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Low incidence of twinning in the loggerhead sea turtle

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Abstract. Twinning has been reported to be much rarer in sea turtles than in freshwater turtles. However, data from sea turtles were inferred from unhatched eggs only. We hypothesized that the difference in twinning events among turtle taxa resulted primarily from the methods used for recording data. In this study, data have been recorded from 727 clutches of eggs of loggerhead sea turtles *Caretta caretta* laid between 2002 and 2009 at the Pelagie Islands, Italy (n = 36 clutches) and at Dalaman Beach, Turkey (n = 691 clutches). Twin embryos were found in seven out of 12160 unhatched eggs (twinning rate per unhatched egg: 0.058), but not a single case of hatched or emerged twins was recorded in 3571 eggs. The twinning rate recorded, based on post-hoc procedures, did not underestimate the occurrence of the phenomenon: the odds of obtaining a pair of twins in unhatched eggs were as large (0.92 times) as the odds of obtaining a pair of twins in the total eggs. Compared with other loggerhead nesting sites, and other sea turtle species, no statistical differences were found. However, twinning in *C. caretta* was found to be 6.9 times rarer than in freshwater turtle species.

Keywords: embryos, hatchlings, eggs, nesting success, *Caretta caretta*

Introduction

Twinning in reptiles is a rare but widely known phenomenon that includes both the development of separate individuals of equal or unequal size, as well as different degrees of the malformation known as axial bifurcation. Twinning has been reported in turtles (Table 1), tortoises (Crooks & Smith 1958, Obst 1976, Tucker & Funk 1976, Cohen 1986, Heimann 1993, Messinger & Patton 1995, Mähn 1996, Stumpel 2007), crocodylians (summarized in Ferguson 1985), lizards (Willis 1932, Shaw 1954, Darevsky 1966, Carpenter & Yoshida 1967, Speer & Bayless 2000, Mendyk 2007) and snakes (Kinkaid 1996, Wallach 2007). Twinning has been found in a fossil choristoderan (Buffetaut et al. 2007) as well, providing evidence that such events have occurred in reptiles since at least the Early Cretaceous period. Completely separated twins have been observed to

survive hatching in captivity and under laboratory conditions in freshwater turtles (Table 1), tortoises (Crooks & Smith 1958, Obst 1976, Heimann 1993, Messinger & Patton 1995), crocodylians (Ferguson 1985), lizards (Shaw 1954, Darevsky 1966) and snakes (Marion 1980, Kinkaid 1996). No data are available on sea turtles, except for a record by Hewavisenthi (1989) about the hatching of the larger twin of a pair found in the nest of a green turtle, *Chelonia mydas*, at post-hoc examination.

Conjoined twin embryos in sea turtles usually die before pipping (Miller 1985) in the early stages of development (Kaska & Downie 1999), and their occurrence has been revealed by the post-hoc examination of nest contents. However, there was a report of a conjoined pair of green turtles that lived in captivity for some months (Haft 1994). In freshwater turtles, more cases have been reported (Table 1),

including one pair of conjoined twins of snapping turtle *Chelydra serpentina* (Cederstrom 1931) that lived in captivity for at least three months.

It is still debated if the lower rate of twinning in sea turtles is accurate or if it is a consequence of the method used to collect data. In the majority of studies focusing on sea turtle nesting activities, the basic parameters for evaluating the success of reproductive effort, such as clutch size and hatching success, have been inferred from nest remains at post-hoc examinations (Horrocks & Scott 1991, Peters et al. 1994, Margaritoulis 2005, Kamel & Delcroix 2009). The nest is usually excavated after the emergence of hatchlings to count the number of hatched and unhatched eggs, as well as live, but not emerged, and dead hatchlings. Unhatched eggs are opened to ascertain the presence and the developmental stage of visible embryos (Miller 1985, Kaska & Downie 1999) or signs of development, such as the presence of blood traces that indicate a fertilized egg (Fowler 1979, Blanck & Sawyer 1981, Whitmore & Dutton 1985). Only those twins that died before hatching have been counted; thus, the phenomenon of twinning in sea turtles may be underestimated (Crooks & Smith 1958).

