

The effect of invertebrate infestation and its correlation with loggerhead sea turtle (*Caretta caretta*) nest success in Laganas Bay, Zakynthos, Greece

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Loggerhead sea turtle (*Caretta caretta*) nests are vulnerable to predators and scavengers, including invertebrates (Paris *et al.* 2002). Dipteran larvae (Phoridae and Sarcophagidae) have been found to infest loggerhead and green sea turtle (*Chelonia mydas*) nests both in northern Cyprus (Broderick & Hancock 1997; McGowan *et al.* 2001a), and Australia (Hall & Parmenter 2006), green sea turtle nests in Costa Rica and Mexico (Fowler 1979; Lopes 1982), as well as hawksbill (*Eretmochelys imbricata*) (Bjorndal *et al.* 1985) and leatherback sea turtle (*Dermochelys coriacea*) nests in Costa Rica (Gautreau 2007). In the Mediterranean, coleopteran larvae were found to infest loggerhead nests in Turkey (Baran & Türkozan 1996) along with Muscidae larvae (Türkozan 2000; Katılmış *et al.* 2006; Katılmış & Urhan 2007a), Acarina, Nematoda and Oligochaeta (Baran *et al.* 2001; Özdemir *et al.* 2006; Urhan *et al.* 2010). There is currently no information on invertebrate infestation of sea turtle nests laid in Greece despite the significance of the nesting population at Zakynthos (Margaritoulis 2005).

Invertebrates are known to feed on weakened or dead hatchlings (Fowler 1979; Lopes 1982), empty egg shells (Baran *et al.* 2001; Bolton *et al.* 2008; Urhan *et al.* 2010), yolk, and dead tissue (Hall & Parmenter 2006; Katılmış & Urhan 2007a; Hall & Parmenter 2008), although they can attack viable hatchlings (Lopes 1982; McGowan *et al.* 2001a; Özdemir *et al.* 2006; Gautreau 2007) and damage intact eggs (Donlan *et al.* 2004; Özdemir *et al.* 2006; Urhan *et al.* 2010). There is debate as to whether lower hatching success observed in sea turtle nests with invertebrates can be attributed to the presence of the invertebrates, or is simply a case of nests with reduced hatching success having a greater likelihood of infestation because they contain more decaying matter. Understanding the impact of invertebrates is important in understanding the overall

relative threat of this source of predation of sea turtle eggs (Bolton *et al.* 2008).

At the rookery level, infestation may be high, with reports of 90% (Lopes 1982) and 84.6% (Hall & Parmenter 2006) of nests being infested. However, at nest level, infestation is typically much lower, *e.g.*, 10.6% (Broderick & Hancock 1997), 0.8% (McGowan *et al.* 2001a) and 3.6% (Katılmış *et al.* 2006) of eggs within a nest being infested. In terms of nest success, Gautreau (2007) noted that it was not significantly lower for infested leatherback nests in Costa Rica, as did Bolton *et al.* (2008) for spiny softshell turtles (*Apalone spinifera*) in Canada. Infestation is generally not considered a threat to nest success (McGowan *et al.* 2001a; Hall & Parmenter 2008). However, invertebrate predation was linked to a 30% reduction in green sea turtle hatching success in Mexico (Lopes 1982), and a lower success of green, loggerhead and flatback (*Natator depressus*) nests in Australia (Hall & Parmenter 2006) and Nile Soft-shelled turtle (*Trionyx triunguis*) nests in Turkey (Katılmış & Urhan 2007b). To date, the influence of invertebrates on the success of loggerhead nests has been little studied, with the only available data from Moulis's (1997) study on the impact of fire ants (*Solenopsis* spp.) on nests laid in the USA.

McGowan *et al.* (2001b) concluded that three main factors affected infestation; nest depth, distance to the high water mark, and the duration of hatchling emergence. The depth of nests was found to be the most important factor relating to dipteran infestation (McGowan *et al.* 2001b; Bolton *et al.* 2008). Özdemir *et al.* (2004) also reported that the duration of hatchling emergence influenced infestation. The most important factor that influenced infestation by coleopteran larvae, however, was the position of nests in relation to vegetation (Donlan *et al.* 2004; Özdemir *et al.* 2006).

The aims of this study were to identify the relationship between invertebrates and the success of loggerhead sea turtle nests, to understand the factors affecting invertebrate prevalence, and to determine whether invertebrates are acting as scavengers or predators in nests. We also present the first data on the extent of infestation present in Laganas Bay (Zakynthos), one of the largest loggerhead rookeries in the Mediterranean (Margaritoulis 2005).

