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Estimating the fertilization rate of sea turtle nests: comparison of two techniques

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Egg fertilization rate is a defining event in the life history of oviparous species. However, for many terrestrial oviparous species, this parameter is only estimated after hatching and emergence of hatchlings, by studying evidence of embryonic development in unhatched eggs. We compared the accuracy of this method with a second technique based on the careful excavation of sea turtle nests within the first 96 hours of oviposition and calculating the proportion of alive eggs, where a clear white spot is externally visible in the shell. Both methods underestimate the fertility rate but we are not aware of any other alternative on-invasive technique. The study was conducted in a nesting population of loggerhead turtles (*Caretta caretta*) at Boa Vista Island (Republic of Cabo Verde). We found significant differences in the estimation of fertilization rate between both techniques. When inspecting eggs after hatching, researchers significantly overestimated the number of unfertilized eggs and this calculation was not correlated with the estimation based on examination of eggs soon after oviposition. The first estimate was not correlated with hatching success or hatchling phenotype. There was no relationship between the number of viable embryos at oviposition and the hatching success and the hatchling's phenotype. The absence of embryo or its early death had no effect on survival of developing embryos within a nest. Fertilization rate in loggerheads did not show spatial or temporal variation during nesting season. We suggest the implementation in sea turtle nesting monitoring programs of annual less-invasive estimation of egg fertilization rate based on the excavation of a sample of representative nests immediately after the formation of the white spot in the shell of developing embryos.

***Key words*:** embryonic development; fertilization rate; loggerhead turtle; reproduction.

Egg fertility is a defining event in the life history of oviparous species (Schultz & Williams, 2005; Hamdoun & Epel, 2007) and a relevant factor for risk assessment and the design of conservation programs (Hamann *et al.*, 2010; Wallace *et al.*, 2011). For example, the final number of offspring and, thus, the reproduction success of the organism are dependent on the percentage of ova that are fertilized and commence development. However, there are many biotic and abiotic factors that affect hatching success by causing embryo mortality during the incubation on the beach (Patino-Martinez *et al.*, 2014; Marco *et al.*, 2015; Sarmiento-Ramírez *et al.*, 2014; De Andrés *et al.*, 2016). Even, the percentage of unfertile eggs may negatively affect development of viable eggs within the nest, because they usually decompose and are

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colonised by microorganisms that may invade the viable eggs and eventually affect their hatching success (BLANCK & SAWYER, 1981; PHILLOTT & PARMENTER, 2001; SARMIENTO-RAMÍREZ *et al.*, 2010,2014). In this scenario, a logical prediction would be the existence of a positive relationship between egg fertilization rate and hatching success.

Furthermore, the proportion of fertilized eggs of a clutch is a good estimator of male abundance and fertility as ROWE *et al.* (2004) observed in freshwater turtles, of human female stress during mating (CAMPAGNE, 2006) or of physiological alterations of the sperm or ova, as detected by RIE *et al.* (2005) in marine fishes. These factors may have a subsequent impact on hatching success. Several studies suggest that human induced environmental impacts could alter male abundance and fertility in several chelonian species (BERGERON *et al.*, 1994; JANZEN, 1994; HAWKES *et al.*, 2007; DE ANDRÉS *et al.*, 2016). For example, environmental pollution can cause endocrine disruption in marine vertebrates. Exposure to contaminants could change the production of hormones such as estradiol or vitellogenin in females affecting egg production (RIE *et al.*, 2005), and can also alter hormone production, sperm quality and activity in males (VAN LOOK & KIME, 2003; FERBER, 2005; DE ANDRÉS *et al.*, 2016). Moreover, the male population of sea turtles could be severely threatened by problems like male selective fishing activities and alteration of sex ratio in hatchlings due to global warming (PATINO-MARTINEZ *et al*, 2012; ABELLA-PÉREZ *et al.*, 2016). For instance, all sea turtle species have been classified as endangered across their range, and in spite of being an illegal practice, sea turtle meat consumption is a deeply-rooted traditional activity in most nesting areas of the world (KLEMENS *et al.*, 2000; LUTZ *et al.*, 2003). Although capture of females probably has a stronger impact on population dynamics and they are likely to be captured more often than males because they are more accessible during nesting and have more fat for consumption (SMITH & BIRD, 2000), there is an inherited tradition that contemplates the consumption of loggerhead male penis liquor as an aphrodisiac beverage, enforcing a male selective fishing activity (ALVES *et al.*, 2008; MARTINS *et al.*, 2015). This long-term practice could have an important impact in the number of adult males and consequently, in egg fertilization.

