From: Thayer Broili
To: Darrell Echols
Cc: Mike Murray
Subject: Fw: dna information
Date: 03/02/2010 02:09 PM
Attachments: Northern Recovery Unit Loggerhead Genetics Project Overview.pdf
2010 Egg Sampling Protocol (2).pdf

Below is proposal to do some genetic turtle egg testing. See details below. Any thoughts? Darrell may have some technical and managerial knowledge of these type of study. Please feel free to include Britta/Michelle in any response.

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----- Forwarded by Thayer Broili/CAHA/NPS on 03/02/2010 01:56 PM -----

Britta Muiznieks/CAHA/NPS
03/02/2010 09:43 AM

To: Thayer Broili/CAHA/NPS@NPS, Margaret Carfioli/CAHA/NPS@NPS
cc: Michelle Bogardus/CAHA/NPS@NPS
Subject: Fw: dna information

Thayer-
This looks like a good proposal and looks like everyone who needs to be involved has been contacted (i.e. FWS) and is in support of it. I think it has the potential to help us answer some of our renesting questions. Can you think of any other concerns?

Meghan-
What sort of paperwork would need to be completed on our end if we wanted to implement it this year?

Britta Muiznieks
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----- Forwarded by Britta Muiznieks/CAHA/NPS on 03/02/2010 09:37 AM -----

Michelle Baker/CAHA/NPS
03/02/2010 07:14 AM

To: Britta Muiznieks/CAHA/NPS@NPS
cc:
Subject: Fw: dna information

I talked to Matthew yesterday afternoon and let him know our concerns about implementing the project. Apparently the study is covered under his current permit from FWS and therefore would be included in our permit from NCWRC. Even though the project is covered, he is asking for a letter of support from FWS just to document that they know about it and support it.

I have not looked at the below documents yet, but he said he was planning on sending the project proposal and other project documents. Take a look and see what you think. He explained the egg sampling procedure to me and it seems very simple and not time consuming. The eggs do not need to be shipped and there will be a student that will be driving around every couple of weeks to pick up the samples.

Michelle

----- Forwarded by Michelle Baker/CAHA/NPS on 03/02/2010 07:09 AM -----

"Godfrey, Matthew H" <matt.godfrey@ncwildlife.org>

03/01/2010 09:37 PM

To "Michelle_Bogardus@nps.gov" <Michelle_Bogardus@nps.gov>

cc

Subject dna information

Hi Michelle,
Here are two documents – one overviews the project and the other overviews the sampling protocols. I will talk to Sandy M. tomorrow about a supporting letter from FWS (there might be one already somewhere for the project as a whole).
best,
Matthew

Matthew H. Godfrey, PhD
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Get NC Wildlife Update -- news including season dates, bag limits, legislative updates and more -- delivered to your Inbox from the N.C. Wildlife Resources Commission.

Northern Recovery Unit Loggerhead Genetics Project Overview.pdf

2010 Egg Sampling Protocol (2).pdf

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Viable Egg Collection Protocol

1) Because of the extremely small amount of DNA present in the shells, it is necessary to change gloves between nests or wipe hands with hand sanitizer to prevent DNA cross contamination of samples from different females.
2) Pull a single egg from each nest either when the nest chamber is validated.
3) Open the egg and discard its contents. (The goal is to try to keep the yolk and associated embryonic disc and membranes OFF the egg shell to the extent possible. We are trying to avoid having the embryonic DNA contaminate the maternal DNA) Pinching a spot on the shell and opening the egg wide open like a bag of chips is the best way to avoid contamination. The egg contents should be discarded well away from the nest, ideally buried into wet sand. Eggs broken by predators (found the morning after oviposition) or while probing will work, but should be rinsed in the ocean first to cleanse any remaining embryonic tissue/yolk membranes.
4) Place the entire shell in a 30 ml conical tube containing 95% ethanol. (Tubes are flammable, so keep away from heat sources!)
5) Write the year, three-digit beach abbreviation (separate list) and nest number on the blue cap of the vial with a Sharpie.
6) While on the beach, try to keep the sample out of direct sunlight if possible.
7) Store the samples in a cool (room temperature is fine), dark place.

