# Microbial Isolations from Olive Ridley (*Lepidochelys olivacea*) and East Pacific Green (*Chelonia mydas agassizii*) Sea Turtle Nests in Pacific Costa Rica, and Testing of Cloacal Fluid Antimicrobial Properties

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Abstract. – Microorganisms associated with olive ridley and East Pacific green turtle nesting and potential cloacal fluid antimicrobial properties were studied in Guanacaste, Costa Rica. During the 2010–2011 season, bacteria and fungi were isolated from olive ridley cloacal fluid, nest chamber sand, and egg samples. Because of the lack of cloacal fluid bacteria isolated, the focus of the 2011–2012 season shifted to determine whether fluid contained antibacterial properties by using Kirby–Bauer disk diffusion assays, and cloacal fluid and sand samples were taken to see whether bacteria were unique to cloacal fluid. Assays were performed on 34 olive ridley and 5 East Pacific green cloacal fluid samples, yielding no zones of inhibition. In the second season, *Corynebacterium* sp., *Bacillus* sp., *Klebsiella* sp., as well as genera documented in previous studies, were found unique to cloacal fluid. *Citrobacter freundii* and *Serratia odorifera* are potential contaminates and were common in cloacal fluid and nest chamber sand samples on all beaches. Fungi unique to cloacal fluid included *Fusarium* sp. and *Geotrichum* sp., with no previous record of *Geotrichum* sp. associated with sea turtle nesting. Our results suggest antimicrobial properties either are absent or undetectable by these methods. Future studies should use molecular techniques for bacterial analysis and alternative approaches for detecting antimicrobial properties.

Key Words. – Reptilia; Testudines; olive ridley sea turtle; antimicrobial properties; cloacal fluid; East Pacific green sea turtle; bacteria; fungi

The presence of nutrients, warm temperatures, and high humidity in sea turtle nests provide an ideal environment for microorganism growth both in the sand and on the eggs. Microorganisms have been found in association with reduced sea turtle hatching success (Wyneken et al. 1988; Mo et al. 1990; Santoro et al. 2006; Craven et al. 2007; Foti et al. 2009), and some species have even been known to cause up to 100% mortality of nests (Sarmiento-Ramírez 2010). Al-Bahry et al. (2009) found that some eggs are laid with potentially harmful bacteria already inside of them at the time of oviposition, suggesting that contaminates in the egg come from the female turtle prior to oviposition. However, it is also possible that eggs become contaminated from the female at the time of oviposition as they pass from the oviduct through the cloaca or the become contaminated while incubating in the sand. Little is known about the effects of microorganisms, especially bacteria, on developing embryos. Under experimental conditions, bacteria applied externally were able to penetrate to the yolk of green turtle eggs within 30 min of exposure, but the mechanisms by which they affected embryo development were not determined (Al-Bahry et al. 2009). Solomon and Baird (1980) found that fungi developing on sea turtle eggs could potentially affect the embryos in three ways: 1) impeding gas exchange of embryos, 2) calcium depletion of egg shells affecting embryo development, and 3) transfer of fungal spores from the allantois to the embryonic tissue.

Nest chamber sand contamination is a potential source for microbial infection of eggs. Sea turtle nests occur in the top meter of sand where bacteria can range from $10^8$ to $10^{12}$ cells per gram of dry sand (Dale 1974). Furthermore, these microorganism concentrations increase in finer sediments because of an increase in the available surface area (Dale 1974; Meyer-Reil et al. 1978; Mazure and Branch 1979). The presence of microorganisms in the sand is also heavily influenced by environmental factors such as rain and runoff (Santoro et al. 2006), wave action (Riedel and Machan 1972), and the presence of organics (Koop and Griffiths 1982).

