pseudemys scripta*) (Moll and Legler 1971). Previous studies of sea turtles have reported that larvae infested a single (McGowan *et al.* 2001a) and a few moribund hatchlings (Andrade *et al.* 1992). In this study, larvae were observed on several occasions feeding on live hatchlings and the action of the larvae would have lead to their death if the maggots would not have been removed. However, when the total number of live hatchlings recovered from nest excavations is considered, only a small proportion of them were attacked by maggots (Table 3-3). This suggests that the flies are not primary, obligate parasites feeding only on the tissues of living hatchlings, but rather secondary facultative species, only infesting wounded and dying animals. Most observations of maggots infesting live hatchlings were made during post-emergence nest excavations, but on a few rare occasions, healthy hatchlings from the main emergence group were being eaten alive by dipteran larvae. To our knowledge, this is the first reported case of myiasis in viable sea turtle hatchlings. Generally, the hatchlings that remain in the nest after the main emergence have a lower chance of survival and the ones recovered probably would have died inside the nest without human intervention. The larvae presumably preferred feeding on dead eggs and hatchlings, but sometimes started feeding on moribund hatchlings in the vicinity. Why some young were attacked and others were not remains unknown. Hatchlings can wait several hours and even days after hatching before emerging from the nest (Balazs 1974, Christens 1990). The hatchlings waiting near the sand surface prior to emergence may be easier to detect by flies and this may make them more prone to infestation. Some hatchlings were covered with residue from rotting eggs

and emitted a smell of decay, and as a result the larvae may have found these young more appealing as a food resource. Similarly, hatchlings containing large amounts of undigested yolk might have been more attractive than young that retracted the yolk sac completely.

In conclusion, dipteran larvae are not considered to be an important source of mortality for Leatherback embryos and hatchlings on Gandoca Beach. The high rookery infestation levels in combination with low larval prevalence per nest suggest that the larvae were scavenging nest remains and not significantly reducing nest success. If the larvae were functioning as active predators of sea turtle eggs and hatchlings, one would expect to see higher numbers of propagules affected in each clutch. However, the fact that some viable hatchlings were attacked confirms that larvae can be carnivorous and are sometime associated with hatchling mortality in addition to being scavengers within nests. This is of potential significance because of the endangered status of many Leatherback turtle populations and actions should be initiated to reduce incidental hatchling mortality caused by fly larvae. Appropriate mitigation measures to increase hatchling protection will be discussed in Chapter III.

CHAPTER II

MECHANISMS OF DIPTERAN LARVAE INFESTATION OF LEATHERBACK TURTLE NESTS: WHO, WHEN AND HOW?

4.1. Introduction

Diptera infesting sea turtle clutches typically are from the Sarcophagidae and the Phoridae. Several species of sarcophagid flies have been recorded (Lopes 1982, Andrade *et al.* 1992, Iverson and Perry 1994, Broderick and Hancock 1997, McGowan *et al.* 2001a, Phillott 2005) and the ubiquitous *Megaselia scalaris* (Fowler 1979, Bjorndal *et al.* 1985, Whitmore and Dutton 1985, Broderick and Hancock 1997, McGowan *et al.* 2001a). One sarcophagid species, *Eumacronychia sternalis*, was reported as a predator of Green turtle (*Chelonia mydas*) clutches in Michoacán, Mexico (Lopes 1982, López Barbosa 1989) and of Eastern box turtle (*Terrapene carolina*) clutches in Central Georgia, USA (Iverson and Perry 1994). *E. sternalis* was also recorded from Green turtle nests in the Galapagos Islands (P. Zárate, pers. comm.) and from Leatherback turtle (*Dermochelys coriacea*) nests in Costa Rica (T. Pape pers. comm.). Another species from the same genus, *E. nigricornis* (Allen), is a known predator of lizard eggs (Mullen *et al.* 1984, Trauth and Mullen 1990). In Australia, two Platystomatidae species were recorded in Green and Loggerhead turtle (*Caretta caretta*) nests (Hall and Parmenter 2006). Muscid species were collected from Loggerhead turtles in Turkey (Baran and Türkozan 1996, Baran *et al.* 2001, Katlmş *et al.* 2006) and in Cyprus (McGowan *et al.* 2001a).

Dipteran infestations of Leatherback turtle nests in Costa Rica have never been studied in detail prior to the current investigation. T. Pape (pers. comm.) identified *E. sternalis* from Leatherback turtle eggs in Tortuguero. *M. scalaris* was recorded in Costa Rica from Green (Fowler 1979), Hawksbill (*Eretmochelys imbricata*) (Bjorndal *et al.* 1985) and freshwater turtle nests (Acuña-Mesén and Hanson 1990). Fowler (1979), did not quantify the damage caused by the flies, but stated that the larvae were found in great numbers in 50 rotten clutches and in nests from which the young had emerged. Bjorndal et al. (1985) only mentioned the presence of *M. scalaris* larvae inside the clutch.

Sea turtle nests could be infested by fly larvae at any stage of incubation. At the time of turtle oviposition, the eggs are exposed and cloacal secretion may emit odour molecules attracting gravid flies. Flies have been seen infrequently landing on eggs at this time (Broderick and Hancock 1997), but they could access the nests any time during embryonic development. For most studies, larvae were observed during post-emergence nest excavations, suggesting that infestation takes place near the end of or after egg incubation. The exact time of dipteran infestation could be assessed by excavating nests several times during incubation, but multiple incursions into the same nest may compromise hatch success and such practices are not recommended for endangered species. An alternative method for estimating the time of clutch infestation is by back-calculating from the known development timeframe for fly larvae at post-emergence nest excavation. This forensic method is extensively used to estimate time of death of dead bodies (Mann *et al.* 1990, Byrd and Castner 2001) and can easily be applied to approximate when dipteran infestation of sea turtle nests is taking place. Research using

this methodology estimated that sarcophagid larvae infested sea turtle nests shortly before and after hatchling emergence (McGowan *et al.* 2001a, Hall 2005). Knowledge of when the maggots infest nests may elucidate on what the flies were attracted to and further clarify their association with sea turtle clutches.

It is presumed that female sarcophagids are initially attracted to turtle nests because of decay and that they deposit active first instar larvae which subsequently burrow through the sand to reach the nest contents (Vogt 1981, Lopes 1982, Broderick and Hancock 1997, McGowan *et al.* 2001a, Hall and Parmenter 2006). This has been demonstrated experimentally with two sarcophagid species, *E. sternalis* (López Barbosa 1989) and *Sarcophaga australis* (Hall 2005). Larvae were capable of burrowing to average sea turtle nest depths when exposed to a rotting food source, showing that flies could access sea turtle nests in this matter. Some authors have suggested that female flies may use ghost crab (*Ocypode* spp.) burrows to gain access to turtle nests since they would give direct access to the decomposing eggs and hatchlings.

In this study we recorded the species of Diptera infesting Leatherback turtle clutches on Gandoca Beach in the 2005 and 2006 nesting seasons. In addition, we determined the life cycle of the dominant species, the stage at which infestation is occurring and elucidated on how the flies gain access to the nests.

4.2. Materials and Methods

Dipteran species recorded

Dipteran larvae found infesting turtle eggs and/or hatchlings were collected from turtle nests during post-emergence excavations (methodology detailed in Section: 2.5). They were then placed in small plastic containers and the contents covered with humid sand. Larvae were fed dead hatchlings and/or dead embryos recovered during nest excavations. The containers were covered with fine meshed fabric and a lid with aeration holes to prevent further infestation while providing air exchange and relatively constant humidity levels. The containers were brought to a field laboratory, placed in a sand filled box and covered with a lid. Incubating larvae were checked daily by emptying the contents into a metal tray. Any puparia found were removed, placed in individual vials and covered with a lid. Checking and removal of puparia continued daily until approximately twenty puparia were collected from each nest. Adult flies were left alive for 24 hours to allow the body cuticle and wings to harden. Some flies were then transferred to two rearing cages (in 2006 only) and the remaining flies were frozen and preserved for identification.

Rearing cages were wooden frames that measured 50 cm by 20 cm and were covered in white mosquito netting. Each rearing cage was provided with an inverted vial containing a mixture of water, honey and yeast as food for the adult flies. A larvi- or oviposition substrate was supplied in the form of a medium-sized vial with a small piece of dead hatchling on the bottom, and the contents were covered with sand. This medium was chosen to best simulate natural nest conditions on the beach. Fly behaviour and

presence of first instar larvae were observed daily. Colonies of flies were maintained for two generations to determine the complete development time of the larvae (from larviposition to formation of the puparium and from pupation to adult eclosion). Second-generation adults were identified to assure that the rearing cages and containers were not contaminated by other fly species. To prevent ants and other non-flying insects from contaminating the samples, all larvae and flies were placed on top of a table with the table legs immersed in water. Larvae and fly colonies were maintained at the field laboratory at ambient temperature.

To examine if the dominate fly species infesting nests on Gandoca Beach was an obligate feeder of turtle eggs, fly larvae were collected from buried carrion traps placed on the beach. Traps were buried on the 1st of June 2006 for the first trial and on the 20th of June 2006 for the second trial. This time period coincides with peak hatchling emergence. Five pieces of beef liver (approximately 20g) were buried randomly on the beach under 60 cm of sand to simulate a Leatherback turtle nest and checked daily for 5 days. Average depth of the top of a Leatherback turtle egg chamber in 2006 was 57.8 cm. Larvae found consuming the carrion were removed and transferred to the field laboratory. Larvae were reared using the methods described above, with the exception that they were fed beef liver instead of hatchling carcasses. Emergent adult flies were frozen for identification.