Data were collected between 05 August - 03 September 2013. We sampled 106 loggerhead nests between East Laganas and Kalamaki beaches, located within the National Marine Park of Zakynthos, Greece (37.73° N, 20.93° E, Fig. 1). See Margaritoulis (2005) for a full description of the sample sites.

Nests were excavated ≥ 14 days after first hatchling emergence, to ensure that natural incubation or emergence was undisturbed, in accordance with ARCHELON (the Sea Turtle Protection Society of Greece) and National Marine Park of Zakynthos (NMPZ) protocol. The majority of nests (75) were excavated 14 days after first emergence, 31 were excavated between 16-38 days after first emergence.



Figure 1. Sample sites; East Laganas beach (LAG) and Kalamaki beach (KAL) in Laganas Bay, Zakynthos, Greece.

Nests were excavated following standard practices (Broderick & Hancock 1997; McGowan *et al.* 2001a). Once the top egg was exposed, distance from sand level to the top egg was measured, and from the top egg to the nearest vegetation; this was recorded as “0 m” if roots were present. The interval between first hatchling emergence and excavation was also noted.

Observations were made for each egg within a nest, with eggs / hatchlings being removed in order they were found. Hatchlings were treated as the final stage of egg development. Egg position within the nest was recorded with the uppermost numbered 1, then 2, 3, and so on. Each egg was categorized into one of the following categories: hatched ($\geq 50\%$ empty shell), non-viable (unhatched with no sign of embryological development), dead or alive embryo (placed into subcategories of early, middle, late stages of development), dead or alive pipped hatchling with shell, and dead or alive hatchling. Eggs were inspected for infestation; defined by at least one invertebrate larva or adult inside, or on, the egg, or the presence of puncture holes in the egg shell - these had perforated edges, and were too small to be caused by the pipping process (Fig. 2).

The total clutch count was divided by two and individually numbered eggs were allocated to the top or bottom half as they would have been found on excavation; if the clutch count was uneven, a middle egg was discarded from analyses to avoid bias when comparing halves of the same nest. The number of invertebrates infesting each egg was counted or estimated in instances of heavy infestation (>50 individuals). Adult specimens were preserved in alcohol and larvae were transported to be raised to adults. Following inspection and sampling, eggs were returned to the egg chamber and re-buried to minimise the attraction of predators and scavengers to nearby nests.

Invertebrates were raised to adulthood for identification, and preserved using standard practice (McGowan *et al.* 2001a).

Specimens were identified to family level using keys (Unwin 1984; Chinery 1993; Pearce & Waite 1994; Gibb & Osteto 2005; Checklist of the Collembola of the World. Available from: www.collembola.org), with the aid of a low-powered microscope. Statistical analyses were performed using SPSS (v. 20.0, IBM Corporation, Armonk, New York, USA). The α level used was 0.05. One-way chi-squared tests were used to investigate associations among invertebrates, the stage of development and position within nests. Mann-Whitney U tests were used to compare infested and non-infested nests. A Wilcoxon signed-rank test was used to compare top and bottom halves of nests. The influence of ecological factors on infestation was investigated using Generalized Linear Models with a negative binomial model and log link, with data rounded to the nearest integer for these analyses. This model removed the need to transform the data (O’Hara & Kotze 2010). The ecological factors tested included: distance to vegetation, nest depth (sand level to top egg), clutch size, number of dead (hatchlings and embryos), number of non-viable eggs, and the interval between initial nest hatching and excavation. Standard success (%) was calculated as

$[(\text{hatched eggs} + \text{hatchlings}) / \text{total clutch}] \times 100$.

Embryonic success (%) was calculated as

$[(\text{hatched eggs} + \text{hatchlings}) / \text{viable eggs}] \times 100$.