Because of the presence of microorganisms in the egg chamber sand, it has been suggested that sea turtle cloacal fluid contains antimicrobial properties to protect the developing eggs (Phillott 2002; Soslau et al. 2011). This clear, sometimes viscous, fluid contains glycoproteins (Phillott 2002) and coats eggs as it is secreted from the cloaca during egg deposition into the nesting chamber. Although the fluid provides lubrication during egg deposition, its exact purpose is unknown. It has been demonstrated that the fluid creates a state of hypoxia prior to deposition, which may suspend embryo development until all embryos are at the same stage prior to deposition

(Rafferty and Reina 2012). Interestingly, Ewert (1985) found that if the glycoprotein cloacal secretions of freshwater turtles were washed off the eggs, they succumbed to infection more easily, suggesting antimicrobial properties may be present. As such, olive ridleys deposit 250–500 ml of fluid on a single nest (Silas and Rajagopalan 1984), which could potentially provide protection if antimicrobials are present in the fluid.

Antimicrobials are commonly found in nature, including those that either are used to defend embryos during development or are passed from mother to offspring to help protect them from microorganisms. Animals can obtain antimicrobials in the form of peptides (Pelegrini et al. 2011) by sequestering them from their diets, through mutualistic relationships with their normal microflora (Dawson 2011) or even through the production of antibodies (Harris et al. 2006). It is possible that any antimicrobial present in the cloacal fluid could be from one or more of these sources.

This study aims to survey the bacteria and fungi present in the cloacal fluid and surrounding sand of olive ridley sea and East Pacific green sea turtle nests in the Guanacaste region of Costa Rica. Because it is suspected that the cloacal fluid contains antimicrobial properties, fluid samples were surveyed for bacterial growth inhibition properties.

## METHODS

This study was conducted from October to March in 2010–2011 and 2011–2012. For both seasons, Playa Grande within Parque Nacional Marino las Baulas (PNMB) was patrolled nightly for olive ridley sea turtles. During the 2011–2012 season, the beaches Playa Cabuyal and Playa Ostional were also patrolled on select nights. Sampling on Playa Ostional occurred on the second night of the February arribada in 2012, and only olive ridleys were sampled on this beach. Sampling both solitary olive ridleys (PNMB and Playa Cabuyal) as well as arribada olive ridleys (Playa Ostional) allows for a comparison of the bacteria and any potential antimicrobial properties in sea turtles that exhibit 2 types of nesting behaviors. East Pacific green turtle samples were collected opportunistically only on Playa Cabuyal during the 2011–2012 season. All samples were collected in the same manner; however, during the first season, samples were frozen until they could be processed. Because of the potential loss of microorganisms from freezing, the second season samples were processed after night patrols, with most being processed with 6 hrs.

Samples were collected after digging was complete, and just prior to oviposition, sand samples were aseptically taken by scraping a sterile 1-dram vial from the bottom to the top of the egg chamber during the brief pause before egg deposition. Cloacal fluid samples were collected after approximately 20 eggs were deposited, allowing for the cloaca to be flushed of contaminants that may have been introduced while emerging and digging the nest. Two sterile 1-dram vials were placed underneath the cloaca to aseptically collect the cloacal fluid drips secreted between bouts of deposited eggs. Each sample contained approximately 1 ml of cloacal fluid, and multiple samples per turtle were taken to minimize sample loss attributable to sand contamination.

To process the sand samples, 2 ml of sterile distilled water was added to each vial and agitated vigorously for 30 sec to dislodge microorganisms from the sand grains. Water has been shown to be a good alternative to the more commonly accepted method of using phosphate buffer solution for extracting bacteria from sand samples (Lee et al. 2006) and was more accessible in the field. A sterile cotton swab was placed in this solution and used to inoculate 2 plates each of Sabouraud (first season), nutrient (second season), and MacConkey (both seasons) agars using the quadrant streak method (Benson 2002). Cloacal fluid samples were swabbed and plated in the same manner, but without the addition of sterile water. MacConkey and nutrient agars were used for the isolation and identification of bacteria, whereas Sabouraud agar was used for fungal growth and isolation. Plates were placed in Styrofoam incubators (Hova Bator model 1602 N, Savannah, GA) at 30°C and 37°C. MacConkey and nutrient agar plates incubated at 37°C for bacterial growth were incubated for 24 hrs, standard laboratory temperature and time for incubation (Benson 2002). Plates incubated at 30°C (approximate average nest temperature on Playa Grande during October and November 2010 when the highest density of olive ridley nests were laid, unpubl. data) were incubated for 48 hrs because lowering incubation temperatures a few degrees has been known to slow the growth rates of enteric bacteria (Bronikowski et al. 2001).