For all flies from the Sarcophagidae, male genitalia were carefully exposed to help with identification. Adult flies of the Family Phoridae were identified by Dr.

Matthias Buck, and the flies of the Families Sarcophagidae, Muscidae and Calliphoridae were identified by Dr. Mehrdad Parchami-Araghi of the University of Guelph. Vouchers of the flies collected were deposited in the University of Guelph Insect Collection in Ontario, Canada and selected sarcophagid specimens were sent to Dr. Thomas Pape at the Diptera Collection of the Zoological Museum of the University of Copenhagen, Denmark.

Timing of infestation

The development period of the two dominate fly species recorded from turtle nests on Gandoca Beach were calculated in the field laboratory under ambient temperature conditions. First instar larvae (less than 24 hours old) were collected from the adult rearing cages and the larvae were subsequently reared. A mean larval development timeframe was calculated for each species, thus providing the number of days from larviposition to formation of puparium. The development rates measured were then compared to durations between field samples collected from post-emergence nest excavations. To yield the elapsed time since nests had been infested by maggots, an estimated age of the larvae at the time of collection from turtle nests needed to be calculated. This was done by subtracting the number of days the larvae collected from nests took to pupate from the known total larval development period. Then, the number of days between hatchling emergence and nest excavation for each nest was subtracted from the estimated age of the larvae at the time of collection. This calculation allowed for discernment of nest infestation timing. Negative results indicate that infestation took place after hatchling emergence.

Nest entry mechanisms

The assumption that dipteran larvae infested nests by burrowing through the sand was tested experimentally by conducting burrowing trials with larvae collected from turtle nests. Three experimental burrowing tunnels were constructed using three circular foam buckets stacked on top of each other. Each bucket had a diameter of 24 cm and was 20 cm high. Humid sand was collected from the beach and sifted with a 0.25 cm grid to remove any debris that might obstruct passage of the larvae. Dead hatchlings were collected during nest excavations and frozen for 24 hours to assure they were free of larvae. Once thawed, two dead hatchlings were placed at the bottom of each burrowing tunnel and they were covered with 60 cm of sand. This depth was used to simulate natural Leatherback nest depths. The tunnels were closed with a lid, sealed with Duct Tape to prevent infestation by other insects and they were left for 48 hours to allow for the permeation of chemical cues through the sand column. Attempts to induce larviposition from gravid *E. sternalis* females were unsuccessful, so larvae were collected from sea turtle nests on the beach. Only first and second instar larvae were selected for the trials. To imitate presumed female larviposition behaviour, 40-50 larvae were placed as a group on top of the burrowing tunnels. The tunnels were shut with a lid and sealed with Duct Tape. After 48 hours had elapsed, the sand was removed from the tunnels down to the turtle remains. All larvae that had reached the decaying matter were collected and reared to adulthood using the methodology described in Section: 0. Adult flies were frozen for identification.

4.3. Results

Dipteran species recorded

The dominant dipterans found infesting Leatherback sea turtle nests on Gandoca Beach were identified to species. In total, 10 species of Diptera were recorded during the course of this study (Table 4-1). *Eumacronychia sternalis* (Allen) from the Sarcophagidae was the principal species infesting Leatherback turtle nests in 2005 and in 2006. *E. sternalis* was also collected from buried carrion traps (beef liver) placed randomly on the beach. Additionally, three other species from the Sarcophagidae, four species from the Muscidae, one species from the Phoridae and one species from the Calliphoridae were found infesting nests (Table 4-1). In comparison to *E. sternalis*, the other species were present in fewer nests. Maggots causing myiasis in five live hatchlings were reared and identified. Two hatchlings were infested exclusively by *E. sternalis* and one hatchling by *Synthesiomyia nudiseta* (Wulp). Two hatchlings were infested by *E. sternalis* in addition to a second species of fly: one by *Musca domestica* (Linnaeus) and the other by an unidentified species from the Muscidae.

Table 4-1**:** Frequency of dipteran species collected from Leatherback turtle nests on Gandoca Beach in the 2005-2006 nesting seasons.

Order	**Family**	**Species**	**Number of nests**	
			2005	**2006**
Diptera	Sarcophagidae	*Eumacronychia sternalis* (Allen)	22	25
		Argoravinia sp. (Townsend)	1	0
		Unidentified sp. 1	0	1
		Unidentified sp. 2	0	2
	Muscidae	*Synthesiomyia nudiseta* (Wulp)	2	2
		Musca domestica (Linnaeus)	0	1
		Musca sp. (Linnaeus)	2	0
		Unidentified sp. 3	0	1
	Phoridae	**Megaselia scalaris* (Loew)	11	5
	Calliphoridae	*Chloroprocta* sp. (Wulp)	1	1

* Possible contaminant species

Identification for all members of the genus *Eumacronychia* is difficult except by reference to the male terminalia. Within the sub-family Miltogramminae, only members of the genera *Eumacronychia* and *Gymnoprosopa* possess a scape raised above the lunule (Figure 4-1) and a phallus with a distinct ventromedian plate (Figure 4-2 and Figure 4-3) (Pape 1996). *Eumacronychia* is also distinguished by its micropubescent arista and proclinate frontorbital bristles in both sexes (Figure 4-1); males possess a broad front, and females have two complete genital tergites, both with marginal bristles (Figure 4-2) (Lopes 1982). *E. sternalis* was first described by Allen (1926). Since then, Reinhard (1965) and Lopes (1982) have provided revisions and given full morphological descriptions of the species. According to Reinhard (1965), *E. sternalis* is distinguished from other species of the genus because of the following characters: abdominal pollen disposed in defined bands; parafacial bare or at most with minute hairs (Figure 4-1); in males, first genital segment glabrous, fifth sternite lobes acutely toothed on inner margin, outer forceps (surstylus) nearly straight in profile (not strongly recurved) with elongated claws, and pulvilli is longer than first tarsal segment (Figure 4-2 and Figure 4-3) (Lopes 1982).

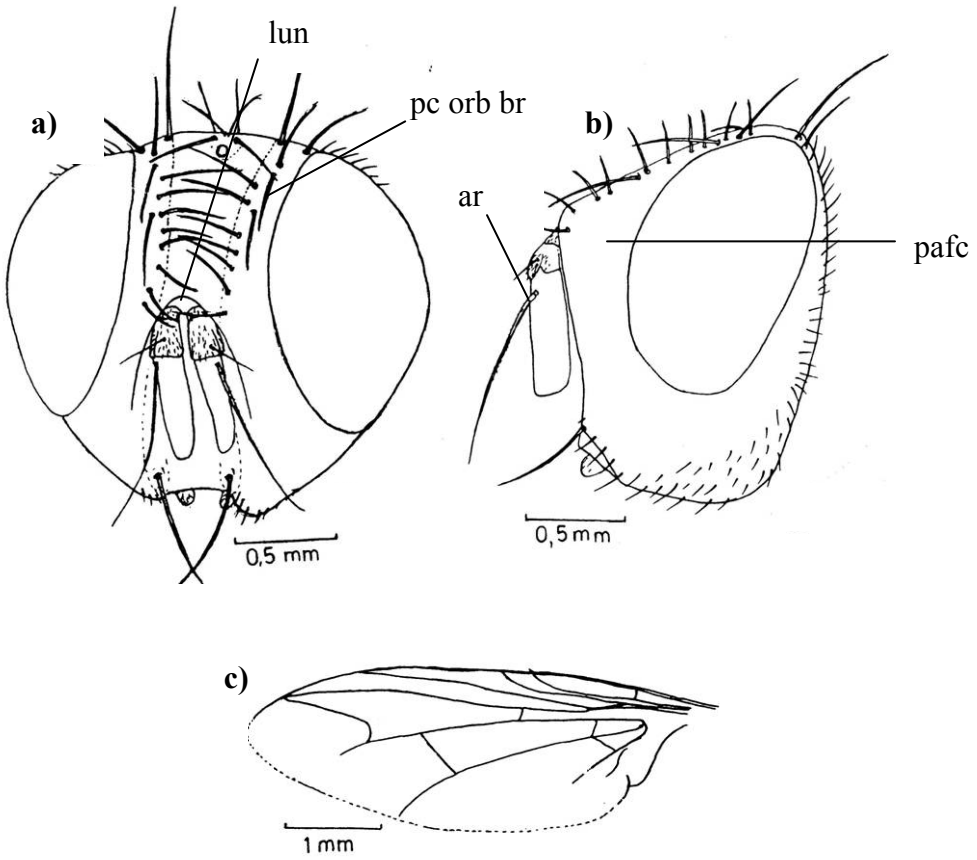


Figure 4-1: E*umacronychia sternalis*. a) head, anterior view; b) head lateral view; c) wing (Source: Lopes, 1982).

Abbreviations: lun, lunule; pafc, parafacial; pc orb br, proclinate orbital bristles.