The reproductive biology of freshwater turtles was largely investigated in the laboratory through the experimental incubation of clutches of eggs obtained from fresh nests or from gravid females (Bull et al. 1982, Brooks et al. 1991, Janzen 1993). Yntema (1970) first reported quantitative information on twinning in *C. serpentina* and then investigated the incidence of separated twins in the same species (Yntema 1971). The incidence of separated and conjoined twins in *C. serpentina* and in the red-eared turtle *Trachemys scripta elegans* was also investigated by Tucker & Janzen (1997).

The goals of this paper were to estimate the incidence of twinning in the loggerhead sea turtle *Caretta caretta*, and to evaluate whether the difference in twinning rate between this species and freshwater turtles is real or is a result of bias related to methods of data collection. We hypothesized that the difference in twinning events among taxa may result primarily from the methods used for recording data. Through evaluating the authenticity of our methods, we provided the first dataset allowing the objective comparison of twinning in sea turtles with that of other reptile taxa.

Material and Methods

Data were recorded on three loggerhead nesting beaches during monitoring programmes carried out from 2002 to 2009: Conigli Beach, Lampedusa, Pelagie Islands, Italy, 35°30'47" N, 12°33'27" E, 6000

m²; Pozzolana di Ponente Beach, Linosa, Pelagie Islands, Italy, 35°51'48" N, 12°51'17" E, 1000 m²; and Dalaman Beach, Dalaman, Muğla, Turkey, that extended from 36°42'02" N, 28°41'31" E in the west to 36°40'33" N, 28°48'01" E in the east, 520000 m².

In the Pelagie Islands, each nest was identified at oviposition and, once the clutch of eggs had been laid and the female had left the beach, the nest was caged and numbered (Boulon 1999). The mesh size of the cages (2 × 2 cm) was selected to prevent hatchlings escaping. For the entire nesting season, the two beaches were monitored at least twice a day by small groups of volunteers (up to 3 people) led by trained personnel. During hatching, a field lab was set up on each of the beaches. Nests were monitored hourly day and night and hatchlings were weighed, measured and inspected for abnormalities (i.e. supernumerary scutes). This also allowed us to check for the eventuality of small dead twins still attached to the yolk sac of large living twins. Nests were excavated eight days after the last emergence of a hatchling (Giacoma & Mari 2003).

In Dalaman Beach, daily night and morning patrols were provided by three groups consisting of 2-3 people. All *in situ* nests under threat from land predators such as foxes (*Vulpes vulpes*) were screened off with a metal grid with a 9 cm mesh, placed above the nest at a depth of 20 cm from the surface above the center of the egg chamber. Nests were both marked with GPS and nest number and date written on a bamboo cane buried in the sand just behind the egg chamber. Nest data regarding clutch size, laying and relocation dates were noted individually for each nest. Eight or 10 days post first emergence of the hatchlings, nests were opened and checked. Undeveloped eggs and dead embryos were identified according to Kaska & Downie (1999).

For all three sites, we recorded the clutch size, the number of hatched and unhatched eggs and the length of the incubation period of each nest. The incubation period was calculated as the number of days from oviposition to the emergence of the first hatchling, and the number of unhatched eggs was counted during post-hoc examination. The clutch size in the Pelagie Islands subset of nests was evaluated by counting eggs at oviposition or at relocation as suggested in Miller (1999); the number of hatched eggs was obtained by subtracting the number of unhatched eggs from the clutch size. In the Dalaman Beach subset, the clutch size was calculated according to Başkale & Kaska (2005) by summing the number of unhatched eggs, the number of predated eggs and the number of hatched eggs, which was inferred by counting the

Table 1. Reports of twinning in turtles in alphabetical order of genus according to habitat. Type of twinning and stage of individual development: *Con twin emb* = conjoined twin embryos, *Con twin hatch* = conjoined twin hatchlings, *Sep twin emb* = separated twin embryos, *Sep twin hatch* = separated twin hatchlings, *Sep twins* = separated twins: one dead at embryological stage and one hatched.