For the first season, all bacterial colonies were identified by biochemical properties using the API 20E systems (Bio-Merieux) at the field site. However, because of problems with the identification systems in the field, for the second season, all morphologically distinct bacterial colony types from the cloacal fluid samples were sent to Laboritorio Clínico y Banco de Sangre San Jose (Costa Rica [www.laboratoriosanjose.com]) for identification. There they were subcultured onto sheep blood agar, MacConkey agar, and mannitol salt agar, as well as Gram stained. Identifications were completed by the laboratory using semi-automated API 20 and API Staph systems (Bio-Merieux) with IdBact software v. 1.1 (G. Kronvall, Sweden). Because of limited resources, only colonies from the sand samples that looked morphologically similar to those obtained from the corresponding cloacal fluid samples were sent to the laboratory for identification. The focus shifted from identifying all bacteria in the sand and cloacal fluid in the first season to determining whether cloacal fluid contains bacteria unique to the female turtle or whether the bacteria is the result of contamination from the sand.

For the fungi in the first season, Sabouraud agar plates were kept at 30°C in Styrofoam incubators for 30 d to allow for the appearance of both slow-growing and fast-growing fungi. Fungal identification was conducted using methods used in Larone (1995). Clear tape was applied to colonies on the plate, pulled off, and placed on a slide containing a drop of lactophenol cotton blue. Genera were identified visually by looking at fungal morphology and structures under a microscope, along with information about initial color and growth time. If the tape method disrupted fungal structures, the slide culture method described in Benson (2002) was used to grow cultures directly on the slides to limit distortion of the physical structures.

During the 2010–2011 season, unhatched olive ridley eggs recovered from nests were sampled 2 d after hatchling emergence or at 65 d in accordance with current olive ridley project protocols. Unhatched eggs were opened to identify whether the egg was fertilized and, if so, then at what stage the embryo ceased to develop. The outside of each egg was thoroughly brushed and rubbed with an alcohol swab. Gloved hands were used to gently pull apart the egg shell, and a single sterile cotton swab was swiped through the yolk, albumen, inner shell casing, and on the surfaces of failed embryos. Each cotton swab was stored in a sterile dram vial, frozen, and processed the next day, after thawing in the same manner as the sand and cloacal fluid.

The Kirby–Bauer disk diffusion assay (Bauer et al. 1966) was performed to determine whether there were any antimicrobial properties in the cloacal fluid samples during the 2011–2012 season. For this, bacterial suspensions of *Enterobacter cloacae* ATCC 700323 (Gram-negative), a penicillin-resistant strain of *Staphylococcus aureus* ATCC 25923 (Gram-positive), and *Pseudomons aeruginosa* ATCC 27853 (Gram-negative), were used as control strains. These species were chosen for the experiments based on their prevalence in the eggs during the first season. After standardization with a 0.5 MacFarland standard, the control strains were plated on both Mueller-Hinton and nutrient agar for confluent growth followed by aseptic application to antibiotic disks containing 5 μg ciprofloxacin, 15 μg erythromycin, a blank disk, and a dried cloacal fluid disk. The dried cloacal fluid disks were prepared from sterilized filter disks created using a standard hole punch on Whatman No. 1 filter paper, submerged in each fluid sample until saturated. Disks were then allowed to dry in a covered, sterile, disposable Petri plate before use in the assay. The prepared plates were incubated at both 30°C (48 hrs) and 37°C (24 hrs) as described above, after which, the zones of inhibition were measured to the nearest millimeter.