The average number of days between observed first hatchling emergence and nest excavation was 15 ± 0.37 SEM, range: 14-38 days). Of the 106 loggerhead nests examined, 44 (41.5%) were infested by at least one invertebrate group. Infestation was greater on East Laganas beach (46.9%, $n = 31$), compared with Kalamaki beach (32.5%, $n = 13$). Of all eggs sampled ($n = 10,223$), 3.1% were infested. Nine invertebrate taxa were recorded infesting nests (Table 1). Sarcophagidae (Diptera) (97.5% larvae, 2.5% adults) (Fig. 2) were the most prevalent, found in 25.5% of nests and had a strong significant association with the top half of nests ($\chi^2_1 = 93.633$, n

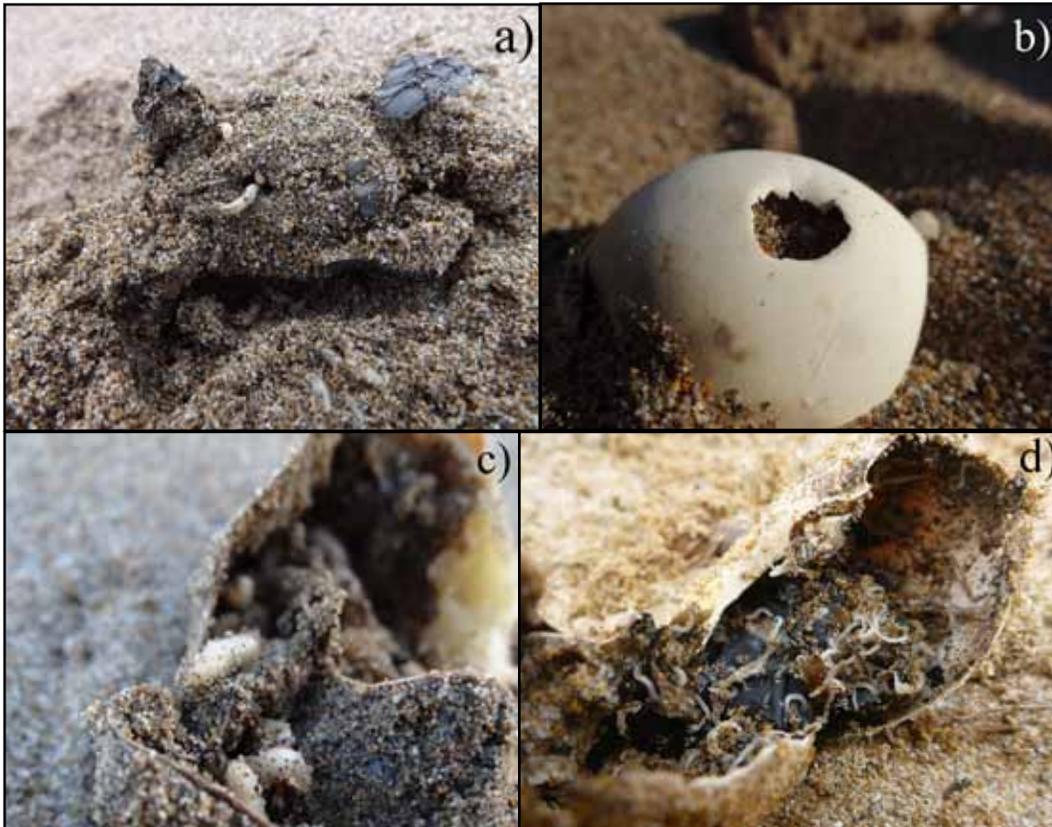


Figure 2. Sarcophagidae penetrating dead hatchling (a), punctured no visible embryo egg (b), Sarcophagidae present in non-viable egg (c), and Nematoda spp. present in late stage embryo egg (d). Photographed by Adam J. Andrews.

Invertebrates	Infested nests	% nests sampled	Infested eggs in top half of nest	Infested eggs in bottom half of nest	Invertebrate individuals per egg
Sarcophagidae (Diptera)	27	25.5	113	7	13.8
Punctured eggs	22	20.8	79	5	n/a
Tenebrionidae (Coleoptera)	6	5.7	4	2	2
Elateridae (Coleoptera)	5	4.7	6	2	3.2
Nematoda spp.	4	3.8	5	11	5.9
Isotomidae (Collembola)	3	2.8	21	33	12.5
Formicidae (Hymenoptera)	3	2.8	5	20	4.7
Histeridae (Coleoptera)	2	1.9	1	1	2
Scarabaeidae (Coleoptera)	1	0.9	0	2	2
Rhinotermitidae (Isoptera)	1	0.9	0	1	1

Table 1. Invertebrate groups (including punctured eggs) observed in infested nests.

= 120, $P < 0.001$). Only a single live hatchling was categorised as infested, where Formicidae numerously covered the hatchling on its (assumed) journey to the surface.