The goal is to collect a freshly laid (< 48 hours old ideally) eggshell from every nest on your beach. We need some sort of genetic sample from every nest laid- if a fresh egg wasn't collected because the nest was originally called a false crawl- then we can use a dead hatchling or embryo flipper collected at inventory. Each and every nest needs to be represented, with the order of preference being:

1) freshly laid egg
2) depredated eggshell (if available from ghost crab depredation, etc. and only if fresh egg wasn't taken)
3) dead hatchling tissue at inventory (fresher the better, only if fresh egg fails)
4) hatched egg shell at inventory (only if no dead hatchlings/embryos available and fresh egg wasn't collected)

Fresh eggs contain by far the best DNA compared to egg shells from various stages of incubation, so please make every effort to get a fresh egg from each and every nest. I will attempt to run all egg samples with sufficient lead time to identify any problem samples. In this case, we can use dead hatchling tissue as a back-up insurance sample. I will notify you of specific nests if this is the case, so don't worry about collecting anything extra if a fresh egg was collected unless you hear from me.

The ultimate goal of the project is to track site fidelity and clutch frequency of northern subpopulation loggerheads nesting from NC to the FL border. We do this by assigning nests to biopsied females or by matching multiple nests from unseen females. We have genetically "tagged" ~ 1000 nesting females in GA since 2005, and it will be interesting to see how many and where these turtles may show up! Getting a viable genetics sample from every nest is critical for producing accurate clutch frequency data with minimal bias.

Thanks so much for all of the help! This is obviously a massive undertaking with respect to scale, and the data we will produce is only as good as the sampling effort. So thank for participating! If you have any questions, concerns, or need additional supplies, please contact me:

Brian Shamblin
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Genetic mark-recapture of Northern Recovery Unit (GA, SC, NC) nesting female loggerheads using DNA derived from eggs

There are several basic loggerhead nesting ecology questions that need to be answered in order to help us understand population dynamics and recover loggerhead populations. These questions include:

- How many females are nesting along the North Carolina, South Carolina, and Georgia coasts each year?
- How many nests does each female lay in a season and does the number change annually?
- What is the average remigration interval?
- How close together or far apart does each individual lay her nests? What proportion of females regularly use multiple islands…multiple states for nesting?
- For how many years is a nesting female reproductively active?
- How precisely does a daughter return to her hatching site to lay her nests…within 10 km, 100 km?
- Are all of the turtles nesting from North Carolina through the Georgia/Florida border just one big subpopulation, or multiple populations?
- What are the primary sources of mortality for adult female loggerheads?

Many of these questions have been addressed by flipper-tagging studies. Unfortunately, now matter how hard you try, you can rarely catch all of the females all of the times they nest on a particular study beach. Tagging studies generally fail to intercept 10-20% of females nesting on a beach each season. And we know from multiple tagging studies that individual females may nest on several different beaches over the course of a season, resulting in biased estimates of site fidelity and clutch frequency. Night monitoring is not feasible on most beaches, so the identities of turtles nesting over most of the coast are unknown. Additionally, flipper tag loss (flipper tag does not stay on the flipper) is very high so you often end up measuring tag loss rather than the actual biological parameter(s) you intended to measure. In short, tagging studies on individual beaches have provided imperfect (biased) estimates of these important reproductive parameters.

In order to get a more complete picture of loggerhead nesting in the northern subpopulation, we need a means of identifying the mother of every nest over a large area that reflects the scale of female nest site fidelity. Recently, scientists at University of Georgia developed the tools to extract and analyze maternal DNA from viable loggerhead eggs. By collecting a single fresh egg shell from each nest, we can accomplish this without ever seeing the females!

Obviously, some of the questions above cannot be answered by a short-term project. But by identifying the mothers of all recorded nests on the northern recovery unit beaches, we can take a snapshot of the nesting population for 2010. Because of differences in site fidelity and clutch frequency among females, there may be many more or many less nesting turtles out there than we think. By identifying each female, we can take a near census of the annual nesting population.

We can also examine nest site fidelity on the recovery unit scale, not just the range of behavior but also determine what proportion of the population is displaying particular behaviors. Understanding the relationship between clutch frequency and nest site fidelity is critical to generating better estimates of nesting female population size and building better population models.