## RESULTS

During the 2010–2011 season, samples were taken from the nest chamber sand and cloacal fluid of 43 olive ridleys in PNMB. Of these samples, only 25 cloacal fluid samples were visibly uncontaminated by sand and used for bacterial and fungal analysis. Of the 25 nests that did not have contaminated samples, only 18 nests (a total of 75 eggs) could be recovered. Four of the unrecovered nests were not observed to have hatched, and thermocouples marking them were pulled. Two nests had to be relocated because of position on the beach; and 1 nest was infiltrated with fire ants at hatching, and an emergency excavation had to be conducted to pull out remaining live hatchlings. The most common bacterium identified from olive ridley sea turtle eggs during the first season was *E. cloacae*; no bacteria were found in the cloacal fluid, and only *Serratia marcens* could be identified confidentially in sand samples (Table 1).

Fungi were found in association with the cloacal fluid, nest chamber sand, and unhatched eggs of olive ridley sea turtles (Table 2). Most of the fungi identified in this study were molds isolated from the cloacal fluid. Although sand samples contained the most fungal isolates, many could not be identified. None of the sand isolates were similar in structure to the *Aspergillium* sp., *Penicillium* sp., *Fusarium* sp., *Geotrichum* sp., or *Mucor* that were successfully identified. *Cladosporium* sp. was found in all 3 sources and was the most commonly identified fungal isolate from olive ridley eggs. Both *Fusarium* sp. and *Geotrichum* sp. were unique to cloacal fluid with only 1 isolate of each identified; they did not

**Table 1.** Bacteria associated with egg chamber sand, eggs, and cloacal fluid from olive ridleys during the 2010–2011 season at Playa Grande, Parque National Marino las Baulas. Number of isolates for each species from each source are shown. The 75 individual eggs were taken from the 18 recovered nests.

| Species | Sand (n = 25) | Egg (n = 75) | Fluid (n = 25) | Total |
|---|---|---|---|---|
| *Citrobacter freundii* | | 1 | | 1 |
| *Citrobacter youngae* | | 2 | | 1 |
| *Enterobacter cloacae* | | 8 | | 8 |
| *Enterobacter sakazakii* | | 2 | | 2 |
| *Pseudomonas aeruginosa* | | 4 | | 4 |
| *Serratia marcescens* | 1 | | | 1 |
| Total | 1 | 17 | 0 | |

**Table 2.** Fungi associated with egg chamber sand, eggs, and cloacal fluid from olive ridleys during the 2010–2011 season at Parque National Marino las Baulas. Number of isolates for each genus from each source are shown.

| Species | Sand (n = 25) | Eggs (n = 75) | Fluid (n = 25) | Total |
|---|---|---|---|---|
| *Aspergillium* sp. | | 1 | 8 | 9 |
| *Cladosporium* sp. | 2 | 4 | 1 | 7 |
| *Penicillium* sp. | | 3 | 3 | 6 |
| *Fusarium* sp. | | | 1 | 1 |
| *Geotrichum* sp. | | | 1 | 1 |
| *Mucor* sp. | | 2 | | 2 |
| Inconclusive | 69 | 3 | 5 | 77 |
| Total | 71 | 13 | 19 | 103 |

**Table 3.** Bacteria associated with egg chamber sand and cloacal fluid from olive ridleys during the 2011–2012 season at Parque National Marino las Baulas (PNMB), Playa Ostional, and Playa Cabuyal. Number of isolates for each species from each source are shown.

| Species | PNMB (n = 17) | | Ostional[a] (n = 14) | | Cabuyal (n = 3) | | Total (n = 34) |
|---|---|---|---|---|---|---|---|
| | Sand | Fluid | Sand | Fluid | Sand | Fluid | |
| *Acinetobacter baumannii* | 1 | | | | | | 1 |
| *Corynebacterium* sp. | 1 | | 2 | | | 1 | 4 |
| *Serratia liquefaciens* | 1 | 3 | | | | | 4 |
| *Serratia odorifera* | 4 | 2 | 4 | 4 | 3 | 3 | 20 |
| *Citrobacter freundii* | 9 | 12 | 6 | 6 | 2 | 4 | 39 |
| *Pseudomonas aeruginosa* | | 1 | | | | | 1 |
| *Serratia plymuthica* | | 1 | | | | | 1 |
| *Enterococcus faecalis* | | 1 | | | | | 1 |
| *Enterobacter cloacae* | | 1 | | | | | 1 |
| *Klebsiella* sp. | | 1 | | | | | 1 |
| *Salmonella* sp. | | 1 | | | | | 1 |
| *Bacillus* sp. | | 1 | | 1 | | | 2 |
| *Staphylococcus epidermidis* | | | | 1 | | | 1 |
| *Enterobacter sakazakii* | | | | | | 1 | 1 |
| Total | 16 | 24 | 12 | 12 | 5 | 9 | 78 |