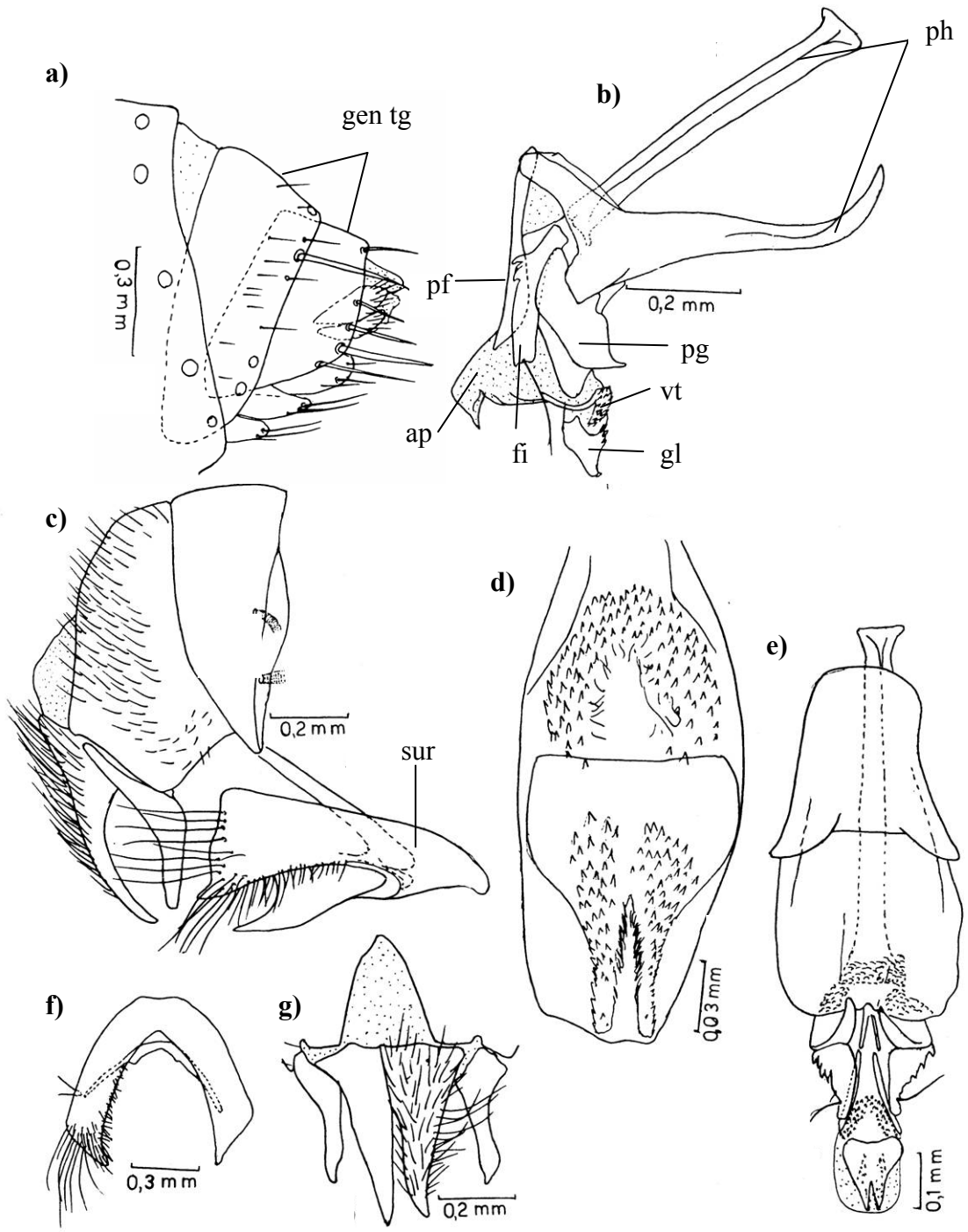


Figure 4-2: *Eumacronychia sternalis* a) female genitalia, lateral view; b) male, phallic organs; c) male, genital segments; d) male, apex of penis; e) male, idem, ventral view; f) male, fifth sternite; g) male, cerci and surstyli (Source: Lopes, 1982).

Abbreviations: ap, apical plate; fi, forcipes interiores; gen tg, genital tergites; gl, glans; pf, paraphallus; ph, phallus; pg, palpi genitalium; sur, surstylus; vt, ventralia.

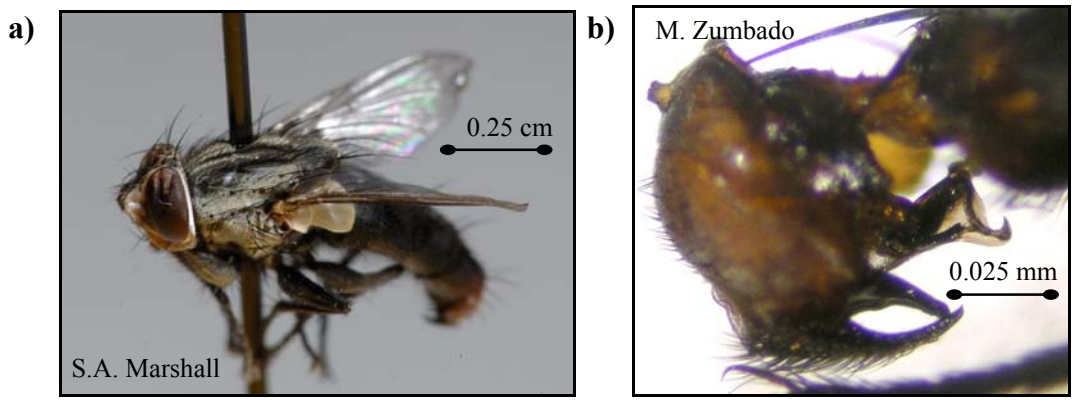


Figure 4-3: *Eumacronychia sternalis* a) full view of male; b) male terminalia

Upon first inspection, *M. scalaris* was the second most important species infesting turtle nests. However, we suspect that *M. scalaris* was contaminating our larval colonies and was not collected from Leatherback turtle nests. This species is a known ubiquitous pest of laboratory colonies. This conclusion was based on the following observations:

1) *M. scalaris* adults were found dead on top of the fabric covering the rearing containers.
2) Extremely small larvae (presumably *M. scalaris*) were seen penetrating the fabric covering the samples.
3) The recorded development timeframe of *M. scalaris* larvae was longer (8.7 days and 9.2 days for 2005 and 2006, respectively) (Table 4-3) than immature development rates for the species established in the literature (5.4 days at 32ºC and 6.9 days at 27ºC) (Trumble and Pienkowski 1979)
4) When larvae were collected from turtle nests, they were noticeably larger than all instars of *M. scalaris*.

Therefore, females of *M. scalaris* were most likely attracted to the decaying matter inside the samples, oviposited on top of the containers and subsequently the larvae entered through the fabric. It is not believed that the other species collected from sea turtle nests were contaminants. The fabric used to cover samples was very finely weaved and would not allow passage of bigger larvae. Additionally, the other species identified from the nests are not known to be pests of laboratory colonies.

Adult flies successfully emerged from all samples collected from turtle nests. Mortality for the larval stage was extremely low and most of the maggots pupariated. However, only 35.6% and 37.9% of the pupae collected produced emergent adult flies for 2005 and 2006, respectively. High levels of mortality after pupariation have previously been reported for sarcophagids reared in vitro. Farkas et al. (2005) recorded mortality rates of 61.2-100% for one sarcophagid species at this stage. Samples failing to produce flies in our study were often infested with fungus. These pests seemed to invade the samples when humidity levels were high, often after several days of rain. Although more larvae were taken from nests than emerged successfully from samples, the gross numbers presumably represent the actual ratios of fly species in infested nests and demonstrate the dominance of particular species.

The majority of infested nests contained only a single species of Diptera and the pattern was similar for both years (Table 4-2). However, two species were recorded in nine and six nests for 2005 and 2006, respectively. Only one nest in 2005 and three nests in 2006 exhibited greater than two species of Diptera simultaneously.

Table 4-2: Frequency of the number of dipteran species collected from infested Leatherback turtle nests on Gandoca Beach in the 2005-2006 nesting seasons.

	Number of nests	
	2005	**2006**
1 species present	18	17
2 species present	9	6
3 species present	1	3
Total number of samples	28	26

Timing of infestation

The development timeframes of the larvae reared from infested nests were recorded. These timeframes are highly dependent on the incubation temperature of the laboratory. The temperatures of sea turtle nests on the beach were recorded in both nesting seasons. In 2005, nests (n=15) recorded an average temperature of 30.6ºC ± 0.1 (n=988, 17.7ºC-42.2ºC) and in 2006, the average temperature of nests (n=39) was 29.4ºC ± 0.1 (n=2135, 16.3ºC-37.6ºC). In the laboratory, flies were incubated at ambient temperature with an average of 27.9°C ± 0.2 (n=48, 24°C-31°C) in 2005 and an average of 27.1°C ± 0.2 (n=67, 24°C-30°C) in 2006. Larvae in turtle nests would have been experiencing slightly warmer temperatures; nonetheless it was considered that the development timeframes could be estimated with sufficient accuracy to permit extrapolation to infestation times.

A mean duration was calculated for each period of development for each species recorded infesting nests (Table 4-3). The mean development period from the time of collection to imago emergence for *E. sternalis* was 20.8 ± 0.4 days (n=80, 12-28) and 23.0 ± 0.3 days (n=158, 16-32) in 2005 and 2006, respectively. For *S. nudiseta*, the

timeframe was 21.8 ± 0.3 days (n=24, 20-23) in 2005 and 18.4 ± 0.2 days (n=38, 17-21) in 2006 (Table 4-3).