Since we will be using genetic data to identify individual turtles, we can also use the same data to examine relatedness among nesting females. Analysis of Georgia turtles suggests that we have some mother/daughters pairs and sister pairs on the nesting beaches. We can use the nesting behavior of relatives to understand how discrete or inter-connected females nesting on different beaches are. This comes back to determining if the northern recovery unit is really acting as a single population unit or if there are multiple demographically discrete rookery units.

Finally, the database of unique genetic tags collected from South Carolina nesting loggerheads may be used to help identify important sources of mortality that are hampering population recovery. For example, tissue samples from adult loggerhead strandings may be compared with the genetic database to identify sources of mortality (e.g., commercial fisheries, boat collisions, channel dredging) affecting local nesting populations.

Frequently Asked Questions

Why is it important to understand how the nesting population dynamics and how does this help recover turtles?
Loggerheads are listed under the Endangered Species Act and therefore have Recovery Objectives and Criteria. One

Objective is to ensure that the number of nests in the Northern Recovery Unit is increasing and that this increase corresponds to an increase in the number of nesting females (estimated from nests, clutch frequency and remigration interval). The inverse is also true for the case of a decreasing nesting population. There are several significant data deficiencies with respect to knowledge of basic loggerhead biology, demography, distributions and movements. To comprehensively conserve and recover loggerhead turtles in the Atlantic, these data gaps need to be addressed (NMFS and USFWS 2008). The primary threats to sea turtles are from human activity. Population models and identification of sources of mortality will allow us to assess whether management that we employ is effective in actually recovering populations.

Why is it important to understand the genetic structure of the nesting population? Recovery units are subunits of a listed species that are geographically or otherwise identifiable and essential to the recovery of the species and are individually necessary to conserve genetic robustness, demographic robustness or some other feature necessary for long-term sustainability of the species. Current research suggests four distinct nesting assemblages based on analysis of haplotypes from mtDNA. Geographic boundaries of U.S recovery units may need to be refined in order to correctly apply population status assessments, threats assessments, and recovery actions. Genetic analysis including continuous spatial sampling of nesting females, are needed to fully describe spatial population structure and identify recovery boundaries based on breakpoints in nesting occurrence and female relatedness. To fully describe female relatedness, additional analyses are needed using the non-coding regions of the mtDNA and more precise analyses of nuclear DNA (NMFS and USFWS 2008).

What part of the egg is the maternal nuclear DNA being extracted from? The maternal nuclear DNA is actually contained between layers of the inner shell membrane.

Why can't we use egg shells from hatched or unhatched eggs or tissue from dead hatchlings that can be collected during inventory so that an egg does not have to be sacrificed? There are several important reasons that a fresh egg shell needs to be collected within 24 hours of oviposition. (a) Within 24 hours, the maternal DNA can be extracted because the embryonic DNA which contains DNA from both parents has not migrated into the inner membrane of the shell. The goal is to genetically identify the nesting female, not her offspring. Moreover, tissue taken from the flipper of a dead hatchling at inventory is only informative if it matches an already known maternal genotype. If you cannot match the embryonic DNA from inventory and you did not sample an eggshell, the opportunity to genetically identify the female that laid the nest has been lost; (b) if the nest does not make it through incubation (nest is washed away or cannot be found), no sample will be taken from this nest and the opportunity to genetically identify the female that laid the nest has been lost; (c) DNA in decomposing tissue is not always useful because the DNA has degraded. The success rate is significantly higher (>90%) with fresh egg shells than with decomposed hatchling tissue.

How are we going to address data gaps in SC that will exist because shells were not collected on beaches that do not have nest protection projects? No research project is perfect, but that does not mean you shouldn't do it. For example, the error rate for identifying nests during nesting beach patrols is about 10% (based on Witherington et al. 2009), but that doesn't mean the volunteers should stop patrolling beaches. Essentially, an estimate of the percent of missed nests in SC will be generated. Early discussions proposed that aerial survey data during the next three years will help derive the percentage of missed nests. It won't be perfect, but it will be by far the best big picture anyone has had up until now.