Dead hatchlings were the most frequently infested stage of development, with 32.5% of samples infested (Table 2). Infestation occurred in 4% of non-viable eggs. Puncture holes were observed in 1.8% (181) eggs and hatchlings, in 46.4% (84) of these cases, no invertebrates were present. Punctured eggs had a significant association with the top half of nests (one-way chi-square: $\chi^2_1 = 66.18$, $n = 85$, $P < 0.001$), and with non-viable eggs ($\chi^2_5 = 164$, $n = 84$, $P < 0.001$). Six eggs infested by Sarcophagidae were punctured, and one egg by Tenebrionidae. Sarcophagidae were the only group to penetrate hatchlings, and were significantly associated with them ($\chi^2_4 = 157.167$, $n = 120$, $P < 0.001$).

There were more late stage embryos infested (3.1%) than any other stage of dead embryo eggs (Table 2.). Only 1.4% of hatched eggs were infested, although this was a large number of eggs (Table 2). *Nematoda* spp. ($\chi^2_2 = 21.125$, $n = 16$, $P < 0.001$), Isotomidae ($\chi^2_2 = 80.333$, $n = 54$, $P < 0.001$), and Formicidae ($\chi^2_3 = 11.960$, $n = 25$, $P = 0.008$) were significantly associated with hatched eggs.

In this study there was no significant difference between the success of non-infested nests compared to infested nests (mean =

69.4% vs. 62.5%) (Mann-Whitney U test: $U = 1108.5$, $n_1 = 44$, $n_2 = 62$, $P = 0.101$) (Fig. 3: a), which was also the case for embryonic success (mean = 85.2% vs. 84.9%) (Mann-Whitney U test: $U = 1320.5$, $n_1 = 44$, $n_2 = 62$, $P = 0.780$) (Fig. 3: b).

Within infested nests, the proportion of infested eggs was significantly greater in the top half of the nest (mean = 9.8% vs. 3.2%) (Wilcoxon signed-rank test: $T = 811$, $n = 106$, $P < 0.001$). Up to 42.5% of the eggs in the top half of a nest were infested, compared to a maximum of 33.3% in the bottom half of nests, where infestation was more variable (Fig. 4). For *Nematoda* spp., Isotomidae, Formicidae, Histeridae and Scarabaeidae, observed infestation was greater in the bottom half of nests.

There was a significant negative correlation between a nest's distance to vegetation and the proportion of infested eggs (GLZM_(b): $\chi^2_1 = 10.181$, $n = 106$, $P = 0.001$) (Fig. 5: a) and between a nest's depth and the proportion of infested eggs (GLZM_(b): $\chi^2_1 = 106.561$, $n = 106$, $P < 0.001$). There was a significant positive relationship between clutch size and the proportion of infested eggs (GLZM_(b): $\chi^2_1 = 18.459$, $n = 106$, $P < 0.001$) (Fig. 5: c) and between the number of non-viable eggs and the proportion of infested eggs (GLZM_(b): $\chi^2_1 = 11.061$, $n = 106$, $P = 0.001$) (Fig. 5: e).

The proportion of infested eggs was not significantly correlated with either the interval between hatching and excavation (GLZM_(b): $\chi^2_1 = 1.317$, $n = 106$, $P = 0.251$) or the number of dead in a nest (GLZM_(b): $\chi^2_1 = 0.012$, $n = 106$, $P = 0.913$).

In this study, nests were excavated 14 days (or longer) after the first hatchling emergence. However, in the majority of other studies nests were excavated much sooner (within 24 hours (Acuña-Mesén & Hanson 1990; Gautreau 2007; Hall & Parmenter 2008): within 48 hours (McGowan *et al.* 2001a; McGowan *et al.* 2001b): within seven days after hatchling emergence (Katılmış *et al.* 2006; Özdemir *et al.* 2006; Hall & Parmenter 2008; Urhan *et al.* 2010)). In just one other study (Baran & Türkozan 1996) were nests excavated 14 days after the first hatchling emergence. In the majority of cases, studies with a short interval to excavation noted lower levels of infestation. However, since McGowan *et al.* (2001b), Gautreau (2007) and Hall & Parmenter (2008) suggest that infestation occurs during and shortly after hatchling emergence, the interval between hatchling emergence and nest excavation should not significantly influence the degree of infestation observed. We found that at least after a

Stage of Development	Eggs infested	Eggs infested/ stage (%)
Hatched (empty egg shells)	93	1.4
Non-viable	96	4
Early	0	0
Middle	1	1.9
Late	35	3.1
Pipped	2	4
Dead Hatchling	90	35.2
Live Embryo	0	0
Live Hatchling	1	1.3
Total	308	

Table 2. Observed stages of development infested by invertebrates.