Freshwater	Con twin emb	Con twin hatch	Sep twin emb	Sep twin hatch	Sep twins
<i>Chelydra</i>	Yntema 1970	Cederstrom 1931	Yntema 1970, 1971	Yntema 1971	Yntema 1970, 1971, Tucker & Janzen 1997
<i>Chrysemys</i>		Barbour 1888, Girard 1891, Blatchley 1906, Hoffman-Derickson 1927	Cunningham 1927		
<i>Emys</i>			Dehnel 1929		Moriarty 2006
<i>Glyptemys</i>				Hildebrand 1938	
<i>Malaclemmys</i>		Hildebrand 1930			
<i>Pseudemys</i>		Hildebrand 1938, Mitchell 2003			
<i>Sternotherus</i>				Lehmann 1984	
<i>Trachemys</i>	Tucker & Janzen 1997	Hildebrand 1938, Obst 1995, Tucker 1996, Tucker & Janzen 1997	Plymale et al. 1980	Tucker 1995, 1996	Tucker & Janzen 1997
<i>Trionyx</i>			Carpenter 1981		
Sea					
<i>Caretta</i>	Caldwell 1959, Fujiwara 1964, Peters et al. 1994, Peters et al. 1994		Caldwell 1959, Fujiwara 1964, Blanck & Sawyer 1981, Limpus 1985, Peters et al. 1994, Peters et al. 1994		
<i>Chelonia</i>	Fowler 1979, Kaska & Downie 1999, Diong et al. 2003		Glaesner 1924, Fowler 1979, Kaska & Downie 1999		Hewavisenthi 1989
<i>Dermochelys</i>			Deraniyagala 1930, 1932, Chan 1985, Eckert 1990		
<i>Lepidochelys</i>			Hewavisenthi 1990		

remains of broken shells of which over 50 % were still intact (Miller 1999).

Obvious twin embryos were detected through a visual inspection of the contents of every unhatched egg at post-hoc examination. Twinning in hatchlings was defined as the difference between the number of hatchlings and the number of hatched eggs (Crooks & Smith 1958). This was carried out in the Pelagic Islands subset of nests only. Hatchlings were corralled at their emergence from the nest, so that the exact number per nest was recorded. This allowed us to consider the eventuality that live twins hatched from the eggs, a possibility that was not considered in previous studies. We also checked for sets with a large living twin and a small dead twin visually examining all the hatched eggs for small dead twins. We also carefully examined the sand inside the nest in a range of 10 cm around the egg chamber and around the emerging area.

The Mann-Whitney U-test was run for two independent samples and the Kruskal-Wallis test for more than two independent samples. To deal with unbalanced tables resulting from the comparison of a rare phenomenon (twinning) with a common state (no twinning), we estimated p-values and their 95 % confidence intervals with the Monte Carlo permutation method (10000 sampled). The tests were performed with SPSS version 16.0 software. To investigate differences in the incidence of twinning we ran Fisher's Exact tests for count data with R Software (R Development Core Team 2009).

Results

A total of 727 nests of loggerhead sea turtles were monitored: in the Pelagic Islands, 21 nests oviposited at Conigli Beach and 15 nests oviposited at Pozzolana di Ponente Beach; 691 nests in Dalaman Beach.

Unhatched eggs

An estimated 58354 eggs were investigated, among which all 12160 unhatched eggs were dissected. Twinning was recorded in seven cases. Embryos for five completely separated pairs of twins were found in five different nests in Dalaman Beach (Fig. 1A, B), one pair of conjoined twins was found in Pozzolana di Ponente Beach (Fig. 1C), and one pair of separated twins was found in Conigli Beach.