Table 4-3: Duration of development periods of Dipteran species collected from Leatherback turtle nests on Gandoca Beach in the 2005-2006 nesting seasons. Values are presented as Mean ± SE (n, range).

Species	Number of days from collection to formation of puparium		Number of days from formation of puparium to imago emergence	
	2005	**2006**	**2005**	**2006**
Eumacronychia sternalis	8.2 ± 0.3 (n=81, 3-15)	9.6 ± 0.2 (n=164, 5-15)	12.5 ± 0.2 (n=83, 9-18)	13.5 ± 0.2 (n= 158, 8-20)
Megaselia scalaris	8.7 ± 0.2 (n=67, 5-14)	9.2 ± 0.4 (n=11, 6-11)	8.3 ± 0.2 (n=63, 6-12)	9.0 ± 0.3 (n=9, 8-11)
Synthesiomyia nudiseta	12.8 ± 0.3 (n=24, 11-14)	8.4 ± 0.1 (n=38, 8-9)	9.0 ± 0.0 (n=24, 9-9)	10.0 ± 0.1 (n=38, 9-12)
Chloroprocta sp.	8.0 ± 0.0 (n=6, 8-8)	11.0 ± 0.0 (n=1, 11-11)	5.0 ± 0.0 (n=6, 5-5)	3.0 ± 0.0 (n=1, 3-3)
Musca domestica	-	8.4 ± 0.4 (n=5, 8-10)	-	5.4 ± 0.4 (n=5, 4-6)
Musca sp.	8.8 ± 0.3 (n= 4, 8-9)	-	5.0 ± 0.0 (n=4, 5-5)	-
Argoravinia sp.	3.0 ± 0.0 (n=2, 3-3)	-	9.0 ± 0.0 (n=2, 9-9)	-

Detailed development data from first instar to adult emergence were recorded for *E. sternalis* and *S. nudiseta* in 2006 (Table 4-4). *E. sternalis* was observed mating three days after entering the rearing cage and mating took place frequently during the process of the study. However, the females only deposited larvae once. *S. nudiseta* was observed mating for the first time nine days after entering the rearing cage. Eggs of *S. nudiseta* were never observed, but first instar larvae were found on two occasions on the oviposition substrate.

Table 4-4: Duration of development periods of *E. sternalis* and *S. nudiseta* incubated in the field laboratory in 2006.

Species	Days from larviposition to pupation	Days from pupation to eclosion	Number of specimens	Mean incubation temperature (°C)
E. sternalis	10	13	5	27.9
S. nudiseta	6.6	9.5	74	28.0

No data were available in the literature for development times of *E. sternalis*. The total time in the laboratory for *E. sternalis* to complete its larval development was ten days (Table 4-4). Back-calculations from this timeframe revealed that nest infestation by *E. sternalis* was taking place immediately after hatchling emergence up to 1.4 days after (Table 4-5). The supposition that female flies deposit larvae during turtle oviposition is unlikely because no flies were observed near female turtles while they were laying eggs. On the other hand, sarcophagids were frequently observed on top of nests during and after hatchling emergence.

Table 4-5: Estimation of the timing of nest infestation by *E. sternalis* estimated by doing back-calculations of known larvae developmental rates for the species. Larvae were collected from Leatherback nests on Gandoca Beach in 2005-2006 (18 nests in 2005 and 25 nests in 2006). Negative values represent that infestation happened after hatchling emergence. Values are presented as Mean ± SE.

Species	Year	Mean age of larvae collected from nest excavations	Mean number of days from hatchling emergence to nest excavation	Estimation of the timing of nest infestation	Number of individuals
E. sternalis	2005	1.8 ± 0.4	3.1 ± 0.2	-1.4 ± 0.4	69
	2006	0.4 ± 0.2	1.1 ± 0.1	-0.7 ± 0.2	164

The same calculations were attempted for *S. nudiseta*, but the recorded number of days from larviposition to pupation for different samples was very variable. Larvae collected from nests were slower to pupate (12.8 days in 2005 and 8.4 days in 2006) than larvae collected from rearing cages (5.0 days and 8.0 days), and this made it impossible to do back-calculations. Larval development timeframes from the literature for *S. nudiseta* also show variation: 12.5 days (Krüger *et al.* 2002), 10.1 days (Rabinovich 1970) and 8.0 days (Siddons and Roy 1942). These discrepancies may be caused by differences in temperature during incubation, variability in the quantity of food provided to the larvae and variation in the density of larvae in each colony.

Nest entry mechanisms

Burrowing trials showed that first and second instar larvae followed emanating odours from decaying hatchlings and could burrow through 60 cm of sand. The average depth to the top egg of Leatherback turtle nests recorded from excavations in 2006 was 57.8 ± 0.8 cm (n=176, range=29-93). The maggots that were collected from the bait were subsequently identified as *E. sternalis*, demonstrating that it could enter sea turtle nests in

this fashion. On the beach, sarcophagid flies were seen entering ghost crab burrows leading to turtle egg chambers. It was therefore assumed that female flies could also use the crab holes to reach the nest contents and larviposit directly on the food source. Whenever available, these burrows may have been used by flies as an alternate mode of entry to the nests.

4.4. Discussion

Dipteran species recorded

The present study established the first detailed description of the association between Leatherback turtle nests and dipteran larvae. There were 10 species of flies, from four different families, found in Leatherback turtle nests on Gandoca Beach during the 2005 and 2006 nesting seasons (Table 4-1). At least four of these species had never been previously recorded infesting sea turtle nests, but several studies found *Eumacronychia sternalis* (Lopes 1982, López Barbosa 1989, T. Pape pers. comm., P. Zárate pers. comm.) as well as *Megaselia scalaris* (Fowler 1979, Bjorndal *et al.* 1985, Whitmore and Dutton 1985, Broderick and Hancock 1997, McGowan *et al.* 2001a). In this discussion, only the most common flies collected from nests will be reviewed in detail.

E. sternalis from the Sarcophagidae was the principal species infesting nests in all seasons. In this study, we report the first case of myiasis for *E. sternalis*. Members of the Family Sarcophagidae are relatively common and have been identified infesting sea turtle nests in Mexico, Ecuador, Costa Rica, Cyprus and Australia (Lopes 1982, López Barbosa 1989, Andrade *et al.* 1992, Broderick and Hancock 1997, McGowan *et al.* 2001a, Hall 2005, Phillott 2005, T. Pape pers. comm., P. Zárate pers. comm.). Sarcophagid flies may

be better adapted to exploit sea turtle nests than egg laying flies because they lay first instar larvae that can start feeding immediately. The genus *Eumacronychia* (Townsend) is part of the sub-family Miltogramminae for which the large majority of species are parasites of solitary bees and wasps, feeding on the food stored for the host progeny (Pape 1996). To date, *Eumacronychia* includes 22 New World species, of which nine have been recorded from Central America. All members of the genus *Eumacronychia* are difficult to distinguish except by reference to the male terminalia (Figure 4-2 and Figure 4-3). Species of *Eumacronychia* show considerable structural diversity and they often exhibit sexual dimorphism (Pape 1996). Limited information exists on this genus and the breeding biology is still partly unknown. A modern revision of the genus is therefore needed to confirm its classification and its general biology. Several species have been recorded visiting vertebrate and invertebrate carrion (Reinhard 1965, Cornaby 1974). Some individuals have been collected from decomposing carcasses of fish, crabs and shrimps in coastal areas and some adults have been found feeding on plant nectar (Reinhard 1965). Some studies suggest that *E. sternalis* (Lopes 1982, Iverson and Perry 1994) and *E. nigricornis* (Mullen *et al.* 1984, Trauth and Mullen 1990) are predators of lizards and turtle eggs, respectively.

Geographic collection records for *E. sternalis* suggest that it has a Nearctic and a Neotropical distribution (Reinhard 1965, Lopes 1982, Iverson and Perry 1994, Pape 1996, P. Zárate pers. comm.) (Table 4-6). Most of the specimens collected originate from coastal regions, but one record from Atlanta, Georgia (USA) confirms that the species also occupies inland habitats (Iverson and Perry 1994). So far, *E. nigricornis* has only

been collected in the USA (Reinhard 1965, Mullen *et al.* 1984, Trauth and Mullen 1990, Pape 1996) (Table 4-6). Both *E. sternalis* (Reinhard 1965, Iverson and Perry 1994, Pape 1996) and *E. nigricornis* (Reinhard 1965, Pape 1996) have been reported from several states in the Southern USA, and therefore appear to be sympatric species for at least part of their distributions (Figure 4-4). *E. nigricornis* was recorded infesting lizard eggs (*Sceloporus undulatus*) in Alabama and Arkansas (Mullen *et al.* 1984, Trauth and Mullen 1990), and *E. sternalis* was recorded infesting freshwater turtle eggs (*Terrapene Carolina*) in Georgia (Iverson and Perry 1994). These results suggest that these flies may be dividing resources, *E. sternalis* infesting turtle nests and *E. nigricornis* infesting lizard nests. However, it is also possible that *E. nigricornis* was misidentified and that it was really *E. sternalis*. Further research where both species occur is needed to clarify this.

Table 4-6: Collection records for *E. sternalis* and *E. nigricornis*.

