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Population Ecology

Reproductive Output and Ultrasonography of an Endangered Population of East Pacific Green Turtles

GABRIELA S. BLANCO,[1] *Department of Biology, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104, USA*
STEPHEN J. MORREALE, *Department of Natural Resources, Cornell University, 122A Fernow Hall, Ithaca, NY 14853, USA*
ELIZABETH VÉLEZ, *Kelonian Conservation Society, Apartado 473-3000, Heredia, Costa Rica*
ROTNEY PIEDRA, *Parque Nacional Marino Las Baulas, Ministerio de Ambiente, Energía y Telecomunicaciones Apartado 10104-1000, San José, Costa Rica*
WILDER M. MONTES, *Goldring Marine Biology Station, Playa Grande, Guanacaste, Costa Rica*
FRANK V. PALADINO, *Department of Biology, Indiana-Purdue University, 2101 East Coliseum Boulevard, Fort Wayne, IN 46805-1499, USA*
JAMES R. SPOTILA, *Department of Biology, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104, USA*

ABSTRACT Reproductive output is one of the most relevant aspects of life history. We analyzed the reproductive output of the endangered East Pacific green turtle (*Chelonia mydas*) nesting in Nombre de Jesús, Costa Rica. We supplemented beach patrols with ultrasonography to estimate clutch frequency. With ultrasound scans, we classified the stage of turtle ovaries as: early stage (2 or more clutches), late stage (1 clutch), and depleted ovaries (no clutches). We calculated mean ($\pm$SD) estimated clutch frequency (ECF) to be 3.7 ± 1.8 ($n = 24$) and an adjusted frequency considering individual stage (ECF_U; ECF + number of clutches remaining as observed in the last ultrasound) as 5.1 ± 1.3. This is greater than previously described for East Pacific green turtles. Greater individual output could be representative of a healthier population; but could also indicate a decrease in the estimate population numbers previously reported.

KEY WORDS *Chelonia mydas*, Costa Rica, East Pacific green turtle, Guanacaste, reproductive output, ultrasound.

Reproductive output is one of the most influential aspects of life history and fundamental to the understanding of the reproductive biology of a species, which is essential for developing effective conservation and management plans for individual populations. For sea turtles, many key aspects of reproduction have been difficult to study in the wild. As a result, little information is available regarding mating systems, lifetime reproductive output, and reproductive strategies. This lack of knowledge frequently hampers conservation efforts. Social systems often limit which individuals can breed, determining the reproductive success of an individual and consequently population size. Understanding how the environment influences mating systems and reproductive strategies is a major concern in conservation (Komdeur and Deerenberg 1997).

As a result of some thorough earlier studies, we know some key steps in the reproductive biology of sea turtles. In general, mature female sea turtles have a pair of ovaries where follicles develop and become enlarged prior to mating (Owens 1980). Usually sometime after mating, enlarged follicles are ovulated into the paired oviducts where they are fertilized and then sheathed in albumen and shell material (Owens 1980). The ovaries and oviducts function synchronously during the reproductive season (Rostal 2007), in which a single female will deposit 2 or more clutches of eggs over a period of many weeks. Where and when the different steps in the female reproductive process occur has direct bearing on female reproductive strategies. Undoubtedly, these strategies are directly relevant to conservation plans for individual species and populations of sea turtles. For example, if mating is immediately followed by follicular development and occurs in waters surrounding nesting beaches, these areas should be a priority for conservation efforts and population management. Contrarily, if mating takes place prior to migration to nesting beaches and egg development starts later in the season, different management plans should be applied. If these strategies vary within populations, special care should be dedicated to where and when researchers should focus energies and resources in terms of conservation.

Adult female sea turtles acquire all the energy needed for reproduction in the foraging grounds prior to migration. Eight to 11 months before the breeding season, turtles start a period where ovarian follicles start developing, and, at the same time, lipids are allocated in preparation to migration and reproduction (Hamann et al. 2002). This is the process of vitellogenesis, which consists of the enlargement of ovaries

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[1]*E-mail: gsb22@drexel.edu*

and follicles before reproduction, during which yolk proteins incorporate with the oocyte within the follicle (Rostal 2007). At this stage, the females do not have fully mature follicles, but the ovary has several size classes of vitellogenic follicles that represent the number of clutches the turtle will lay during the season (Owens 1980, Etches and Petitte 1990, Rostal et al. 1998). These follicles continue to increase in size during premating and mating periods (Rostal et al. 1990). As female sea turtles get closer to the nesting season, they become receptive to the mating advances of males. At this stage, the ovaries are completely developed (Rostal et al. 1998) and the final stages of follicular maturation begin (Owens 1980). Mating apparently occurs immediately prior to and at the beginning of the nesting season (Pearse and Avise 2001). Previous research suggests that the first ovulation of the nesting season takes place several weeks after mating in loggerhead (*Caretta caretta*) and green turtles (*Chelonia mydas*; Owens 1997). However, Manire et al. (2008) indicated that after successful intromission, ovulation may occur within a few days.