Additionally, during the last decades, climate change has caused a worldwide significant increase of air and seawater temperatures, affecting animals and plants (WALTHER *et al.*, 2002; PARMESAN & YOHE, 2003), and models suggest that temperatures will continue increasing during the 21st century. In sea turtles, incubation temperature defines the proportion of males and females that are annually produced on nesting areas (YNTEMA & MROSOVSKY, 1982). For *Caretta caretta*, 29ºC is the pivotal temperature, in which 50% females and 50% males are produced (MROSOVSKY & YNTEMA, 1980; WIBBELS, 2003). At higher temperatures, hatchling sex ratio is skewed toward females and 100% female hatchlings are produced over 32ºC (WIBBELS, 2003). A strong female biased sex ratio has already been documented in many loggerhead nesting areas (GODLEY *et al.*, 2001; WIBBELS, 2003). Increasing tem-

peratures in the last decades could have diminished the production of male hatchlings, affecting the potential for egg fertilization (GLEN & MROSOVSKY, 2004).

For all these reasons, it is very important to estimate adult male abundance and its sexual health. However, since males are migratory and stay in the ocean during their life, it is difficult to estimate their population number without in-water studies. In this context, the estimation of egg fertilization rate could be an indirect assessment of the status of adult males. This is another relevant reason to implement the evaluation of fertility rate in endangered turtles monitoring programs (GLEN & MROSOVSKY, 2004).

Regarding how to directly assess the egg fertility rate there are severe difficulties, mainly because it is highly invasive since fertilization is an intrauterine process when females are in the sea (MILLER, 1985). Ova fertilization in terrestrial oviparous species is usually internal and occurs within the first 72 hours after oviposition. In these species, embryonic development starts in the female oviduct and eggs are laid when embryos are several days old (MILLER, 1985). At egg laying, some fertilized eggs could have already died. Moreover, fertile eggs at laying have no external signals of embryonic development and egg dissection is necessary to record the fertilization, but this is a very invasive technique, especially for endangered species.

For all these reasons, and due to the additional difficulty to estimate adult male abundance, and male and female sexual health, the indirect estimation of the egg fertilization rate is very important and should be implemented in monitoring programs (GLEN & MROSOVSKY, 2004). An accurate estimation of the egg fertilization rate can also permit a better estimation of embryo mortality during incubation.

For many terrestrial oviparous species, this parameter is only estimated after hatching, studying evidences of embryonic development in unhatched eggs. However, in that moment, dead eggs are decomposing and it is usually difficult to discriminate between unfertile eggs and eggs whose embryos died early in their development. Thus, this estimation of fertilization rate can be very inaccurate and underestimate the real value.

We propose in the present study a less invasive alternative method to estimate egg fertility short time after oviposition.

For many oviparous reptiles, embryonic development can be detected externally after the first 24 hours of incubation by observing the appearance of a white spot in the eggshell. With this technique, we are estimating together the fertility rate and the early embryo death (between fertilization and the estimation of the white spot) and it is not possible to discriminate the contribution of each factor. Thus, this technique would also underestimate the real value of fertility as the other technique does. However, early embryo mortality is likely very low because the available time is very short, the egg is most of the time fully protected within the oviduct and the embryo is so small with very low requirements. Anyway, it is highly probable than variability in early embryo mortality is also very low. For these reasons, we consider than the white spot technique can be a better estimator of the fertilization rate,

and most of the variability found on this parameter is due to the fertility rate. We have estimated the egg fertilization rate using two non-invasive techniques in an endangered and important loggerhead turtle population from Cabo Verde (MARCO *et al.*, 2011,2012), with three objectives: 1) to compare the efficiency of the two techniques in order to recommend the most appropriated for sea turtle nesting monitoring programs; 2) to estimate the rate of egg fertilization in the endangered studied population and its temporal and spatial variation; 3) to test the hypothesis that the percentage of unfertile eggs can affect the survival of the eggs with developing embryos within a nest.