Why does additional information on clutch frequency and remigration interval need to be collected? We don't have a good handle on clutch frequency or remigration interval because the survey area of tagging studies generally represents less than half the area used by nesting females. Clutch frequency for loggerheads has been reported as 3 – 5.5 nests per female per season and remigration interval has been reported as 2.5 – 3.7 years (see Table 3 in NMFS and USFWS 2008). However, using DNA tags from a single egg is a powerful way to learn which individual females have come up on which beaches, and the information will help us know exactly how many individuals are nesting, how often, and where. No other research project has been done on this scale before, and it will be extremely beneficial for management purposes and conforms to Recovery Plan action items.

What are the limitations of flipper tagging studies?
Unfortunately, no matter how hard you try, you can rarely catch all of the females all of the times they nest on a particular study beach. We know from multiple tagging and satellite telemetry studies (Scott 2006) that individual females may nest on several different beaches over the course of a season. Night monitoring is not feasible on most beaches, so the identity

of turtles nesting over most of the coast is unknown. Additionally, flipper tag loss is very high so you often end up measuring tag loss rather than the actual biological parameter(s) you intended to measure.

What is the estimated percentage of eggs to be taken out of the total eggs laid during the three years of this study in SC? If we look at the numbers for 2009, 2183 loggerhead nests were laid. The estimated number of eggs laid was 237,081. If we had conducted this genetics project in 2009, 2183 eggs would have been taken for this research project. This equals less than 1% (0.92%) of the eggs laid. Additionally, not all of the 2183 eggs taken for this project would need to have been sacrificed. There were 512 nests in 2009 that had at least one documented egg loss. This means that the egg needed for research could in most instances have been taken from these losses that already occurred. The limiting factor is that egg losses that occur after 24 hours of deposition cannot be used. So, if you factor this into the 2009 scenario, 2183 minus 512 eggs would have been sacrificed = 0.70% of the eggs laid.

How many total eggs were lost to probing in SC during 2009? In 2009, there were 504 eggs lost to probing in 144 nests.

What was the total documented and estimated egg loss in SC during 2009? In 2009, 10,328 (4.3%) eggs were actually documented as lost. This does not include eggs from *in situ* nests that were lost to tide/storm events where the nest was washed away or could not be found. If you estimate this number, there were 100 *in situ* nests lost to tides in 2009 (the number of eggs in the nest were never counted). Using a mean clutch count of 120, this is an additional 12,000 eggs lost to tides and storm. This gives us an estimated egg loss of 22,328 in 2009 which is 8.6% of the estimated eggs laid (the estimated number of eggs laid was increased by the same amount).

How have GA nest protection participants responded to this study?
Georgia has been conducting this research for two years and although there was some initial reluctance on the part of a few cooperators, all of the GA projects are now enthusiastic participants. The University of Georgia (Brian Shamblin) provides each of the projects with an overview of the identities of females laying eggs on their beach and also nesting events on other islands.

What information has Georgia gained from the first two years of the study?
Approximately 800 individual females have been genetically tagged over the first two years of complete coverage sampling along the Georgia coast, ~450 in 2008 and ~350 in 2009. Genetic tagging actually began on a limited basis in 2005, so there have been over 1000 individual females genetically tagged in Georgia. Of the 64 females that were directly intercepted and sampled in 2005, 35 have been detected nesting since, most without actually observing the nesting female. Among these are turtles that nested on Sapelo and Blackbeard and had satellite transmitters fitted in 2005. Of the 12 females originally sampled, 9 have been recorded upon remigration, nesting very close to their original tagging locations: Sapelo Queen, Zapala, Georgia, Maureen, Pearl, Gypsy, Seaweed, Queen Anne, and Cabretta. Tissue sampling of a decomposed stranded turtle confirmed its identity as Sapelo Queen, who was killed after laying the first nest on Sapelo in 2008. The fate of most of these turtles would not be known without the genetic tagging as most were not observed nesting on a flipper tagging beach.