Species	Distribution	Collection Records	Source
E. sternalis	Nearctic	USA (California, Georgia, New Mexico, Texas) and Mexico (Baja California)	Iverson and Perry (1994), Pape (1996), Reinhard (1965)
	Neotropical	Mexico (Michoacán), Guatemala, Nicaragua, Costa Rica and Ecuador (Galápagos Islands)	Lopes (1982), Pape (1996), Reinhard (1965), P. Zárate (pers. comm.)
E. nigricornis	Nearctic	USA (Alabama, Arizona, Arkansas, California, Georgia, Missouri, Nevada, New Jersey, New Mexico, Ohio, Texas, Virginia)	Mullen *et al.* (1984), Pape (1996), Reinhard (1965)

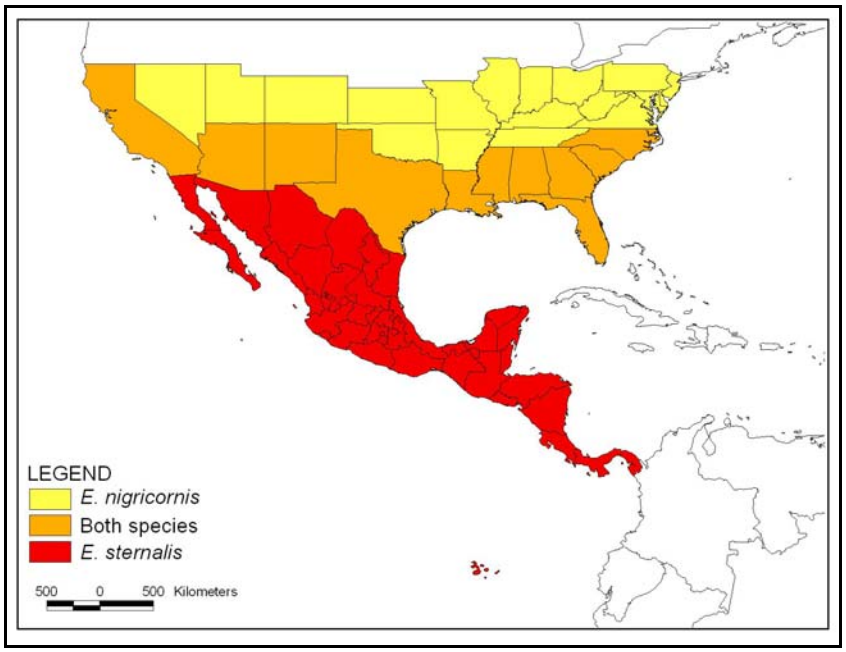


Figure 4-4: Estimated distributions of *E. sternalis* and *E. nigricornis* based on collection records for both species.

Many Sarcophagidae have preferred breeding mediums. Field observations of *Peckia gulo* suggest that it uses dead crabs as a larviposition medium in nature and attempts to rear it on beef and fish in the laboratory were unsuccessful (Méndez and Pape 2002). The fact that *E. sternalis* was collected in buried carrion traps and successfully reared on beef liver suggests that it is not an obligate feeder of turtle eggs and that it is probably an opportunist. Flies with a generalist life strategy spread their progeny between many available decaying materials, and hence their effect on sea turtle nests would be minimal compared to a species that would specialize on sea turtle eggs. During the sea turtle nesting season, decomposing turtle eggs and hatchlings would be readily available on the beach and could be an important food resource for species able to reach the remains. In this study, larvae of *E. sternalis* were present during the entire time of hatchling emergence (from May to August). It is not known whether or not this species

breeds throughout the whole year, but its generalist characteristics implies that it could use decomposing carcasses of sea animals as a food source when no dead eggs and hatchlings are available.

On first inspection, the omnipresent *M. scalaris* (Loew) was found to be the second most important species infesting Leatherback turtle nests in our study (Table 4-1), but this was not the case when correction for presumed post-collection contamination was considered. Phorid flies are notorious for entering places trough the smallest of openings and are ubiquitous pests of laboratory colonies (Disney 2005). Therefore, it was not surprising that *M. scalaris* could enter the rearing containers. *M. scalaris* can lay eggs almost anywhere even through quite fine gauze (Disney 2005). Moll and Legler (1971), reported two species of phorids invading plastic bags of turtle eggs by crawling through the knots tied in the necks of these bags. Hall (2005) also found that *M. scalaris* was a post-collection contaminant and that it was not collected from sea turtle nests. This was concluded because of the development timeframes for *M. scalaris* calculated from the laboratory being much longer than the development periods established for the species, and because of the observations of small holes in the plastic wrap covering several samples. It is possible that some of the *M. scalaris* reported in the literature could also have been contaminants, just as they were in Hall (2005) and almost certainly in this study. Proper precautions against contamination of samples by other polyphagous saprophage species must be taken when rearing larvae. In our study, samples were covered with finely weaved fabric and with punctured plastic lids. In the future, muslin or fine gauze could be used to exclude laboratory culture pests. Despite their ubiquitous

nature and our observations in the laboratory, several adaptations possessed by *M. scalaris* might allow it to infest sea turtle nests. *M. scalaris* is able to burrow through the soil and the larvae are extremely opportunistic, feeding on a large variety of organic material (Disney 1994). Furthermore, it was found causing myiasis in one freshwater turtle species (Moll and Legler 1971) and in one species of snake (Silva *et al.* 1999).

We collected *Synthesiomyia nudiseta* in two Leatherback turtle nests in 2005 and in two nests in 2006 (Table 4-1). Other researchers have reported muscid species from turtle clutches (Baran *et al.* 2001, McGowan *et al.* 2001a, Özdemir *et al.* 2004, Katlmş *et al.* 2006) but despite having been recorded from several turtle nests, muscids were only considered the principal dipterans in studies conducted in Turkey. *S. nudiseta* is the only known species of the genus *Synthesiomyia* (Siddons and Roy 1942, Skidmore 1985, Huckett and Vockeroth 1987). It is common in the Neotropical region (Siddons and Roy 1942, Almeida *et al.* 1997) and has been associated previously with vertebrate carrion (Jirón *et al.* 1983, Omar *et al.* 1994). *S. nudiseta* is considered a cosmopolitan and a semidomestic species (Rabinovich 1970) and may be a vector of human pathogens (Almeida *et al.* 1997). The larvae also cause secondary human myiasis (Coia, in Aubertin and Buxton 1934). Despite its close association with humans, few researchers have studied the species. Jirón et al. (1983) recorded *S. nudiseta* in a mammal carcass in Costa Rica and Omar et al. (1994) described their importance for carcass decomposition in Malaysia. In Gandoca, *S. nudiseta* was not commonly found in nests, however this species is considered important because we found that it attacked Leatherback turtle

hatchlings. To assess the impact of this species on hatchling mortality, we suggest that larvae infesting sea turtle nests on other beaches be assessed.

We found one calliphorid species, *Chloroprocta* sp. (Wulp), from two Leatherback turtle nests. This is the first reported association between the Calliphoridae and sea turtle nests. Generally, Calliphoridae larvae are uncommon on buried remains (Lundt 1964, Payne and King 1968). It is possible that *Chloroprocta* sp. may not have been collected from Leatherback nests, but rather from infested dead hatchlings near the surface of the sand.

The presence of multiple species in several nests suggests that inter-specific competition was occurring, which may act as a limiting factor for the number of larvae within nests. The effects of high densities of maggots can be detrimental or fatal to larvae because food is removed quickly and individuals may be prevented from reaching maximum prepupal size (Archer and Elgar 2003b). Food may be scarce in nests that contain just a few dead eggs and hatchlings, and under such constraints larval competition would be intense. Sarcophagidae and Muscidae larvae are larger at every instar stage than Phoridae larvae and based on size alone, they could effectively out-compete phorids. Indeed, the first instar larvae of some species of the Sarcophagidae kill competitors when food is scarce and when competition is high (Blackith and Blackith 1990).

Timing of infestation

Observations of nesting turtles during and shortly after oviposition yielded no sightings of flies near the egg chamber, which is consistent with most studies (Lopes 1982, Hall 2005). Broderick and Hancock (1997) reported that flies were seen infrequently surrounding turtles at oviposition, but they did not mention whether or not these flies were depositing eggs or larvae. Hence, dipterans seem to infest nests sometime after oviposition. In Australia, Green and Flatback (*Natator depressus*) turtle nests examined at mid-development did not show any signs of infestation even if some eggs were dead (Hall 2005). It was suggested that the female flies were not able to detect the nest because the dead eggs were not yet at an appropriate stage of decomposition and they were not emanating sufficient chemical attractants (Hall 2005). Multiple incursions into the same nest can potentially lower hatch success and was considered too invasive to carry out in our study because of the precariousness of Leatherback turtle embryos.

An alternative method to estimate when larvae are infesting nests is by back-calculating from the known immature development timeframe of dipteran species at the time of post-emergence nest excavation. This is a common forensic technique used to approximate post-mortem interval and was used previously to approximate the timing of turtle nest infestation by McGowan et al. (2001a) and Hall (2005). By doing back-calculations, it was discovered that Platystomatidae species infesting nests in Australia appeared to be occupying a specialist niche within turtle nests and thus infested the clutches as much as two to three weeks before hatching of the young (Hall 2005). Previous studies of sarcophagid infestations concluded that nest infestation was taking place a few days prior to or shortly after hatchling emergence (McGowan *et al.* 2001a,

Hall 2005). We found that *E. sternalis* was infesting Leatherback turtle nests shortly before or a few days after hatchling emergence.