MATERIALS AND METHODS

Field studies were carried out in the southeastern beaches of Boa Vista Island, Republic of Cabo Verde (16.18°N, 22.92° W). We estimated the rate of egg fertility using for each nest two non-invasive techniques: 1 – estimating the percentage of eggs with developing embryos between 24 to 72 hours after egg laying using the white spot technique (BLANCK & SAWYER, 1981); and 2 – determining the percentage of dead eggs with no sign of embryonic development after hatching. These estimates were correlated for each nest and also with hatching success, the latter calculated by dividing the number of hatched turtles by the initial number of laid eggs.

In order to select the best moment to estimate fertility early in the incubation through the white spot method, we made an initial evaluation using a small sample of eggs at a time when we could assess the existence of a developing embryo. From 20th June to 2nd July 2005, we studied 60 eggs from eight nests (five eggs from four nests and 10 eggs from the remaining four nests) to assess the fertility estimation using the white spot embryo development method. We kept the eggs out from the nests in plastic containers with natural sand from the beach at environmental temperature of 26-30ºC. All eggs were carefully examined every two hours using surgical globes and moving the eggs very slowly and avoiding any turning or vibration in order to determine the presence and size of the white spot. Eggs were also candled to expose the extent of the vascularized area of the yolk sac (YS) and chorioallantoic membrane (CAM) and their precursor, the area vasculosa (AV) of gastrulae and neurulae. The moment for the detection of the developing embryo for further studies was selected when the number of experimental eggs where embryonic development was detected did not increase after that time. Egg manipulation by inexperienced staff can cause severe movements, which in turn will induce mortality of eggs if they occur between about 12 hours and 14 days after the eggs are laid (LIMPUS *et al.*, 1979), and should not be used in monitoring programs. To estimate the fertilization rate, all eggs of 38 complete nests laid from 6th August to 9th September 2005 were examined after hatching, and 29 of them were also examined early in the development. All nests were marked during egg laying. The eggs were translocated to a beach hatchery between the first 24 and 96 hours after laying and incubated under standard conditions (for details see ABELLA *et al.*, 2007). The hatchery was located on the beach of

Benginho at less than 100 m apart from the beaches of origin of the studied nests. Predation or inundation, the main threats to eggs, was avoided. To estimate fertilization rate early in the development, at the beginning of translocation, nests were excavated carefully and eggs were extracted conserving their original axial position, avoiding rolling or shocking movements (LIMPUS *et al.*, 1979; PARMENTER, 1980). The eggs were classified as fertile if they presented the white spot (BLANCK & SAWYER, 1981, MILLER 1985). Those eggs with a healthy aspect (eggshell turgid, and spherical, with an uniform whitish colour, without coloured spots), and no evidence of the white spot were further examined with a flashlight in order to identify if any signs of development existed; if they did not appear, they were considered unfertile (ABELLA *et al.*, 2007). After the egg examination, the clutch was reburied conserving the original oviposition order. All process was carried out in the minimum possible time (maximum one hour). The careful extraction and relocation of eggs from nests to check for fertilization during the first 96 hours of incubation has no impact on embryonic development (ABELLA *et al.*, 2007). In order to evaluate hatching success, on incubation day 45, a round plastic net was placed around every nest to keep the hatchlings inside, making it possible to count and measure them. After setting the net, all nests were checked every two hours during the night when emergence occurs. Hatchlings were never more than two hours restrained into the net before release. To weight the hatchlings we used a PK-401 (Max. 400g; d = 0.1g) balance, and to measure the Straight Carapace Length (SCL) and the Straight Carapace Width (ACR) a Mitutoyo Absolute Digimatic (0.05 mm) digital caliper.