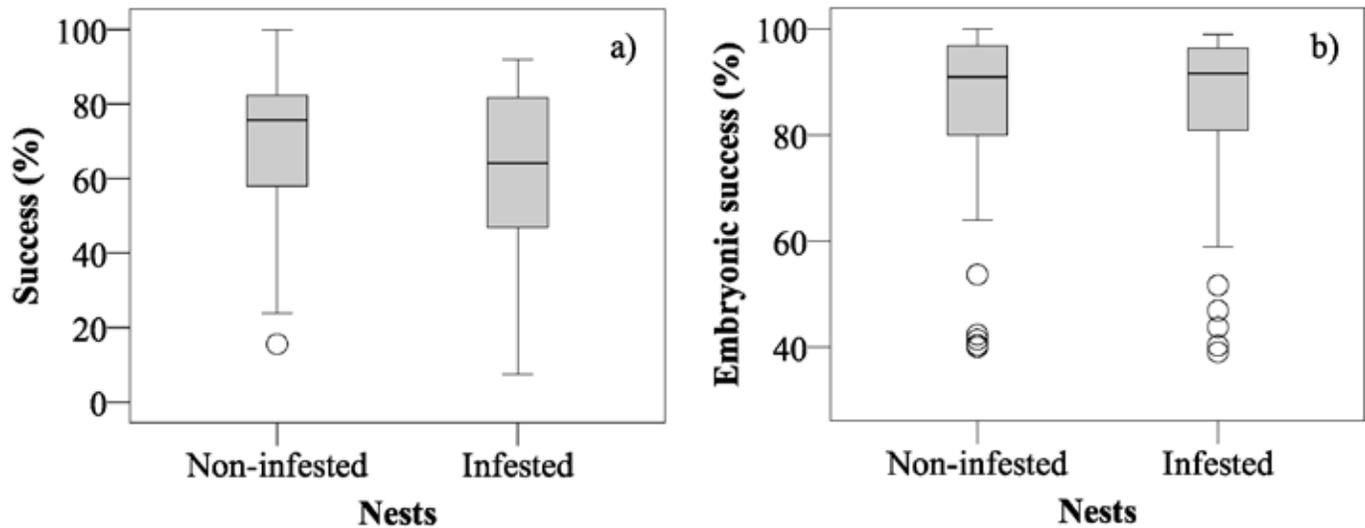


Figure 3. Boxplots of nest success (%) (a) and embryonic success (%) (b), displaying differences between non-infested and infested nests. T bars = minimum and maximum values, excluding outliers. Outliers (circles) display values between 1.5 and 3 interquartile ranges from the 25th and 75th percentiles. Mean value excludes outliers.

14-day delay after emergence, there was no significant increase in likelihood of infestation with increased days between emergence and excavation.

Most sea turtle clutches contain embryos that fail during development (Gautreau 2007). The decomposition of necrotic matter has been associated with invertebrate infestation (Fowler 1979; McGowan *et al.* 2001a; Bolton *et al.* 2008). Therefore most nests can be expected to contain at least a few invertebrates. This will cause the proportion of infested nests in a rookery to be high when an “infested” nest is defined by a single invertebrate infesting a single egg/hatchling (the case in all studies mentioned). However, when infestation is measured as the number of infested eggs within

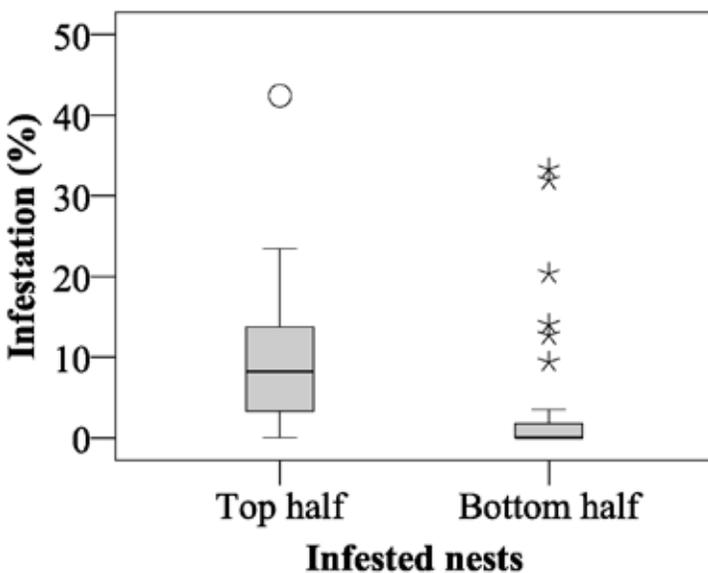


Figure 4. Boxplots displaying differences between the top and bottom half infestation (%). T bars = minimum and maximum values, excluding outliers and extremes. Outliers (circles) display values between 1.5 and 3 interquartile ranges from the 25th and 75th percentiles. Extremes (asterisks) display values greater than 3 interquartile ranges from the 25th and 75th percentiles. Mean value excludes outliers.