[a] Indicates an arribada beach.

appear to infect the eggs and were not found in any sand sample because they were structurally different from isolations that could not be identified.

During the 2011–2012 season, samples were cultured from the nest chamber sand and cloacal fluid of a total of 62 individual females: 40 olive ridleys from PNMB, 14 olive ridleys from Playa Ostional, and 3 olive ridleys and 5 East Pacific green turtles from Playa Cabuyal. *Citrobacter* sp. and *Serratia* sp. were found in both commonly identified in cloacal fluid as well as sand (Tables 3 and 4) on all 3 beaches and both turtle species. *Citrobacter* sp. and *Serratia* sp. were commonly found in corresponding sand and cloacal fluid samples, even though there was no visible contamination of sand in fluid samples, and occasionally found in one sample type but not the other in the same sample set. During the 2011–2012 season, bacteria isolated from the cloacal fluids were dominated by the Gram-negative gammaproteobacteria (*Enterobacteriaceae*); Gram-positive genera (*Corynebacteria* sp., *Bacillus* sp., and *Staphylococcus* sp.) were isolated. Similar bacterial genera were isolated from samples taken from both arribada and solitary nesting beaches, although most of those unique to the cloacal fluid were obtained at PNMB.

Kirby–Bauer experiments were run with all samples from Playas Cabuyal and Ostional and 22 of the Playa Grande samples. Kirby–Bauer disk diffusion assays to

**Table 4.** Bacteria associated with egg chamber sand and cloacal fluid from East Pacific green turtles during the 2011–2012 season at Playa Cabuyal. Number of isolates for each species from each source are shown.

| Species | Sand (n = 5) | Fluid (n = 5) | Total |
|---|---|---|---|
| *Staphylococcus aureus* | 1 | | 1 |
| *Citrobacter freundii* | 2 | 4 | 6 |
| *Serratia odorifera* | 3 | 3 | 6 |
| Total | 6 | 7 | 13 |

assess antimicrobial properties of sea turtle cloacal fluids did not yield any zones of inhibition for the fluids regardless of the bacterial control strain, media, or temperature used (Table 5). The control disks (antibiotics and blank) performed as expected with no variation regardless of the type of media used or the temperature of incubation. There was no difference in inhibition zones based on nesting behavior exhibited.

## DISCUSSION

To better understand the microbial diversity associated with olive ridley and East Pacific green sea turtles at Playa Grande, Cabuyal, and Ostional Beaches in the Guanacaste region of Costa Rica and their potential effects on hatchling success, several bacterial and fungal isolates were obtained from the cloacal fluids and eggs of nesting turtles as well as nest chamber sand. In an effort to distinguish potential environmental biological contaminants from the normal flora of the sea turtle cloacal fluid, only isolates from the sand that were morphologically similar to those isolated from corresponding cloacal fluid samples were identified. Thus, the data provides a qualitative survey and should not be interpreted as measures of abundance. *Serratia* and *Citrobacter* sp. were found in all of the 2011–2012 sample types, on all of the beaches, and with both nesting behaviors (arribada and solitary). Thus, it may be that exposure to *Serratia* sp. and *Citrobacter* sp. by the female sea turtles occurs when they come ashore to nest rather than when they are free ranging, or they are ubiquitous in multiple environments. Both of these genera can be opportunistic pathogens and have been found in the failed eggs of several other sea turtle species (Wyneken et al. 1988; Girondot et al. 1990; Al-Bahry et al. 2009). Alternatively, several Gram-negative gammaproteobacteria of the *Enterobacteriaceae* and 3