To estimate the fertilization rate and the hatching success of the nest after hatchling emergence, each nest was exhumed five days after the last hatchling was observed emerging. Nest contents were classified as shells of hatched eggs, dead emerging hatchlings, dead pipped hatchlings, live emerged hatchlings, live pipped hatchlings, eggs without macroscopic signs of embryonic development, and dead embryos. Eggs classified as "without embryonic development" did not present visible signs of development, however, it was not possible to certify if they were fertile or not because of the decomposition stage of the egg contents in all cases. To estimate egg fertilization rate from the exhumation method, we divided the number of dead eggs without signals of embryonic development by the clutch size for nests that were not predated during the experiment.

Given that hatchlings can accidentally escape from enclosures, hatching success was estimated taking into account for every clutch the highest of the two parameters: number of empty eggshells during exhumation or number of detected emerged hatchlings. The incubation period was defined as the number of days between egg-laying and the time at which the majority of hatchlings emerged from the nest; for 96% of nests this was also when the first hatchling emergence was recorded. Fertility rates were normalized using arcsine transformation prior to statistical testing.

To test whether the technique of early

Table 1: Percentage of loggerhead eggs where embryonic development can be recognized from two techniques: nest exhumation and examination for white spot during the 96 hours of incubation.

Method	Mean	Standard Deviation	Minimum	Maximum	N
Exhumation	82.29	10.59	58.23	100.00	29
White spot	93.76	7.39	75.00	100.00	29

estimation had an impact on hatching success and hatchling phenotype, we compared the nests that were used for the estimation of egg fertilization rate with nests incubated under the same standard conditions on a beach hatchery but that were translocated immediately after egg laying (published in ABELLA *et al.*, 2007).

RESULTS

The detection of a developing embryo with the candling method was successful in all viable eggs after the first 24 hours. The detection of the white spot was successful in all viable eggs after 36 hours and for most of the viable eggs after 38 hours.

Significant differences were found when comparing the two estimates of egg fertilization for each nest (Paired T-test: t = -4.7232; $P < 0.0001$; N = 29; Table 1; Fig. 1). Furthermore, values obtained with both methods were not correlated ($r^2 = 0.0007$; r = -0.026; $P = 0.8930$). Values found using the white spot technique to estimate fertility

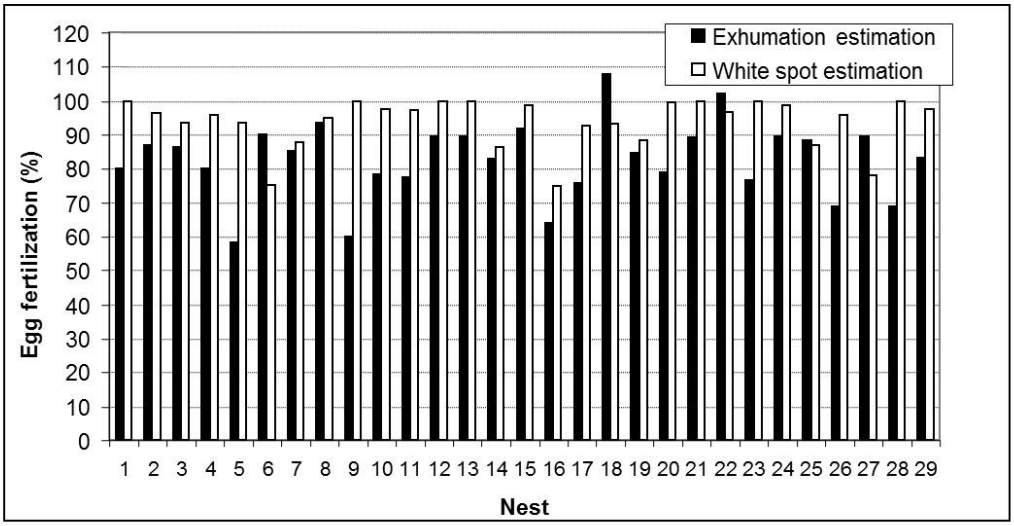

Figure 1: Egg fertilization (%) found in 29 nests of *Caretta caretta* measured with two methods: exhumation estimation and white spot presence assessment on August 2005, Boa Vista Island (Republic of Cabo Verde).