nests, reported infestation levels are lower. Just 3.1% of all eggs and hatchlings sampled during this study were infested, similar to previous studies which reported mainly dipteran infestation, *e.g.*, 0.5-0.8% to 2.1% (Baran *et al.* 2001; McGowan *et al.* 2001a), with the exception of 10.6% from a relatively small sample reported by Broderick & Hancock (1997) for loggerhead and green sea turtle nests.

Sea turtle nests, from deposition to post hatchling emergence, contain a range of potential food sources, intact viable and non-viable eggs at various developmental stages to live and dead hatchlings, and post-hatch egg remnants. The number of invertebrate taxa, from nine taxa (families/ orders), recorded in the excavated nests is similarly broad. Some invertebrates, notably Isotomidae, Nematoda spp., and Rhinotermitidae are known only to feed on readily available decomposing matter (Elke & Sybilla 1995; Myles 1997; Nicholas & Hodda 1999), whilst others such as Sarcophagidae are capable of puncturing eggs and preying on embryos and hatchlings (Lopes 1982).

In the interest of conservation, it is necessary to understand the cause of puncture damage and predation of hatchlings in order to act against any threat posed. However, the only invertebrate group observed attacking a live hatchling was Formicidae, and in only one instance. Formicidae, in all cases fire ants, have been reported to negatively affect hatchlings of a range of species and locations (Allen *et al.* 2001; Paris *et al.* 2002; Wetterer *et al.* 2007). Although, Formicidae capable of stinging hatchlings are not present in Europe (Katılımiş & Urhan 2007b).

In previous studies (Baran & Türkozan 1996; Baran *et al.* 2001; Katılımiş *et al.* 2006; Özdemir *et al.* 2006; Bolton *et al.* 2008) greater numbers of punctured eggs were found where invertebrates were present, therefore damage was linked to mainly tenebrionid larvae and, in some cases, dipteran larvae (Acuña-Mesén & Hanson 1990; McGowan *et al.* 2001a; Özdemir *et al.* 2006; Bolton *et al.* 2008). Our results showed 1.8% of eggs and hatchlings observed with puncture holes, indicative of predation, which is far fewer than that reported by previous studies, *e.g.*, 11.0% (Urhan *et al.* 2010), 8.2% (Özdemir *et al.* 2004) and 3.6% (Katılımiş *et al.* 2006). In these studies, the