Both extremes of nest site fidelity behavior have been recorded. One female laid 7 nests on less than 1 km of beach on St. Catherines Island, whereas a different female nested on both Cumberland and Wassaw the same nesting season. Inter-seasonal nest site fidelity from turtles genetically tagged in 2006 that returned to nest in 2008 and 2009 (n = 44) suggests that a large majority are nesting within 25 km of their nesting locations from 2006. However, a small proportion of females were detected nesting at more distant locations outside the pilot study area. Relatedness analysis suggests that there are some mother/daughter as well as sister/sister pairs of turtles nesting along the GA coast. A particular female from Jekyll Island appears to be the mother of two nesting females, one that uses Jekyll Island and one that has nested on Blackbeard.

Why do we need to expand this project into SC and NC?
The ultimate short-term goals of the project are quantifying nesting female numbers and clutch frequencies. Our ability to achieve this is intimately tied with female nest site fidelity. While the Georgia coast is large relative to any single flipper tagging beach, it still may be too short to accurately capture the variation in female nesting behavior. We need a snapshot of this behavior across longer coastlines to fill in gaps in the data. Several females have been detected laying only one or two clutches in the center of the GA study are and nowhere else along the GA coast. Supplementary data from Hilton Head flippers collected at inventory (thanks Carlos and helpers!) demonstrate that at least two of these turtles also nested

in SC. We need to know how many of these females are really laying only one or two nests (if any), and if females are laying elsewhere, we need to know where.

Nest site fidelity data can also be used to determine if any population structure occurs within the Northern Recovery Unit. Because all these nesting turtles share the same mitochondrial DNA control region marker, turtles from NC, SC, and GA are indistinguishable. But by tracking individual females, it will be possible to essentially generate nesting home ranges and describe the scale of nest site fidelity. Examining nesting home ranges of close relatives is especially helpful for indirectly determining the scale of natal homing by nesting females. There may be cryptic demographic breaks along the coast that could warrant the recognition of additional recovery units.

How did reviewers comment on this research?
The research proposal was evaluated by FWS and NMFS and independent reviewers. All of them acknowledged that the benefits of having this information far outweighed the costs of taking a single egg from each nest. This research is not being conducted because it is an interesting hypothetical research project. This study is critical to our recovery efforts and is a high priority action in the recovery plan.

Grant Proposal

Project Goals and Objectives

1) Estimate annual nesting population size (via near census) for the Northern Recovery Unit 2010-2012.
2) Estimate intra-seasonal clutch frequency.
3) Estimate remigration intervals of Northern Recovery Unit females.
4) Characterize scale of intra-seasonal and inter-seasonal nest site fidelity of all nesting females where possible.
5) Characterize population structure among Northern Recovery Unit rookeries via inferred mother-daughter nesting home range comparisons.

The proposed research addresses priority action items in the Recovery Plan for the Northwest Atlantic Population of the Loggerhead Sea Turtle including: 153) determination and monitoring of clutch frequency, 154) determination and monitoring of remigration interval, and 155) determination of female reproductive life span. Reproductive data are typically derived from flipper tagging projects with limited geographic scope. Nesting female home ranges often exceed the area covered by tagging surveys resulting in incomplete and biased results. In addition, all nesting females are not detected on tagging surveys due to spatial and temporal gaps in coverage.

The least biased approach to generating fecundity parameter estimates is genetic mark-recapture across all monitored nesting beaches within the Northern Recovery Unit. Preliminary data from a statewide genetic mark-recapture study in Georgia suggest that three-year remigration intervals may be more prevalent than previous research has suggested. Moreover, several females have been observed nesting only once or twice on the study beaches in central Georgia and nowhere else along the entire Georgia coast. It is unclear whether these observations represent females truly laying only one to two nests per season, or if these females are simply low site fidelity turtles that have also nested outside Georgia. It is important to ascertain whether decreasing nest counts seen on Northern Recovery Unit beaches (NMFS and USFWS 2008) reflect a decrease in nesting females or may be the result of reduced intra-seasonal clutch frequency and increased remigration intervals.

