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RESEARCH NOTE

Sea turtle hatchling carapace as a source of high quantity and quality DNA

Uso del caparazón de crías de tortugas marinas como fuente de alta cantidad y calidad de ADN

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Abstract.- Hatchling DNA provides valuable information on sea turtles. Samples can be obtained from dead hatchlings or embryo, or, when live animal samples are needed, from blood, flipper or carapace. We compared 120 DNA extractions from flipper and carapace tissue of dead and live hatchlings. There were significant differences in DNA yields from the different tissues, but no significant differences in DNA purity. Some flipper samples yielded low amounts of DNA, while the carapace tissue consistently produced high yields of good quality DNA. This suggests that carapace represents the best option for tissue sampling of hatchling sea turtles in genetic research.

Key words: Carapace, DNA extraction, sea turtle

INTRODUCTION

Genetics research has provided new perspectives on the biology and evolution of sea turtles (Karl & Bowen 1999, Avise 2007, Campbell & Godfrey 2010). For example, it has improved sea turtle taxonomy and systematics (Avise *et al.* 1992, Bowen *et al.* 1996), provided an evaluation of nesting female philopatry (Bowen *et al.* 1992, Bowen & Avise 1996, Plotkin 2003), increased understanding of population structure and juvenile origins (Bowen *et al.* 1995, Dutton *et al.* 2007), and uncovered multiple paternity (Kichler *et al.* 1999, Jensen *et al.* 2006, Joseph & Shaw 2010). Such studies have led to improvements in sea turtle conservation and management techniques, such as the definition of regional management units (Wallace *et al.* 2010).

The starting point for any genetic study is tissue sampling for DNA extraction, and the success of the research depends on the quantity and quality of the DNA that can be obtained. Where hatchlings have been used for sea turtle genetic research (*e.g.*, in multiple paternity studies), DNA has been obtained from various source materials: dead hatchlings or embryos (Kichler *et al.* 1999, Bagda *et al.* 2012), blood samples (Fitzsimmons 1998, Hoekert *et al.* 2002, Jensen *et al.* 2006, Zbinden *et al.* 2007, Sakaoka *et al.* 2011), a small skin snip from the trailing edge of the flippers (Stewart & Dutton 2011, Ekanayake *et al.* 2013), and carapace samples (Moore & Ball 2002, Theissinger *et al.* 2009, Phillips *et al.* 2013, Wright *et al.* 2013). Blood sampling is the most invasive of these approaches, since it requires a high level of technical skill if injury to the individual turtles is to be prevented (Fitzsimmons *et al.* 1999). Various techniques for obtaining blood from sea turtles have been described (Dozy *et al.* 1964, Berkson 1966, Owens & Ruiz 1980, Bulté *et al.* 2006), but some are potentially harmful to the turtle and are not recommended (Dutton 1996), and all of them could cause internal damage to the veins, blood vessels or the dorsal spine as a consequence of an incorrect needle insertion.

Such difficulties could be avoided by using less invasive sampling techniques, such as the removal of a small piece of tissue from the flippers or carapace of hatchlings. These are considered to be easy and low-cost sampling techniques that can readily increase the scale of sea turtle genetic studies (Jensen *et al.* 2013). Both sampling techniques can be performed with a biopsy punch or a scalpel blade, although the biopsy punch is preferred, given that the size of the sample can be controlled to minimize the incision, and the procedure is safer for both the hatchling and the investigator.

The use of dead hatchlings or embryos is a good option when only limited numbers of samples are required, but suffers from the disadvantage that tissues rapidly decompose, and therefore the integrity of any DNA extracted will depend on the nature and freshness of the tissue when sourced (Dutton 1996). Thus, using dead hatchling tissue could reduce the number of samples that are of sufficient quality for molecular analysis and risks compromising the strength of the statistical analysis.

The aim of this study was to evaluate the quality and quantity of the DNA extracted from those tissues that can be collected by techniques that are less invasive than blood sampling, *i.e.*, carapace, skin and muscle.