The cue that attracted this species to the nest was probably hatching and emergence of hatchlings. The presence of the nest was most likely indicated to gravid flies by the smell associated with decomposing eggs and hatchlings inside the nest chamber carried to the surface with exiting hatchlings. Active young inside the nest may break dead eggs which would further increase the release of putrescent odor molecules that could be detected by female flies. Ghost crabs are also attracted to nests after the hatchlings emerge (pers. observ.) and frequently lacerate the dead eggs with their claws. It may also be easier for larvae to burrow through the sand and reach the nest contents after hatchling emergence because hatchlings loosen the sand while exiting the nest.

Nest entry mechanisms

Previous investigations have demonstrated that sarcophagid larvae can burrow to the depth of sea turtle nests (López Barbosa 1989, Hall 2005) and our results further supported this conclusion in the case of Leatherback nests. Sarcophagid flies lay active first instar larvae and therefore can immediately and efficiently start feeding on carrion as opposed to oviparous Diptera. The fact that *S. nudiseta* is oviparous (Siddons and Roy 1942, Rabinovich 1970, Almeida *et al.* 1997, Krüger *et al.* 2002) raises questions on how it was able to infest Leatherback turtle nests. It is possible that gravid females oviposit into the sand and that the larvae then burrow down to the decaying nest content, but this would put the eggs at risk of desiccation. Eggs dry out easily, and consequently they must

therefore remain under moist conditions. The beach sand may be too hot or too dry to provide a suitable substrate for fly eggs to hatch. Under these circumstances, adult females may locate and enter ghost crab burrows leading to the nest and deposit eggs either directly on carrion or into the surrounding moist sand. Field observations suggest that the latter mode of nest entry was used by female sarcophagid flies and this entrance could have been used by *S. nudiseta* as well. Crab holes would provide direct access to the decaying necrotic tissue within the nest, and whenever available would presumably be used by female flies to enter turtle nests. However, even though crab burrows may provide easier access to nest contents, it is probably not the main mechanism used by dipterans to infest turtle nests. These burrows are transient and unpredictable and when the percentage of nests infested by larvae on Gandoca Beach is considered (>75%) it seems unlikely that flies completely rely on such a precarious resource. *S. nudiseta* may be restricted to crab burrows to access sea turtle nests and this may explain why this species was found in fewer clutches when compared to *E. sternalis* who can lay active larvae. Presumably, dipterans use a combination of crab burrows and ovi- or larviposition into the sand to access rotting sea turtle nests.

The fact that *E. sternalis* appeared to be infesting nests after turtle hatching and that it seemed attracted to the clutch because of decay provides further support that dipteran larvae from this study were mostly occupying a scavenger niche and that they are not a serious threat to sea turtle populations. In a broad context, dipteran larvae may actually play an important role on the nesting beach by degrading dead material from nests and by helping to return the nutrients and energy back into the trophic web.

Speculatively, the removal of necrotic material from the sand in high-density rookeries could minimize levels of infection from other sources such as fungi and bacteria. While there is scope for further investigation, the results from this study represent a considerable advance in our knowledge of the mechanisms of dipteran infestation of sea turtle nests and provide new information on the life history of *E. sternalis*.

CHAPTER III

FACTORS AFFECTING DIPTERAN LARVAE INFESTATION RATE OF LEATHERBACK TURTLE NESTS

5.1. Introduction

The choice of an oviposition site by a female turtle influences the microenvironment in which the embryos will develop; it affects offspring sex, phenotype, hatching success (Ackerman 1997), as well as the likelihood of nest depredation (Blamires and Guinea 1998). Ultimately, the choice of a nest site by a female turtle or by a person translocating the clutch will determine the biological and physical factors of the nest and as a result, some sea turtle nests may be more prone to infestation by dipteran larvae. The few studies that investigated factors that influence the rate of fly larvae infestation have reported conflicting results and most have failed to ascertain why some nests become infested while others remain free of larvae. Factors that have been shown to affect dipteran infestation levels of sea turtle clutches include nest depth (Özdemir *et al.* 2004), distance from the high water mark to the nest (McGowan *et al.* 2001b, Hall 2005), hatchling emergence duration (McGowan *et al.* 2001b), number of dead eggs and hatchlings within a nest (Hall 2005) and nest translocation (Andrade *et al.* 1992, McGowan *et al.* 2001b). The interaction between nest depth and the number of dead eggs per nest was also found to predict the incidence of larvae in turtle nests. Shallow nests were more prone to infestation when they contained a higher number of dead eggs and hatchlings (McGowan *et al.* 2001b, Hall 2005). Since flies probably locate nests by detecting chemical cues associated with decay, nests that contain higher numbers of dead embryos and hatchlings would presumably attract more flies. Deeper nests may be more

difficult to reach or odours from decaying tissue may lose potency as they permeate up through the sand column.

A common strategy used to increase hatch success of turtle nests is clutch translocation to a communal egg hatchery. In Michoacán, Mexico, Leatherback (*Dermochelys coriacea*) and Olive Ridley (*Lepidochelys olivacea*) turtle nests relocated to an egg hatchery experienced increased levels of infestation (Andrade *et al.* 1992) compared to *in situ* nests. Conversely, Vásquez Bustos (1994) found no evidence to support that Leatherback clutches in hatcheries are predisposed to dipteran infestation; the location of hatcheries with respect to the high water mark, the number of nests in a hatchery and the density of nests did not affect the rate of infestation (Vásquez Bustos 1994). However, a major drawback from the Vasquez investigation is that it was only carried out at the level of the hatcheries and nests were not individually considered. In this study, we assessed the biological and physical parameters of Leatherback turtle nests that affect the rate of infestation by fly larvae on Gandoca Beach. We hypothesized that factors influencing the intensity of odour molecules emanating from nests, such as the number of dead hatchlings and nest depth, will be important predictors of larval incidence.

Most studies suggest that fly larvae do not pose a serious threat to sea turtle populations (Vásquez Bustos 1994, Broderick and Hancock 1997, McGowan *et al.* 2001a, Hall and Parmenter 2006). Similarly, our results indicate that dipteran infestation does not reduce hatching success significantly, but contrary to the previous

investigations, we found that flies induce incidental hatchling mortality. Because of the endangered status of Leatherback turtles and because their embryos are highly sensitive to disturbances (Bell *et al.* 2003), nests should be protected against infestation by fly larvae. Identifying the nest factors that may be related to infestation is important to develop appropriate nest protection measures and could help improve management strategies. Presently, one of the only tools available to help protect nests from infestation by fly larvae are net baskets that cover the clutches. These baskets presumably prevent flies from larvipositing on the clutch and also help reduce depredation by ghost crabs. On Gandoca Beach, all nests translocated to egg hatcheries are covered with baskets. Here we conduct the first investigation on the effectiveness of these baskets against infestation by flies. We hypothesized that the nest baskets will reduce levels of infestation, but that overall rates of infestation will be higher in the hatchery than on the beach due to the higher densities of nests in hatcheries.

5.2. Materials and Methods

Nest factors affecting infestation rate

Data were collected from Leatherback turtle nest excavations on Gandoca Beach during peak hatchling emergence season, from May to August, in 2005 and 2006. Nests were excavated by hand following the procedures in Section 2.5. All nest contents were removed and all eggs and hatchlings found infested with fly larvae were quantified (Table 2-1). A selection of measurable biological and physical nest parameters was generated based on *a priori* knowledge of fly larvae infestation of sea turtle nests. Variables measured were clutch size, sampling year, number of days from hatchling emergence to nest excavation (0 up to 5 days), nest translocation (*in situ*, relocated, hatchery A and

hatchery B), depth to the top and bottom of the nest chamber, number of eggs with signs of bacteria or fungus invasion, number of undeveloped eggs and embryos, and number of pipped eggs and dead hatchlings (Table 5-1). To infer the impact of rain on fly infestation of nests, rainfall amounts were recorded daily by volunteers at both hatcheries.

To determine what nest factors affect larval incidence within individual nests, the data were analyzed using Generalized Linear Models with negative binomial error distribution. The number of infested eggs and hatchlings per nest was used as the response variable. The negative binomial model was chosen because the response variable was strongly skewed to the right, a common problem with count data, and as a result did not follow a normal distribution. The Poisson model is often considered the standard way of describing count data, but it does not account for overdispersion because it assumes that the mean and the variance are approximately the same (Nødtvedt *et al.* 2002). The negative binomial error distribution is a modification of the Poisson distribution that allows for overdispersion and deals with high number of zero counts (Gardner *et al.* 1995).

The following procedures were used to construct and select the model that best fit the data. Clutch size was included in all of the competing models as a covariate because the number of infested eggs and hatchlings is partially dependent on the number of eggs in the nest. During nest excavations, nest remnants were classified in several categories (Table 2-1). To limit the number of variables included in the models, the number of undeveloped eggs and dead embryos, as well as the number of pipped eggs and dead

hatchlings were pooled together to form two predictive variables: dead eggs and dead hatchlings, respectively. Larvae preferentially fed on pipped eggs and dead hatchlings over undeveloped eggs and dead embryos (Table 3-3), it was therefore more appropriate to analyze the data as two separate categories rather than four. Depth to the bottom of the nest chamber was not included in the models because it was correlated with the depth to the top nest chamber ($r = 0.718$, $n = 275$, $P < 0.001$). The assumption of linearity of continuous predictor variables and the response variable was investigated. The relationships between the response variable and the variable dead eggs, and dead hatchlings were not linear. Continuous quadratic terms were created for both variables to increase model performance. An interaction term between the variables dead hatchlings and depth were incorporated in the models as previous studies (McGowan *et al.* 2001b, Hall 2005) found a significant interaction between number of dead per nest and the depth of the egg chamber.