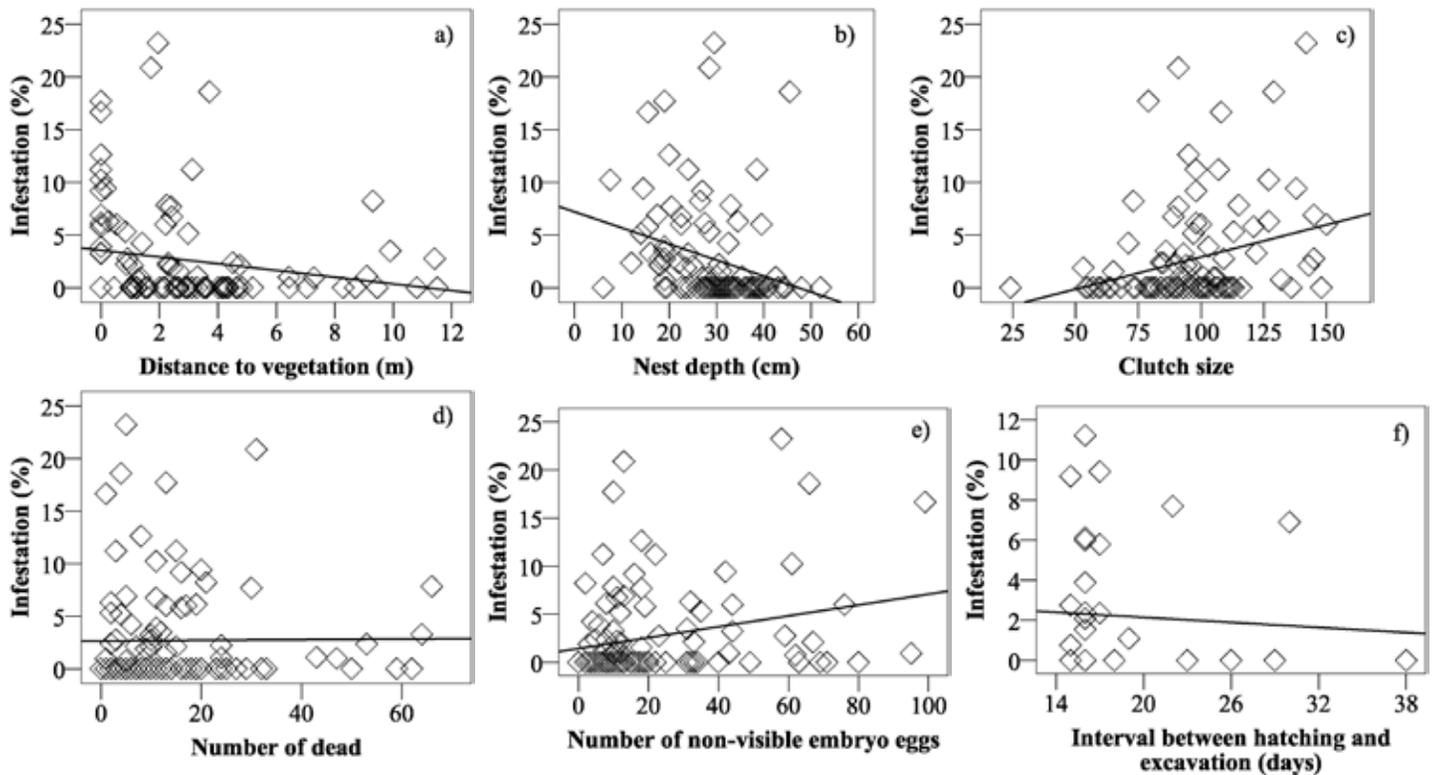


Figure 5. Scatterplots with trend line displaying correlation between infestation (%) and distance to vegetation ($R^2 = 0.035$) (a), nest depth ($R^2 = 0.077$) (b), clutch size ($R^2 = 0.081$) (c), number of dead ($R^2 = 0.8628_{,s}$) (d), number of non-viable eggs ($R^2 = 0.066$) (e), and interval between hatching and excavation ($R^2 = 0.7861_{,4}$) (f).

damage was attributed to coleopteran larvae. However, in the current study, Sarcophagidae, which previous studies have reported to infest relatively low numbers of eggs (Lopes 1982; Broderick & Hancock 1997; Donlan *et al.* 2004; Gautreau 2007; Bolton *et al.* 2008), were more closely associated with the punctured eggs, since they were both observed in greater numbers than Tenebrionidae and infested a similar range of egg stages to those punctured. In agreement with other studies (Baran *et al.* 2001; Bolton *et al.* 2008; Hall & Parmenter 2008), Sarcophagidae were associated with the top half of nests, as were punctured eggs. This increases the likelihood that Sarcophagidae were the cause of egg perforation, and that they accessed nests by burrowing down to them. In our study, invertebrate abundance was not great enough to exploit the entire nest as a food source, which may be why infested eggs were in the top half of nests (McGowan *et al.* 2001a; Katılmış *et al.* 2006; Bolton *et al.* 2008).

Although we cannot state with certainty that Sarcophagidae were not responsible for the death of viable eggs or live hatchlings, their trophic niche is normally that of a scavenger rather than a predator, feeding on, and breeding in, decaying animal or plant matter (Hall & Parmenter 2008). Therefore it is unlikely that Sarcophagidae pose a threat to otherwise viable eggs. If invertebrates were able to penetrate viable eggs, infestation events approaching 100% would be commonly observed (Bolton *et al.* 2008). The greatest infestation in a single nest found during this study was 20.8%. However, in Turkey, Özdemir *et al.* (2004) found that up to 88.4% of loggerhead eggs may be infested, suggesting that although not often the case, extreme infestation events can occur. Our results suggest that in Greece invertebrates principally act as scavengers, have little effect on nest success and pose no threat to the conservation of the species.