All clutches except the two that were oviposited late in the nesting season, successfully completed the incubation period. The average length of incubation was significantly different among the three beaches (Kruskall-Wallis test, $df = 2$, $P < 0.001$), ranging from the shortest period in the dark volcanic sand of Pozzolana di Ponente ($n = 14$, mean = 47.6 days, $SD = 2.3$) to the longest period recorded in the white calcareous sand of Conigli Beach ($n = 20$, mean = 63.3 days, $SD = 6.2$). The analysis to investigate differences in the length of incubation among nests with and without twins was completed for the site with more nests, that is Dalaman Beach ($n = 691$, mean = 48.5 days, $SD = 4.6$). No significant difference was found (Mann-Whitney test, $U = 940$, $P = 0.081$, 95 % CI = 0.076-0.087).

Nests laid in the Pelagie Islands and at Dalaman Beach differed in clutch size (Pelagie Islands: $n = 36$, mean = 99.2 eggs per nest, $SD = 21.6$; Dalaman Beach: $n = 691$, mean = 79.3 eggs per nest, $SD = 22.6$; Mann-Whitney test, $U = 5750$, $P < 0.001$), but the range and the average clutch size were consistent with the range and the average clutch size known for the species in the Mediterranean Basin (range of clutch size means: 65.0-130.4 eggs per nest; range of clutch size individual values: 1-211 eggs per nest; Margaritoulis et al. 2003), so data were combined for the analysis. No differences were found in clutch size among nests

with and without twins (Mann-Whitney test, $U = 1839$, $n = 727$, $P = 0.221$, 95 % CI = 0.213-0.229).

The rate of twinning in unhatched eggs was not significantly different between Conigli Beach and Pozzolana di Ponente (Fisher's Exact test, $P = 1.000$), nor between the Pelagie Islands and Dalaman Beach (Fisher's Exact test, $P = 0.137$), so data were combined for the analysis. No statistical differences in the rate of twinning in dead-in-shell embryos in unhatched eggs were found when we compared data from this study with those reported in the literature for other nesting colonies of loggerhead sea turtles and for green turtles (Table 2).

Total eggs

Not a single case of hatchling twins was recorded from nests oviposited at Pelagie Islands. In fact, knowing the total number of eggs ($n = 3571$), the total number of hatchlings ($n = 2417$) and the total number of unhatched eggs ($n = 1154$), we verified that the ratio for hatchlings and eggs was 1 : 1. In addition, no dead embryos were found in hatched eggs or in the egg chambers. Twinning was recorded in 0.056 % of total eggs laid (two out of 3571 eggs). When we compared this ratio with those reported for clutches of freshwater turtles *C. serpentina* and *T. scripta elegans* (44 out of 11306 eggs altogether from Yntema 1971 and Tucker & Janzen 1997; 0.389 % twins per egg on average), we obtained a statistically significant difference (Fisher's Exact test, one-side $P < 0.001$).

Discussion

The low incidence of twinning that we found in unhatched eggs (0.058 %) was not statistically different to those reported for other nesting colonies of *C. caretta* (0.036 % from Blanck & Sawyer 1981; 0.123 % from Peters et al. 1994), and to that known for the sea turtle *C. mydas* (0.152 % from Fowler

Table 2. Results from Fisher's Exact test for the proportion of unhatched eggs with twins and unhatched eggs without twins (in brackets) for different species (Cc = *Caretta caretta*, Cm = *Chelonia mydas*) and other nesting colonies. None of the results were statistically significant at $\alpha = 0.05$.