Materials and methods

Samples from hawksbill turtle *Eretmochelys imbricata* (Linnaeus, 1766), hatchlings were taken during the nesting season at El Cuyo beach in the Yucatan Peninsula, Mexico. Nests were monitored daily after 55 days of incubation, which is the average incubation period for a hawksbill clutch in the region (Chim-Vera 2009). Once the hatchlings were gone, after an average incubation period of 63 (± 1.43) days, we opened the nests to collect live lagging hatchlings and any dead animals. For live animals, samples were taken from the edge of the marginal scute near the supracaudal scute, or from the edge of the left flipper. In both cases we first cleaned the tissue with betadine and used a 3 mm biopsy punch to take the sample (Fig. 1). Carapace samples were approximately half of the biopsy punch circumference, while for the flippers we took only a very small piece of tissue so that hatchling viability was not affected. For dead hatchling samples, we used a scalpel to obtain a piece of muscle from their rear flippers or a 3 mm biopsy punch in the case of the carapace. All samples were preserved in a 70% ethanol solution, and stored at -20°C according to standard protocols (Dutton 1996, Fitzsimmons *et al.* 1999).

DNAs were extracted from 30 samples of each type of tissue using the DNeasy Blood and Tissue DNA extraction kit (QIAGEN 2006) according to the Animal Tissue Protocol with a 2.5 h lysis period and a final buffer (AE) elution of 100 µl. To determine DNA concentration (ng μl^{-1}) and quality (absorbance $_{260/280}$) we used a Nanodrop 2000c spectrophotometer (Thermo Scientific 2009). Finally, all samples were tested for consistent amplification by PCR of the microsatellite Eim31 target region (Miro-Herrans *et al.* 2008). PCR amplification was assessed by electrophoresis through 3% agarose gels followed by photographic documentation.

Results and discussion

There were significant differences in the yield of DNA from the various tissues sampled (H= 64.71, P< 0.0001), with live hatchling skin tissue giving the lowest quantity of DNA (an average concentration of 8.7 ± 3.11 ng μl^{-1}). For dead hatchling flipper, the average concentration was 29.96 (± 22.07) ng μl^{-1}, which was similar to the average DNA concentration from dead hatchling carapace, *i.e.*, 30.35 (± 15.23) ng μl^{-1}. The highest DNA yield was obtained from live hatchling carapace, with an average concentration of 53.88 (± 23.50) ng μl^{-1} (Fig. 2).

The expected target region of 314-342 bp for the microsatellite Eim31 was amplified by PCR from all carapace samples, except where DNA concentrations were less than 5 ng μl^{-1} (Fig. 3). This suggests that, for the protocol used here, a DNA concentration of 5 ng μl^{-1} represents the minimum required for successful PCR. The dead and live hatchling flipper tissue yielded DNA concentrations of at least 0.50 and 3.60 ng μl^{-1}, respectively. The lower yields from the dead tissue could be due to degradation through natural decomposition and may be influenced by environmental conditions (humidity, temperature, etc.) (Dutton 1996), given the variation in nest locations on the beach profile. In the case of live tissue, the sample size is very