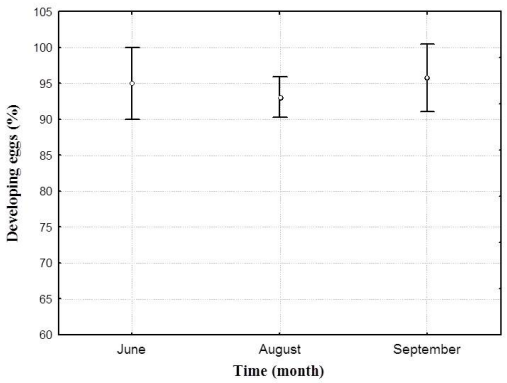

Figure 2: Seasonal variation of the number of fertile eggs in nests of *Caretta caretta* laid in June, August and September 2005 nesting season in Boa Vista Island (Republic of Cabo Verde).

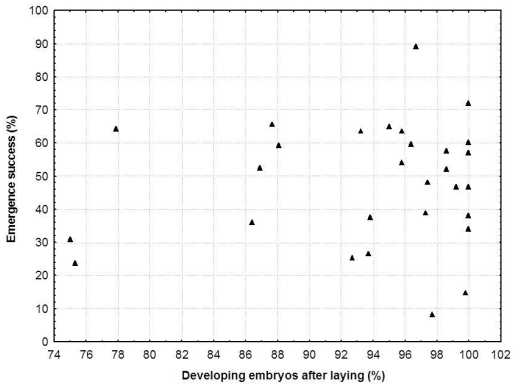

Figure 3: Relationship between the estimate of egg fertility (%) and emergence success (%) resulted of 29 nests of *Caretta caretta*, laid in August 2005 on Boa Vista (Republic of Cabo Verde).

were significantly higher than those estimated with the exhumation method (Table 1).

The best estimation of egg fertilization rate for the species during the 2005 nesting season, exceeded on average 94% fertile (95% confidence limits: 91.9 and 96.2%; N = 29 nests).

The minimum value found in a nest was 75%, but in 11.5% of nests, all eggs were fertile. We did not detect a temporal variation in the number of developing embryos within the season ($F_{2,26}$ = 0.583; *P* = 0.563; Fig. 2) or spatial variation when comparing nests from different beaches ($F_{2,26}$ = 0.532; *P* = 0.592).

Female body size (r = 0.054; *P* = 0.760; N = 29) and clutch size (r = -0.094; *P* = 0.549) were not related to estimates of egg fertilization rate. The mean hatching success of 50 clutches incubated in a hatchery was 52.37% and varied from 0 to 94.57%. When we compare the percentage of alive embryos at oviposition with the hatching success (in the 29 clutches where both parameters were measured) we did not detected a significant correlation (r = 0.097; t = 0.504; *P* = 0.618; N = 29; Fig. 3).

Discussion

We estimated a high egg fertilization rate in the Cape Verdean loggerhead population during the whole 2005 nesting season, that would be even higher if we could exclude early embryo mortality from the estimate. The mean value was higher than 94% and there was variability among nests (75% - 100%). Our results are similar to those found in other loggerhead populations (Miller, 1985; Miller & Limpus, 2003), confirming a high egg fertilization rate for the species. We deduce that current egg fertilization in adult Cape Verdean loggerhead females appears not to be threatened. Nevertheless, in terms of egg fertilization, we have no reasons to think that male production and abundance are not threatened and is enough to guarantee

an optimal reproductive success. Adult males that are now mating with nesting females have hatched several decades ago. Hypothetical current bias on male production (ABELLA-PÉREZ *et al.*, 2016) can have negative effects in the next decades.