Table 5-1: Competing models to predict the number of dipteran infested eggs and hatchlings per Leatherback nest.

Model	Variables
Year	clutch size, year
Exposure	clutch size, days*
Exposure/Dead	clutch size, days, dead eggs^2, dead hatchlings^2
Hatchlings	clutch size, dead hatchlings^2
Eggs	clutch size, dead eggs^2
Dead	clutch size, dead hatchlings^2, dead eggs^2
Dead/Bacteria-Fungus	clutch size, dead hatchlings^2, dead eggs^2, bacteria-fungus
Depth	clutch size, depth
Interaction	clutch size, depth x dead hatchlings
Translocation	clutch size, translocation
Translocation/Depth/Dead	clutch size, translocation, depth, dead hatchlings^2, dead eggs^2
Translocation/Interaction	clutch size, translocation, depth x dead hatchlings
Full	clutch size, year, days, translocation, depth, dead hatchlings^2, dead eggs^2, bacteria-fungus
Full with Interaction	clutch size, year, days, translocation, dead eggs^2, bacteria-fungus, depth x dead hatchlings

*Refers to the number of days between hatchling emergence and nest excavation.

Variables were considered significant when the p-value for the Wald-test (Z-test) was lower than 0.05 (Dohoo *et al.* 2003). One-tail deviance χ^2 goodness-of-fit tests were computed to assess overall model fit (Dohoo *et al.* 2003). Overdispersion was assed by calculating $(1 + \alpha\mu)$, this formula was used because the variance in negative binomial models is a function of both alpha and the mean (Dohoo *et al.* 2003). Overdispersion factors should be less than four to assure the model structure is correct (Burnham and Anderson 1998).

The model that best fit the data was selected using Akaike's Information Criteria (AIC). The value of AIC is calculated as follows:

$$AIC = -2 (\text{log-likelihood}) + 2K$$

where K equals the number of parameters in the model. The model with the minimum AIC is considered the best approximating model given the data. To calculate the difference in information lost between a model of interest and the best approximating model, I used Akaike's distance (ΔAIC_i):

$$\Delta AIC = AIC_i - \min AIC$$

To measure the strength of evidence for each model and represent the ratio of ΔAIC_i values for each model relative to the whole set of R candidate models, I calculated the Akaike model weight (ω_i) as follows:

$$\omega_i = \frac{\exp(-\Delta AIC_i/2)}{\sum_{r=1}^{R} \exp(-\Delta AIC_r/2)}$$

Finally, evidence ratios were calculated to assess the probability that a certain model was the best among the whole set of competing models:

$$\text{Evidence ratio} = \omega_1 \text{ - } \omega_i$$

where ω_1 is the weight calculated for the best model and ω_i is the weight for the model of interest. All analyses were done using Statistical Software for Professionals (Stata), version 9 (Stata Corporation) and Microsoft Excel (Microsoft Corporation).

The effectiveness of protective baskets

All nests translocated in egg hatcheries on Gandoca Beach were covered with net baskets immediately after turtle oviposition (Figure 2-2) to protect the clutch from ghost crabs and avoid infestation by fly larvae (Section 2.3). To test the effectiveness of these baskets against dipteran infestation, a random sample of nests distributed randomly outside of hatchery B (within 50 meters) were also covered with baskets. The number of

fly infested eggs and hatchlings recorded from excavations of the covered nests outside of the hatchery was compared to the number from uncovered nests located within 200 meters of the hatchery. Nests within this distance were considered to be under similar beach conditions. The baskets advertise the exact location of the clutches, so to avoid nest poaching only the nests close to the hatcheries, which were under constant surveillance, could be covered. The number of infested eggs and hatchlings per nest did not follow a normal distribution; therefore a Mann-Whitney (U) test was used to conduct the analysis. Results were considered significant at the 0.05 level. The data were analyzed using the Statistical Package for the Social Sciences, version 12 (SPSS, Inc.).

5.3. Results

Nest factors affecting infestation rate

Data were collected from 341 Leatherback turtle nests on Gandoca Beach during the 2005 and 2006 nesting seasons. The distribution for the number of infested eggs and hatchlings per nest was heavily skewed to the right and contained a high proportion (21.7%) of zero counts. The overall variance was 84.4 and the mean 7.2; hence a Poisson model was not considered appropriate since it assumes the mean and the variance to be approximately the same. The AIC values for the competing models were very similar, suggesting that no model was outstandingly better at predicting larval incidence per nest (

Table 5-2). Deviance χ^2 goodness-of-fit tests revealed that all competing models fit the data well (p-value>0.05). The full model including the interaction term (depth x dead hatchlings) had the lowest AIC value and was considered the best at approximating the data. Although several of the variables in this model are not significant, they appear to

have confounding effects with other variables and help to explain the variability in the response variable.

Table 5-2: AIC values for the competing generalized linear models with negative binomial error distributions to predict the number of dipteran infested eggs and hatchlings per Leatherback nest. Models with p-values greater than 0.05 fit the data well. A total of 341 nests were assessed in the 2005 and 2006 nesting seasons.

Model	χ^2 Goodness-of-fit	P-value	Number of Parameters	AIC	ΔAIC	Weight	Evidence Ratio
Full with Interaction	419.88	0.100	13	5.17	0.00	0.08	1.000
Full	418.35	0.999	12	5.18	0.00	0.08	1.002
Exposure/Dead	414.35	0.998	6	5.19	0.02	0.08	1.011
Dead/Bacteria-Fungus	414.91	0.998	6	5.19	0.02	0.08	1.011
Dead	414.01	0.998	5	5.19	0.02	0.08	1.012
Translocation/Depth/Dead	414.09	0.999	9	5.22	0.05	0.08	1.023
Hatchlings	414.49	0.998	3	5.23	0.05	0.08	1.028
Interaction	403.82	0.993	3	5.36	0.18	0.07	1.097
Translocation/Interaction	404.53	0.996	7	5.37	0.20	0.07	1.103
Year	388.18	0.969	2	5.80	0.63	0.06	1.370
Eggs	387.87	0.971	3	5.84	0.67	0.06	1.396
Exposure	387.85	0.968	2	5.84	0.67	0.06	1.398
Depth	387.78	0.968	2	5.84	0.67	0.06	1.399
Translocation	387.00	0.971	4	5.85	0.68	0.06	1.407

The variables measured that best predicted larval incidence in nests were: clutch size, sampling year, the number of eggs with signs of bacteria or fungus invasion, and the interaction between depth to the top egg chamber and number of dead hatchlings (Table 5-3). The number of infested eggs and hatchlings per nest was higher in 2005 (9.0 ± 0.8, n=174) than in 2006 (5.2 ± 0.6, n=167). This may be related to the environmental conditions for each nesting season. The daily average rainfall amount recorded from April to July was lower in 2005 (9.5 ml ± 2.5) than in 2006 (16.5 ml ± 4.3). The relationship between larval incidence and nest depth varied with the number of dead

hatchlings within the nest. For example, nests with similar depths that contained many dead hatchlings had a higher incidence of larvae than the nests with few dead hatchlings. Therefore, shallow nests were found to be highly infested only if they contained many dead hatchlings; nest depth alone did not predict the number of infested eggs and hatchlings in a nest.

Table 5-3: Coefficient (± SE) and p-values for the variables included in the five best competing models to predict the number of dipteran infested eggs and hatchlings per Leatherback nest. * Significant at the 0.05 rejection level.

Models		**Full with Interaction**	**Full**	**Exposure/Dead**	**Dead/Bacteria-Fungus**	**Dead**
Variables	**Level**	**Coefficient ± SE (P-value)**	**Coefficient ± SE (P-value)**	**Coefficient ± SE (P-value)**	**Coefficient ± SE (P-value)**	**Coefficient ± SE (P-value)**
Clutch Size	-	0.01 ± 0.00* (0.013)	0.01 ± 0.00* (0.012)	0.01 ± 0.00* (0.018)	0.01 ± 0.00* (0.018)	0.01 ± 0.00* (0.020)
Year	2005	Baseline	Baseline	-	-	-
	2006	-0.44 ± 0.12* (<0.001)	-0.44 ± 0.12* (<0.001)	-	-	-
Days	-	0.01 ± 0.04 (0.872)	0.00 ± 0.04 (0.970)	0.06 ± 0.04 (0.118)	-	-
Translocation	*In situ*	Baseline	Baseline	-	-	-
	Relocated	-0.09 ± 0.15 (0.547)	-0.08 ± 0.15 (0.599)	-	-	-
	Hatchery A	-0.29 ± 0.18 (0.106)	-0.29 ± 0.18 (0.109)	-	-	-
	Hatchery B	-0.28 ± 0.17 (0.091)	-0.27 ± 0.17 (0.106)	-	-	-
Depth	-	-	0.0 ± 0.00 (0.922)	-	-	-
Bacteria-Fungus	-	0.02 ± 0.01* (0.037)	0.02 ± 0.01* (0.034)	-	0.02 ± 0.01 (0.142)	-
Dead Eggs^2	-	-0.00 ± 0.00 (0.082)	-0.00 ± 0.00 (0.101)	-0.00 ± 0.00 (0.263)	-0.00 ± 0.00 (0.222)	-0.00 ± 0.00 (0.244)
Dead Hatchlings^2	-	-	-0.00 ± 0.00* (<0.001)	-0.00 ± 0.00* (<0.001)	-0.00 ± 0.00* (<0.001)	-0.00 ± 0.00* (<0.001)
Depth x Dead Hatchlings	-	-0.00 ± 0.00* (0.054)	-	-	-	-

The effectiveness of protective baskets

In 2006, a random sample of nests within 200 meters of hatchery B were covered with baskets immediately after turtle oviposition. The covered nests had significantly less dipteran infested eggs and hatchlings (5.0 ± 1.6, n=23) than nests left uncovered (1.9 ± 1.4, n=14) ($U_{(1)\ 22,\ 14} = 84.5$, $p = 0.02$). Although the sample size was small, this provides evidence that the baskets are effective in protecting the clutch against infestation by fly larvae.