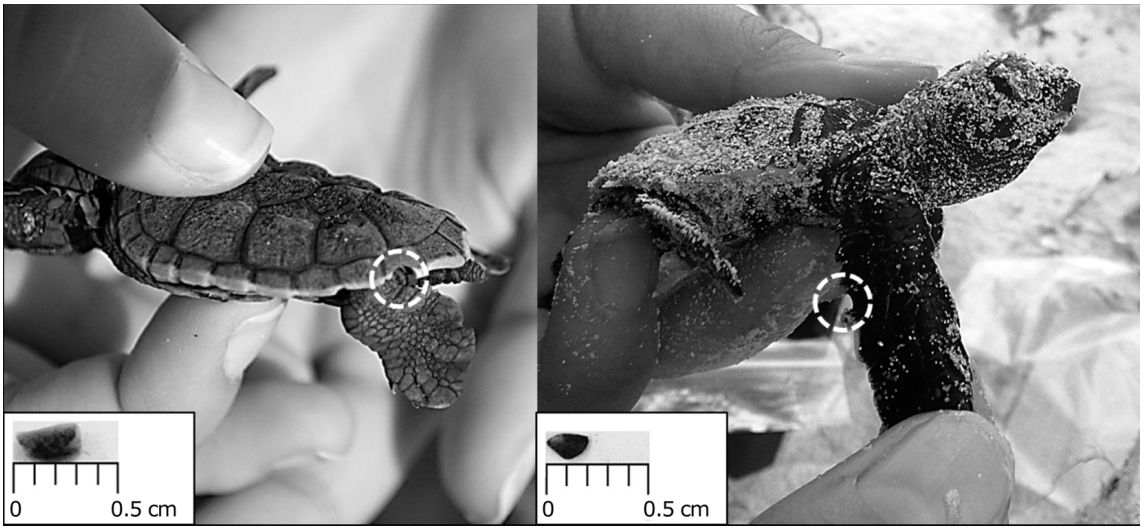


Figure 1. Live hatchling with a marginal scute (left) and a front flipper (right) notch after taking a tissue sample using a 3 mm biopsy punch / Cría viva con una muesca en el escudo marginal (izquierda) y en la aleta frontal (derecha) después de haber tomado una muestra de tejido usando un sacabocados para biopsia de 3 mm

small (range: 0.40-1.30 mg; Fig. 2), which sets an upper limit on the amount of DNA that can be obtained.

For carapace samples from both live and dead animals, the minimum amount of DNA obtained was significantly higher than for flipper tissues. Nevertheless, dead hatchling carapace yielded less DNA than that of live animals, suggesting that dead hatchling carapace is also affected by natural decomposition processes. However, because the hard carapace tissue (bone) decomposes more slowly than soft tissues such as skin and muscle, it represents a better option when sampling dead hatchlings.

There was no significant difference in DNA purity between the different types of sample (H= 6.80, P= 0.0779), but DNA from the carapace samples showed less variable absorbance $_{260/280}$ ($Abs_{260/280}$) values (Fig. 2). DNA with an $Abs_{260/280}$ ratio of ~1.8 is generally accepted as sufficiently pure for molecular analysis. While higher values do not indicate problems with the sample, if the ratio is appreciably lower, either protein or other residues may be present, or the nucleic acid concentration could be very low (<10 ng μl^{-1}) (Thermo Scientific 2009). The $Abs_{260/280}$ ratio was usually higher than 1.8, demonstrating excellent DNA purity in all samples.

Given that carapace samples from both dead and live hatchlings provide good yields of DNA, and that the DNA concentration obtained is considerably higher than that from live hatchling flipper tissue, the use of carapace as a DNA source offers several advantages: (1) it does not suffer the soft tissue degradation found in dead sea turtles, which may compromise DNA integrity; (2) high yields of DNA can be guaranteed; (3) minimal training of personnel is required. In addition, the simplicity of the technique means it is ideal for obtaining and preserving samples in remote areas.

Another advantage of carapace sampling is that the cut made may be useful as a permanent tag: the notching of a marginal scute or combination of scutes can be used to identify year-classes of hatchlings, although large number of hatchlings need to be sampled (Balazs 1999) given the high mortality in the first stages of the sea turtle life cycle (Spotila 2004). In Africa, marginal scutes are cut in a particular pattern as a tagging system; this tagging method helps to reveal when turtles reach sexual maturity, since the carapace does not regenerate and it is possible to identify turtles with a carapace 'scar' at the nesting beaches (Nel *et al.* 2011).

In conclusion, given the increasing interest in the genetics of sea turtles, there is a clear need for a sampling method that not only guarantees DNA yields of sufficiently high quantity and quality for molecular analysis, but that also limits injury to this critically endangered species. Sampling the carapace of either dead or live hatchlings results in DNA solutions of good concentration and quality and avoids the possibility of sample loss due to soft tissue degradation. Carapace sampling is simple and inexpensive, and large numbers of samples can be collected as easily as flipper tissue from live hatchlings; in addition, the relatively large size of the carapace sample results in more DNA than from flipper tissue, and allows multiple molecular analyses to be performed with the same sample. Carapace sampling should therefore facilitate collection of the more robust and accurate data required to underpin international conservation strategies for sea turtles.