In addition to assessing fecundity parameters, the proposed research addresses action items 111) refinement of boundaries of recovery units and 113) development of new techniques for refining population genetic structure. Encalada et al. (1998) hypothesized that additional demographic partitioning likely occurs among Northern Recovery Unit rookeries, but that lack of resolution with the genetic marker prevented further analysis. Traditional population subdivision and assignment approaches based on microsatellites perform poorly (Shamblin unpublished), presumably due to moderate to high levels of male-mediated gene flow (Bowen et al. 2005, Shamblin 2007) that erode any genetic signature of fine scale female natal homing. However, parentage assignments are proving useful at detecting recruitment and dispersal and characterizing marine population connectivity in cases where gene flow is too high to detect structure using traditional approaches (e.g., Planes et al. 2009). Preliminary parentage analysis of the approximately 1000 nesting females genotyped from Georgia suggests the presence of approximately 20 mother-daughter pairs (Shamblin unpublished), with additional detection expected with a substantial increase in individuals genotyped. We will use spatial data from nests of inferred mother-daughter pairs to determine whether any additional structure occurs within the Northern Recovery Unit, and if so, at what geographic scale.

Project Narrative

During the 2010-2012 nesting seasons, a single viable egg will be obtained from each nest during clutch validation by project collaborators on all surveyed beaches in North Carolina, South Carolina, and Georgia. We will extract DNA based on methods described in Shamblin et al. (in prep) that have proven capable of generating high quality maternal DNA. We will attempt to genotype each sample across 17 microsatellite loci isolated from loggerhead turtles (Shamblin et al. 2007, Shamblin et al. 2009). The non-exclusion probability of identity of the combined microsatellite panel based on individual females genotyped to date is 6.99×10^{-31}. Based on these results, we are confident that we can identify unique genotypes (genetic tags) for all individual nesting females in the Northern Recovery Unit. Nests will be matched for unseen females and assigned to tagged females where skin samples are available. Intra-seasonal clutch frequency will be estimated based on the number of nest matches. Nest site fidelity data (extent of beach utilized, average distance between nests, sequential distance and directionality between each nest) will be calculated by treating each nest as a route event along the coast. The most likely mother of each nesting female will be inferred using program CERVUS, and confidence in each pair of assignments will be calculated using novel methods specifically designed for large datasets with no pedigree information (Christie 2009).

Benefits or Results Expected

Given the geographic coverage and intensive nature of sampling, the proposed research should provide the most robust parameter estimates available for vital reproductive parameters such as intra-seasonal clutch frequency, remigration intervals, and nest site fidelity within and between nesting seasons for the genetically distinct Northern Recovery Unit. Fecundity data are critical inputs to demographic models and important for translating nest counts into estimates of nesting female numbers. As the proposed research represents nearly complete coverage of Northern Recovery Unit nesting beaches (less unmonitored areas in South Carolina) it offers an unprecedented opportunity to directly estimate nesting female population size and clutch frequency. Project success will be evaluated on the basis of acquisition of high quality genotypes for > 90% of sampled nests.

Federal, State and Local Government Activities

The proposed research is an extension of ongoing section 6 funded research. This proposal would extend egg genotyping in Georgia by two years (2011-2012) and expand sampling coverage into South Carolina and North Carolina for all three years. The project has genetically tagged approximately 1,000 nesting females to date with analysis of clutch frequency, nest site fidelity, and remigration intervals ongoing. The genetic mark-recapture technique has proven robust, with ten of thirteen 2004-2005 satellite tagged turtles detected during remigrations and forty-four non-invasively inferred females from the 2006 egg pilot study on Ossabaw and St. Catherines detected nesting during the 2008 and 2009 seasons.

Environmental Impacts

The proposed research will require lethal take of a single egg per nest. Several attempts at non-destructive means of DNA extraction have been explored, and none have proven capable of producing maternal DNA of sufficient quality and quantity to consistently amplify genotypes. The take of a single viable egg per nest will not influence overall reproductive success or hinder population recovery.

Project Management

The principal investigators of the project are Mark Dodd (Georgia DNR), DuBose Griffin (South Carolina DNR), and Matthew Godfrey (North Carolina WRC). These individuals oversee sea turtle research and management activities for their respective state agencies. Dr. Joe Nairn, University of Georgia, will serve as a cooperating researcher. Dr. Nairn specializes in wildlife genetics in the Warnell School of Forest Resources. Dr. Nairns's students have examined population structure in loggerhead sea turtle populations in the southeastern U.S. and have developed 17 unique microsatellite markers for individual genotyping in loggerheads.

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