The finding that overall nest success was not significantly lower in infested nests is in agreement with the findings of Gautreau (2007) and Bolton *et al.* (2008) but in contrast to those of Lopes (1982, cited in Broderick & Hancock 1997), who reports a 30% decrease in nest success. However the sample size and method from this latter study are unknown. That infested nests did not have a significantly lower embryonic success rate is in agreement with Hall & Parmenter (2006). As the embryonic success of Greek loggerhead sea turtle nests was not significantly influenced by invertebrate infestation, it suggests that the majority of eggs infested were either those that were non-viable or had already hatched. Therefore the number of viable eggs affected was small, indicating that the main food source within nests is not necrotic tissue, but non-viable eggs. Although, we acknowledge that hatchlings may have been infested whilst alive and died before sampling was carried out.

While live hatchlings were found inside nests, only one was recorded as infested. Even considering the late excavation of nests in this study, it may be assumed that if live hatchlings were present in infested nests, more observations would be made of invertebrates feeding on, or at least attempting to predate them. Although several studies have recorded invasion of live hatchlings, this has never exceeded more than a few hatchlings in a nest (Fowler 1979; McGowan *et al.* 2001a; Paris *et al.* 2002; Özdemir *et al.* 2006; Gautreau 2007; Holcomb & Carr 2011). Therefore, excavating at hatchling emergence with a risk to disturbing still incubating eggs may not be worthwhile considering the low risk posed to hatchlings by invertebrates.

Several studies have suggested that invertebrates are only aware of nests during the period of hatchling emergence. Disturbance of

sand by the hatchlings is thought to advertise nest position through the release of olfactory cues, thus attracting invertebrates to feed on the decaying matter (McGowan *et al.* 2001b; Gautreau 2007; Bolton *et al.* 2008; Hall & Parmenter 2008). This may explain why a delay in excavation did not influence infestation, because hatchling emergence had already ceased. In contrast, other studies found the timing of nest excavation to influence the level of infestation (McGowan *et al.* 2001a; Gautreau 2007). Our results suggest that hatchling emergence was more closely related to the infestation of nests rather than the decomposition of matter, although more study is needed to verify this during the first two weeks after first hatchling emergence.

The depth of nests had a significant influence on infestation rates, which agrees with the findings from two main studies (McGowan *et al.* 2001b; Hall & Parmenter 2008). Chemical odour traces likely lose potency as they permeate up through the sand column (McGowan *et al.* 2001a). Therefore deeper nests are more difficult for invertebrates to locate and infest. In agreement with the current study, Hall & Parmenter (2008) reported that as clutch size increased, so did the infestation level. This suggests that invertebrates are attracted to decaying matter as larger clutches represent a greater food source. However, contrary to several studies (McGowan *et al.* 2001b; Gautreau 2007; Hall & Parmenter 2008), the number of dead in this study did not significantly affect infestation levels. This may be because invertebrates are attracted by decaying non-viable eggs (Broderick & Hancock 1997; Acuña-Mesén & Hanson 1990; Saumure *et al.* 2006; Holcomb & Carr 2011). Therefore infested nests were those with a larger number of non-viable eggs, and a larger clutch size (Katılmış & Urhan 2007a; Bolton *et al.* 2008). We found that as the number of non-viable eggs increase, so did the level of infestation.

While other studies noted predominantly dipteran infestation (McGowan *et al.* 2001b; Hall & Parmenter 2006; Bolton *et al.* 2008), distance to vegetation significantly influenced infestation in the current study. This suggests that these invertebrates as well as coleopterans may be associated with vegetation (Katılmış *et al.* 2006; Özdemir *et al.* 2006; Katılmış & Urhan 2007a). Because coleopterans have a greater ability to cause damage to nests (Katılmış *et al.* 2006), nest relocation and hatchery position may be considered for sites with abundant Coleoptera if a large proportion of nests are laid close to vegetation.

The infestation rates at nest level for Zakynthos were similarly low when compared with previous studies (Baran *et al.* 2001; McGowan *et al.* 2001a; Katılmış *et al.* 2006). It is likely that invertebrates infested nests shortly before, or during hatchling emergence, with invertebrates acting as scavengers, feeding mainly on non-viable eggs or dead hatchlings. Infestation appears to be more closely related to hatchling emergence than the amount of decaying matter, as such, we did not detect an influence in delaying excavation >14 days on infestation rates. More work is needed to accurately define the arrival times of invertebrates, as this remains poorly understood. Our study suggests that invertebrates were attracted to nests with a lower hatching success, and were not the cause of reduced success. It is thus suggested that in the Mediterranean, invertebrates pose little threat to sea turtle nests.

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