**Table 5.** Kirby–Bauer disk diffusion assay results using olive ridley and East Pacific green turtle cloacal fluids. Average diameter (mm) and standard deviation of the zones of inhibition are reported. Nutrient agar (NA) and Muller-Hinton agar (MH) plates incubated at 30°C and 37°C. The letters F, C, E, and B represent cloacal fluid, ciprofloxacin (5 µg), erythromycin (15 µg), and blank filter disks, respectively. SD = standard deviation.

| | 30 NA results | | | | 37 NA results | | | | 30 MH results | | | | 37 MH results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | C | E | B | F | C | E | B | F | C | E | B | F | C | E | B |
| **Olive ridley** | | | | | | | | | | | | | | | | |
| *Enterobacter cloacae* | | | | | | | | | | | | | | | | |
| Mean | 0 | 32.7 | 0 | 0 | 0 | 32.0 | 0 | 0 | 0 | 26.8 | 0 | 0 | 0 | 26.2 | 0 | 0 |
| SD | 0 | 3.8 | 0 | 0 | 0 | 3.3 | 0 | 0 | 0 | 2.2 | 0 | 0 | 0 | 2.0 | 0 | 0 |
| *Staphylococcus aureus* | | | | | | | | | | | | | | | | |
| Mean | 0 | 24.9 | 25.4 | 0 | 0 | 26.2 | 25.0 | 0 | 0 | 25.4 | 25.3 | 0 | 0 | 24.2 | 24.0 | 0 |
| SD | 0 | 5.6 | 4.5 | 0 | 0 | 5.0 | 4.2 | 0 | 0 | 4.3 | 6.3 | 0 | 0 | 4.5 | 6.3 | 0 |
| *Pseudomonas aeruginosa* | | | | | | | | | | | | | | | | |
| Mean | 0 | 29.5 | 0 | 0 | 0 | 28.8 | 0 | 0 | 0 | 29.2 | 0 | 0 | 0 | 27.8 | 0 | 0 |
| SD | 0 | 2.9 | 0 | 0 | 0 | 3.9 | 0 | 0 | 0 | 3.1 | 0 | 0 | 0 | 3.2 | 0 | 0 |
| **East Pacific green** | | | | | | | | | | | | | | | | |
| *Enterobacter cloacae* | | | | | | | | | | | | | | | | |
| Mean | 0 | 29.8 | 0 | 0 | 0 | 33.8 | 0 | 0 | 0 | 26.8 | 0 | 0 | 0 | 26.2 | 0 | 0 |
| SD | 0 | 4.4 | 0 | 0 | 0 | 3.6 | 0 | 0 | 0 | 1.7 | 0 | 0 | 0 | 1.6 | 0 | 0 |
| *Staphylococcus aureus* | | | | | | | | | | | | | | | | |
| Mean | 0 | 22.5 | 22.5 | 0 | 0 | 25.8 | 17.6 | 0 | 0 | 23.5 | 24.0 | 0 | 0 | 23.8 | 17.4 | 0 |
| SD | 0 | 1.3 | 2.4 | 0 | 0 | 5.1 | 10.0 | 0 | 0 | 1.3 | 0.8 | 0 | 0 | 5.9 | 9.8 | 0 |
| *Pseudomonas aeruginosa* | | | | | | | | | | | | | | | | |
| Mean | 0 | 28.8 | 0 | 0 | 0 | 27.8 | 0 | 0 | 0 | 30.0 | 0 | 0 | 0 | 24.8 | 0 | 0 |
| SD | 0 | 3.3 | 0 | 0 | 0 | 3.0 | 0 | 0 | 0 | 0.8 | 0 | 0 | 0 | 1.5 | 0 | 0 |