	Cc	Cc	Cc	Cm
	this study (7-12153)	Blanck & Sawyer 1981 (1-2810)	Peters et al. 1994 (2-1627)	Fowler 1979 (3-1974)
Cc	this study (7-12153)	-	-	-
Cc	Blanck & Sawyer 1981 (1-2810)	1.000	-	-
Cc	Peters et al. 1994 (2-1627)	0.289	0.559	-
Cm	Fowler 1979 (3-1974)	0.154	0.313	1.000

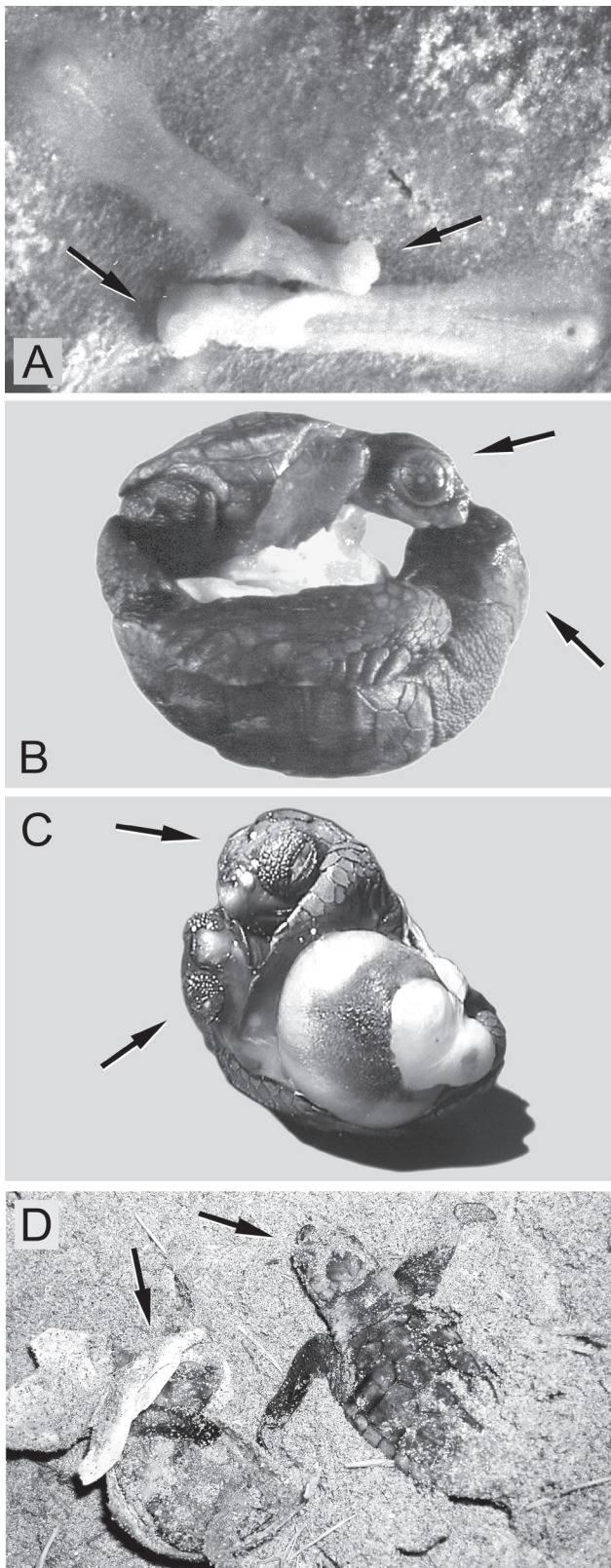


Fig. 1. (A) Separated twins dead at an early embryological stage, Dalaman Beach, Turkey in 2002. (B) Separated twins of unequal size dead late in the embryological stage at Dalaman Beach, Turkey in 2004. (C) Conjoined twins dead late in the embryological stage at Pozzolana di Ponente Beach, Italy, 2004. (D) Pair of twins, one viable hatched and the other, dead and smaller, rested inside the egg shell at Mon Repos, Australia, 2006. Arrows point toward heads. (Credits: photos A, B: J. Kaska; C: L. Taidelli; D: S. Piovano).