5.4. Discussion

Nest factors affecting infestation rate

The incidence of larvae within individual Leatherback nests on Gandoca Beach was significantly higher in 2005 than in 2006. This could be attributed to differing environmental conditions between nesting seasons. The climate, such as excessive rainfall amounts, strongly affects population dynamics of flies, as well as the development of turtle embryos (Kraemer and Bell 1980). In 2005, the average daily rainfall on Gandoca Beach was lower than in 2006. Heavy rainfall compacts and hardens the sand, and that might make it more difficult for maggots to burrow through the sand column. Odour molecules may be carried further when the sand is dry, and consequently could attract more flies to the nests. The quantity of nests on a beach may also influence the levels of infestation. Fly multiplication can be enormous whenever breeding mediums are readily available, it has been suggested that as much as one million saprophagous flies could be produced from the body of a single cow (Bishopp *et al.* 1917). Therefore, beaches with high nest densities might sustain greater numbers of carrion-eating flies because they would have greater amounts of food available for their offspring. On

Gandoca Beach, Leatherback nesting activity was higher in 2005 (642 nests) than in 2006 (419 nests) and this possibly explains the higher levels of dipteran infestation in 2005.

Shallower nests were more prone to fly larvae infestation than deeper nests when they contained a higher number of pipped eggs and dead hatchlings. The interaction between nest depth and the number of dead was also shown to affect infestation of turtle nests in Cyprus (McGowan *et al.* 2001b) and in Central Queensland (Hall 2005). Chemical odours associated with decay probably loose potency as they permeate through the sand, making deeper nests harder to detect. Moreover, decaying matter in deep nests may be harder to reach than in shallow nests which could prevent some fly species from infesting deeply laid clutches. Musca sp. infesting Loggerhead (*Caretta caretta*) turtle clutches in Turkey were found to predominantly infest shallow nests (Özdemir *et al.* 2004) which suggests that different species of Diptera have a high degree of variation in their burrowing abilities. In this study, burrowing trials conducted with *Eumacronychia sternalis* larvae (see Section 4.3) showed that this species can reach average Leatherback egg chambers. Nonetheless, it would be more energetically costly for larvae to dig far or over a long time. Consequently, gravid flies may preferentially choose to larviposit over shallow nests, making these nests more susceptible to infestation. If clutches do not contain necrotic material and flies are not able to detect their presence, depth alone does not seem to affect larval prevalence.

During post-emergence nest excavations, fungal and bacterial growth can often be detected on the shell exterior and in the contents of unhatched eggs. The contamination of

non-viable eggs by mycoflora and bacteria aids in the decomposition cycle and increases the release of putrescent molecules that could be detected by dipterans. Our results showed that as the number of eggs within a clutch containing signs of bacteria or fungus invasion increased, so did the incidence of larvae within the nest. Flies may be able to detect gases released by bacteria or fungus during tissue putrefaction and may use that as a cue to infest sea turtle nests. This is the first reported association between fly larvae infestation of turtle clutches and invasion of eggs by fungus or bacteria.

Some authors have inferred that the timing of nest excavation can influence the level of infestation by flies (Vásquez Bustos 1994, McGowan *et al.* 2001a, Hall 2005) as the number of days after hatchling emergence increases, so does the number of larvae within a nest. Therefore, if excavations are done several days after the emergence of the hatchlings, measures of infestation may be over-represented. Similarly, it was found that as hatchling emergence duration increases, so does the number of infested eggs in a clutch (McGowan *et al.* 2001b). Hatchling emergence potentially advertises the location of nests to flies and the longer this process continues, the more likely infestation becomes. In Cyprus, McGowan et al. (2001a) attributed higher levels of nest infestation in 1997 compared to 1996 to a change in methodological protocol regarding nest excavation. In 1997, nests were left for a longer period of time before excavation commenced. In this study, nests were excavated shortly after hatchling emergence up to five days after to measure the effect of nest excavation timing on infestation rates. The timing of excavation was not found to be a significant predictor of the incidence of maggots in nests. This suggests that even if nests are "exposed" to flies for longer

periods, infestation of the clutch by Diptera will remain low as long as the clutch does not contain decomposing matter. Sarcophagid flies are known to detect and colonize carrion very rapidly, and trials conducted on Gandoca Beach using buried carrion traps showed that *E. sternalis* larvae colonized decaying beef liver within twenty-four hours. The discrepancies found between studies may be attributed to differences in emergence behaviour between turtle species or to the low sample size used in McGowan et al (2001b), they excavated a total of 39 nests of which only 16 nests were infested by flies. Experimental approaches manipulating nest contents and the length of time they remain in the sand would be useful to gain better understanding of the influence of excavation timing on fly infestation of turtle nests.

Nest translocation was not found to be a significant predictor of the prevalence of larvae within a nest. Since larvae seem to infest clutches shortly before or after hatchling emergence, it is unlikely that the action of translocating a nest directly after oviposition would increase infestation rates. In Cyprus, relocated nests had fewer infested eggs than the ones left *in situ*, but the translocated clutches tended to be located at greater depths when compared to natural nests, and it may be that relocation alone was not important in predicting larval incidence (McGowan *et al.* 2001b). Clutch translocation is a common practice used on sea turtle nesting beaches, nests are either relocated to safer sections of the beach or to communal egg hatcheries. Generally, such areas have higher nest densities than other beach sections and flies may be more readily attracted to these areas because they may be releasing stronger chemical attractants. Andrade et al (1992) reported higher levels of fly infestation for Leatherback turtle nests inside hatcheries than

for *in situ* nests and proposed nest density as the main factor involved and not relocation per se. Contrarily, when Vasquez Bustos (1994) measured infestation levels for hatchery nests submitted to different densities, it was concluded that fly larvae infestation was not dependent on nest density. In our study, we did not directly measure the effect of nest density on infestation rates. Nests translocated to communal egg hatcheries did not experience higher rates of infestation than clutches left *in situ* or than relocated clutches even though hatcheries contained many nests in a relatively small area. However, hatchery nests were covered with fine mesh baskets during the entire incubation period and several adhesive fly ribbons were planted inside the hatcheries to catch female flies. This could have biased our results by lowering infestation levels for nests inside the hatcheries and solid conclusions on the effect of nest density can not be made. Nests outside of hatchery B protected with baskets had significantly lower numbers of infested eggs and hatchlings than nests left uncovered. Therefore, the baskets seem effective at excluding fly larvae from infesting sea turtle clutches, and this may explain why hatchery nests did not have higher infestation levels compared to nests left *in situ*.

Recommendations on how to reduce infestation

Our results demonstrate that fly larvae do not significantly reduce hatch success of Leatherback turtle clutches. However, *E. sternalis* and *Synthesiomyia nudiseta* maggots were found to cause myiasis on live hatchlings (Section 3.3) and because of the endangered status of all sea turtle species, protective measures should be taken to reduce any incidental mortality caused by flies. We showed that finely meshed baskets prevent flies from depositing their progeny on top of nests, but their major drawback is that they advertise the exact location of the clutch which limits their usage to areas where egg

poaching is not of concern. Alternate fly excluding devices that could be used anywhere on the beach should be developed to try and minimize the negative impact of flies on turtle nests. Research on the effectiveness of fly traps and adhesive fly ribbon on reducing infestation rates for nests translocated in hatcheries should be initiated.

Our results identified some of the key nest factors that may predispose sea turtle clutches to infestation by fly larvae. Based on our new understanding of the relationship between flies and turtle nests, we recommend that the following actions be taken to try and reduce any negative impact Diptera cause to sea turtle eggs and hatchlings:

1. Fine mesh baskets should continue to be used as nest protectors in areas where egg poaching is not a threat.
2. Hatcheries should be entirely screened to keep gravid female flies from gaining access to nests and to reduce infestation levels.
3. The location of hatcheries should be changed yearly so that the sand does not contain any residual odours from previous years.
4. Nests inside hatcheries should always be adequately separated (at least 80 cm) to prevent larvae from infesting neighbouring nests.
5. When hatchlings are expected to hatch, any dead hatchlings in the sand column should be removed as they may put the rest of the clutch at risk of being infested by flies.
6. Nest excavations should be executed no more than twenty-four hours after the main group of hatchlings have emerged (more than 50% of the eggs) to remove any moribund hatchlings from the nest chamber. These hatchlings

are at higher risk of being attacked by flies and are unlikely to emerge from the nest without human intervention.

7. Nest contents, especially in areas of high nest densities, should be removed and disposed of away from other nests shortly after hatchling emergence to reduce the chance of maggots crossing over to nests located at proximity.

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