Gram-positive genera were found to be unique to the cloacal fluid samples (Tables 3 and 4). Among them were those known to be associated with failed sea turtle eggs or pathogens to free ranging turtles (Glazebrook et al. 1993; Aguirre et al. 1994). *Bacillus* sp., for instance, has been found in association with failed loggerhead (*Caretta caretta*) eggs in Jekyll Island Georgia, failed leatherback (*Dermochelys coriacea*) eggs in PNMB, and some diseases in free-ranging turtles, although is best known as a saprophytic common skin flora of sea turtles (Aguirre et al. 1994). *Enterobacter sakazakii* was found in eggs and in 1 cloacal fluid sample and has been shown to produce enterotoxins, which can lead to neonatal meningitis resulting in mortality in humans and mice, and could potentially have similar impacts on developing sea turtle embryos (Pagotto et al. 2003). Many of the isolates are known opportunists that can become pathogenic to immunocompromized hosts. As such, many of the bacteria found in the sand, cloacal fluids, and eggs of this study have been associated with reduced hatchling success (Wyneken et al. 1988; Santoro et al. 2006; Craven et al. 2007; Foti et al. 2009).

Many of the fungal genera isolated from eggs, sand, and cloacal fluids of olive ridley sea turtles on Playa Grande have also been isolated from other sea turtle species around the world. These fungal genera are predominately saprophytic in nature, but can be opportunistic pathogens, especially in immunocompromized hosts (Ng et al. 1994; Larone 1995). Thus, they could be pathogenic to developing embryos, especially to those under environmental stress. *Fusarium* was the only documented fungus from the cloaca of nesting female olive ridleys prior to this study. This particular fungus has been proven to cause up to 100% mortality of loggerhead nests and is a major factor in the reduction of their population in Cape Verde, Africa (Sarmiento-Ramírez 2010). This study also successfully identified *Aspergillium* sp., *Cladosporium* sp., and *Penicillium* sp., which are known isolates from the cloacas of other sea turtle species, as well as *Geotrichum* sp., which has not previously been documented as a sea turtle cloacal fluid isolate.

Phillott (2002) discovered that fungal growth can be inhibited for a couple of days by the presence of sea turtle cloacal fluids under laboratory conditions. This suggests that the cloacal fluid can briefly protect a clutch from fungal invasion. However, after these first couple of days the fungi rapidly populated the petri plates, suggesting that the fungus could not initially grow 1) because of the anoxic properties of the fluid (Rafferti and Reina 2012); 2) because the fluid contained weak antimicrobial properties; or 3) because the fluid physically washes away the fungi. Although not tested on fungi in this study, the inability of cloacal fluid to inhibit the growth of Gram-positive and Gram-negative bacterial strains in the Kirby–Bauer disk diffusion assay suggests there are no antibacterial properties in the cloacal fluids of olive ridley or East Pacific green turtles of northwestern Costa Rica. Similar results have been found in Costa Rican leatherback turtles (K. Vinette Herrin, unpubl. data, 2012) and Australian green sea turtles (R. Reina, unpubl. data, 2012). An alternative interpretation

could be that the Kirby–Bauer assay was not an effective method for detecting antimicrobials in cloacal fluids, perhaps attributable to inefficient diffusion of the fluid into the media or a low concentration of the antimicrobial if it is indeed present. Interestingly, current research on the antimicrobial properties of cloacal fluid from flatback and loggerhead sea turtles in Australia shows mixed results that vary according to both the species and individuals within the population (K. Vinette Herrin, *pers. comm.*, 25 March 2012). Thus, it is also possible that there is variation in the presence and concentration of antimicrobials within a nesting population's cloacal fluid. Also worth considering is that antimicrobial activity may be the result of antibodies that have built up from exposure of females to bacteria.

This study was able to identify several potential pathogens that could be affecting hatchling success of sea turtles in the Guanacaste region of Costa Rica. Sea turtle eggs have the potential to be with potential pathogens by both the nesting female and the sand in which they incubate. Using disk diffusion assays conducted at average nest incubation temperature and accepted laboratory standards, there was no evidence to suggest that sea turtle cloacal fluids contain antimicrobial agents against the bacterial control strains used. Lack of antimicrobial agents in cloacal fluid has also been supported by other recent studies; thus, purpose of the cloacal fluid is not likely to act as an antimicrobial barrier for the developing eggs.

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