1979). An even lower twinning rate in unhatched eggs (0.03 %) was reported by Eckert (1990) for the leatherback sea turtle *Dermodochelys coriacea*. We estimated the odds of obtaining a pair of twins in unhatched eggs to be as large (0.92 times) as the odds of obtaining a pair of twins in the total eggs, proving that in our case the post-hoc examination did not underestimate the occurrence of twinning. In addition, the number of twin embryos we recorded was much greater than the number of twins that survived to complete the embryonic development and hatch (i.e. none). Our results clearly suggest that sea turtle species are subject to a low incidence of twinning. Turtle conjoined twins usually die at the embryonic stage (Miller 1985), thus the finding that our pair of conjoined twins were dead-in-shell was not unexpected. Many similar isolated cases have been described in detail in the literature (Table 1), but unfortunately they were occasional observations not accompanied by the quantitative data needed to estimate the incidence of the event and make further comparisons.

Among separated twins, it is known from observations of the incubated eggs of freshwater turtles that one individual could be viable and hatch, while the other, usually smaller, remains inside the shell (Table 1). Such an event has been observed in the wild for sea turtles *C. caretta* at Mon Repos Beach, Bundaberg, Australia (S. Piovano unpublished data, Fig. 1D). Tucker & Janzen (1997) suggested that most freshwater turtle eggs containing twins produce at least one living turtle, but twin hatchlings possibly have a lowered fitness. However, Yntema (1970) reported viable twins of *C. serpentina* having the same carapace length as their siblings, and Moriarty (2006) reported of a twin of the wood turtle *Glyptemys insculpta* that lived and bred in captivity for more than 12 years. Our sample of twins was mainly composed of separated embryos of unequal size, none of which hatched. The same fate was shared by the two pairs of twins of equal size. One pair from Dalaman Beach died at an early stage of development (Fig. 1A). The other pair found at Conigli Beach died at a late stage of development. Possibly, this last case was the result of the competition for the resources allocated in the egg, which were not sufficient to assure the complete

development of more than one embryo. Our results suggest that *C. caretta* does not benefit from twinning, as none of our twins survived.

One of the objectives of this study was to determine whether nest parameters such as clutch size and incubation period differ in nests with and without twins. It was shown in *T. scripta elegans* that twin-producing females laid larger clutches than females that did not produce twins (Tucker & Janzen 1997). We did not find a significant difference in the number of eggs laid between nests with and without twins. In the same way, we found no correlation between twinning and the length of the incubation period. These observations support the hypothesis that twinning in sea turtles is a rare and unpredictable event.

The findings from this study showed that the methodology used for data collection cannot be identified as being primarily responsible for the different twinning rates documented between freshwater and sea turtles. Twinning was 6.9 times rarer than in freshwater species. The freshwater turtles data set included data on *C. serpentina* from Yntema (1971), even though they were significantly different from those of Tucker & Janzen (1997) for the same species (Fisher's Exact test, $P = 0.033$) and for *T. scripta elegans* (Fisher's Exact test, $P < 0.001$). We agree with Tucker & Janzen (1997) that there is no reason to exclude those data from the analysis. We are aware that in an ordinary post-hoc examination of a sea turtle nest, particular cases such as a large living twin with a small dead twin may not be detectable. The hypothesis that post-hoc twin counts exclude this class of twins would be supported by comparison with the freshwater turtles twinning rate in Tucker & Janzen (1997) if the number of sets of large living/small dead twins were excluded. This would result in a twinning rate of 0.048 % (*T. scripta elegans* and *C. serpentina*), which is similar to the rate we found

(Fisher's Exact test, $P = 1.000$). We would stress that only an ad-hoc study under laboratory conditions could clarify this point. However, for the nests we examined at the Pelagie Islands, two observations suggest we can exclude the eventuality that this type of twinning occurred and was not detected. The first was that the number of hatchlings plus the number of unhatched eggs was equal to the total number of eggs we counted at oviposition. The second was that we did not find any small dead embryos inside hatched eggs, nor in the surrounding sand neither attached to hatchlings. Based on the within-group consistency of estimates and the difference in the percentage of twins between sea and freshwater species, we consider the documented difference in twinning rates as objective and not a consequence of the methodology employed in studying sea turtles. Indeed, twinning data from post-hoc examinations were demonstrated to be a good proxy of the real incidence of twinning in sea turtles.

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