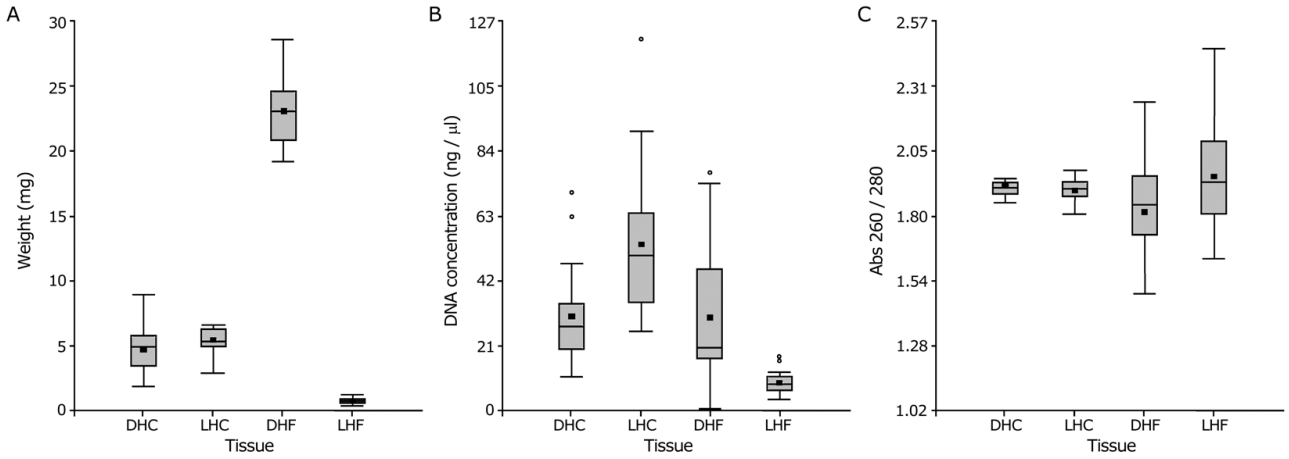
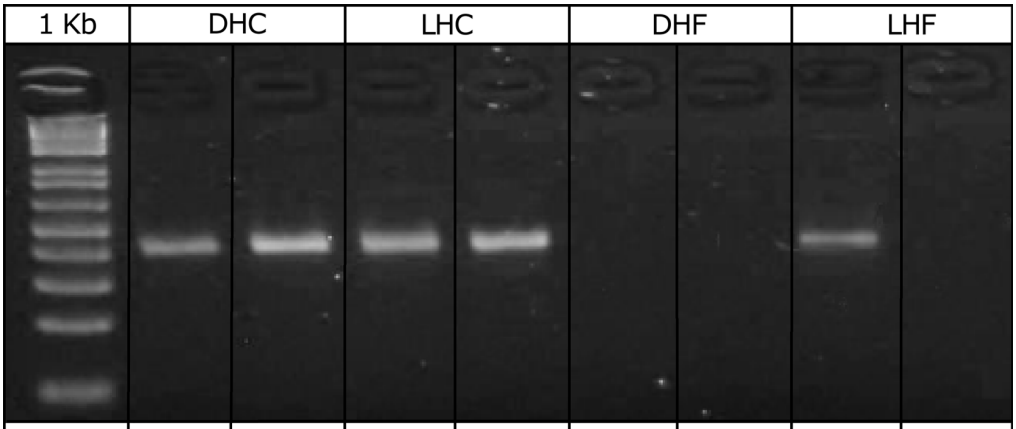


Figure 2. Sample weight (A), DNA concentration (B) and DNA purity (C) with different types of tissue. DHC: dead hatchling carapace, LHC: live hatchling carapace, DHF: dead hatchling flipper, LHF: live hatchling flipper / Peso de las muestras (A), concentración de ADN (B) y pureza (C) obtenida por diferentes tipos de tejidos. DHC: caparazón de crías muertas, LHC: caparazón de crías vivas, DHF: aleta de crías muertas, LHF: aleta de crías vivas



Weight (mg)	4.4	5.5	2.9	5.8	19.4	24.4	0.8	0.6
DNA (ng/μl)	10.9	12	25.8	28.6	1.01	2.4	5.4	3.6
ABS 260/280	2.04	1.93	1.90	1.93	1.09	1.30	1.69	1.92

Figure 3. Gel electrophoresis showing PCR amplification of microsatellite Eim31. Each sample represents the 2 different individuals with the lowest DNA concentration for each type of tissue. DHC: dead hatchling carapace, LHC: live hatchling carapace, DHF: dead hatchling flipper, LHF: live hatchling flipper / Gel de electroforesis de la amplificación por PCR del microsatélite Eim31. Cada muestra representa 2 individuos diferentes con las menores concentraciones de ADN obtenidas por cada tipo de tejido. DHC: caparazón de crías muertas, LHC: caparazón de crías vivas, DHF: aleta de crías muertas, LHF: aleta de crías vivas

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