It is important to consider that sperm storage occurs in loggerhead sea turtle females (HARRY & BRISCOE, 1988). This strategy can assure egg fertilization in case of possible future lack of males for mating (FITZSIMMONS, 1998). Studies about mating systems (sperm storage and multiple paternity of nests) would be necessary in order to estimate the number of effective males in the population, to know their genetic diversity, its biological consequences, and to complete this information (SANZ *et al.*, 2007). There is also no evidence of alterations in fertility of breeding sea turtles of both sexes due to environmental pollution (VAN LOOK & KIME, 2003; FERBER, 2005; RIE *et al.*, 2005).

Both tested methods underestimated the fertilization rate; the exhumation estimation method because from internal fertilization to the examination date early in the incubation, some embryos could die (MILLER, 1985; MILLER & LIMPUS, 2003); and the white spot presence method because some eggs classified as "undeveloped" could had been fertile but embryos might have died very early during incubation. These small embryos could not be identified due to factors as embryonic decomposition, insect infections, extremely egg dryness that hide the presence of an embryo. Data from exhumation also resulted in a rougher approach, because some eggs could have been predated before exhumation. Also, with the exhumation method, mistakes are more likely to occur like with the number of shells found inside the nest chamber; sometimes the number of shells found was higher than the eggs counted during laying (fertility rate exceeded 100% value), so possibly shells of an old nest were considered, or a half shell could be counted as a whole one. Nevertheless, the exhumation method is a widely used technique (BLANCK & SAWYER, 1981; BELL *et al.*, 2003), but it is undoubtedly much less precise and exact than the white spot estimation, and we do not recommend it to estimate egg fertility in management programs. BELL *et al.* (2003) also found in *Dermochelys coriacea* that the exhumation method has very low accuracy in comparison with egg dissection in the laboratory. However, egg dissection is an invasive technique that kills the embryos and requires some degree of sophistication and training. On the contrary, the white spot embryo development method is a simple, useful and non-invasive simple technique that permits measuring fertility accurately in the field, and it does not require egg dissection, and as a consequence, embryo sacrifice. This method can be applied with a very short training and with a very low probability of having mistakes. The evaluation of egg fertilization rate early in the development has no effect on subsequent embryo mortality or hatchling phenotype, and can be efficiently used during delayed translocations of doomed nests to safe beach areas or hatcheries (ABELLA *et al.*, 2007), gathering further information about breeding success and improving the efficiency of nesting monitoring programs. Anyway, many precautions have to be taken with these types of relocations to

avoid damage of the embryos (LIMPUS *et al.*, 1979; WYNEKEN *et al.*, 1988), and should only be implemented when hatching success on the beach is very low, and the efficiency of the relocation has proved to be very successful in the rookery. A periodical monitoring of egg fertilization in the populations would be essential to detect problems in egg fertilization at the time that could permit taking measures to prevent, solve, mitigate or compensate their critical costs on sea turtle population structure. These measures should be implemented in integrated strategies for the conservation of sea turtles, and their results should be periodically evaluated. Egg fertilization monitoring and conservation actions if needed, would be especially important in conservation programs that manage highly threatened populations with few individuals. The final goal of these actions should be the increase of hatchling production.

We did not found correlation between the estimated egg fertility and the emergence success. This lack of correlation could be caused by the huge variability of hatching success. Unfertilized eggs in the nest may experience decomposition and fungus growth that could spread invading viable eggs (BLANCK & SAWYER, 1981; PHILLOTT & PARMENTER, 2001). These authors suggest that nests with higher number of unfertile eggs may favour higher rates of fungus colonization and, thus, possibly lower hatching success. However, the lack of correlation between rate of early developing embryos and hatching success suggests that unfertile eggs are not having a significant impact on the survival of viable eggs in this loggerhead population. In this case, the removal of unfertile eggs early in the incubation would have no benefit for the population. Still, in general the white spot technique can permit the removal of unfertile eggs in species or populations, where they may significantly affect the development or survival of the viable eggs within the same nest.

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