Clutch frequency is required to calculate many population parameters, such as the number of females in a population or estimates of annual reproductive output. Such information is essential to completely understand and assess the status of sea turtle nesting populations and to estimate hatchling production. Without this knowledge, the establishment of effective conservation strategies and management plans are hindered. Generally, sea turtles have high clutch frequencies and high seasonal reproductive output. For green turtles, clutch frequency in the Atlantic has been reported to be as high as 6 clutches per season (Bjorndal et al. 1999). However, a conspecific, the East Pacific green turtle, is reported to have a clutch frequency of 3 or less for a nesting season (Alvarado-Díaz et al. 2003). Even though previous research provides information about estimated number of clutches for all sea turtle species, such information is limited because of incomplete coverage of nesting areas, loss of tags, and loss of individual turtles due to occasional lack of fidelity to nesting beaches (Miller 1997). Additionally, for many of these reasons, the average number of clutches laid per season may have been underestimated for many populations. This may be the case for East Pacific green turtle populations, yet solid information on this life history trait is still rare. Undoubtedly, such a low average number of clutches would equate to a low reproductive output, which might help explain the extremely endangered status of these populations (Alvarado-Díaz et al. 2003).

Information on the reproductive cycle of turtles in the wild is difficult to obtain. Ultrasound imaging has been used to study reproductive cycles in tortoises (Robeck et al. 1990), freshwater turtles (Shelby et al. 2000), female sea turtles (Rostal et al. 1990, 1998), and male sea turtles (Pease et al. 2010), and is an effective modality to study the reproductive cycle and physiology of wild animals. Ultrasonography allows for the analysis of reptile reproductive status without the use of anesthesia or other invasive techniques (Rostal et al. 1990). This technique is an effective method to repeatedly monitor follicular development and egg production (Robeck et al. 1990).

Our objective was to use ultrasonography as a supplement of beach patrol to better estimate mean clutch frequency of the endangered East Pacific green turtles (*Chelonia mydas*) nesting at a beach complex in northwestern Costa Rica. We used this estimate combined with data obtained on the number of females on the beach to provide more accurate information on reproductive output.

STUDY AREA

Nombre de Jesús (10°23′30″N; 85°50′07″W) is a high energy sandy beach, approximately 1-km long located in the north of the Nicoya Peninsula, Guanacaste Province, Costa Rica. Along with its neighboring beach, it hosts a large aggregation of nesting East Pacific green turtles with approximately 15 turtles per night during peak season.

METHODS

We patrolled Nombre de Jesús from October 2008 to January 2009 and from July to November 2009 to intercept nesting female green turtles. After each turtle finished nesting, we set up a portable real-time ultrasound scanner (Aloka SSD-500; Hitachi Aloka Medical, Ltd., Tokyo, Japan) behind the front flippers of the turtle. The scanner ran silently as it was plugged into a portable battery. We scanned the turtle while it was covering the nest. We used a 3.5 MHz electronic convex probe, because longer wavelength penetrates deeper than 20 cm into tissue and is more effective in larger species of sea turtles than shorter wavelengths (5.0 MHz; Rostal et al. 1990). We coated the probe with Aquasonic® gel (Parker Laboratories, Fairfield, NJ), a coupling gel that enhances imaging. We scanned 1 ovary and oviduct at a time by placing the probe in the inguinal region above the hind flipper (Rostal et al. 1990); we recorded every scan using an attached printer (video graphic printer; Sony Corporation, Tokyo, Japan). In addition to ultrasound scanning, we checked each turtle for passive integrated transponder (PIT) tags with a handheld PIT tag reader (AVID Identification Systems Inc., Norco, CA). If the turtle was not tagged, we applied a new PIT tag in the right front flipper for later identification. This study was approved by the Animal Care and Use Committee of Drexel University (Protocol 18466) and by the Ministry of Environment, Energy and Telecommunications (MINAET) in Costa Rica (ACT-PNMB-005-2007; ACT-SASP-PI-195; ACT-OR-D-050).

When we observed turtles during consecutive successful nesting events, we calculated a mean observed internesting period (OIP), which was simply the interval in days between observed successful nesting events averaged among all turtles for which we had this type of data. We excluded turtles observed after an interval greater than 20 days and less than 6 days from the analysis following protocol established by Reina et al. (2002). Similarly, we recorded observed clutch frequency (OCF) as the number of clutches actually observed during the sampling period for an individual. The OCF may underestimate the number of clutches per turtle (Frazer and Richardson 1985, Steyermark et al. 1996) unless a turtle is seen every time it nests throughout the season, and therefore the mean OCF is undoubtedly an underestimate of this key population parameter. To approach a more realistic estimate, we calculated estimated clutch frequency (ECF) using the first and last date of observation of an individual turtle, dividing it by the OIP and adding 1 for the first oviposition (Reina et al. 2002, Limpus et al. 2003).

For turtles with complete information on the reproductive status from ultrasonography, we added 0, 1, or 2 clutches to their ECF depending on the reproductive status they showed the last time they were seen on the beach. In this way, we obtained an even more realistic ECF_U estimate (ECF + number of clutches remaining as observed in the last ultrasound).

To calculate OIP, OCF, ECF, and ECF_U we used turtles encountered from July to November 2009 because the beach surveillance was more complete than in 2008. Although the beach was not completely monitored for the entire 2009 season, the effort was high throughout the season, and enough consecutive encounters existed to provide a solid baseline for estimates of seasonal reproductive output.

As another benefit to the ultrasound technique, we were also able to calculate the percentage of turtles with late-stage or depleted ovaries to have a better idea of the timing of the end of the nesting season for this population, about which little was known previously. In this analysis, we included all the turtles scanned in 2008 and 2009. This was supplemented with occasional surveys of the nesting beaches in March, April, and June to verify the absence of tracks during those months.

RESULTS

We encountered and scanned with ultrasound 96 individual turtles 127 times from October 2008 to November 2009. In all of the scans, we were able to visualize the ovaries of the turtles. Scanning the ovaries, we were able to differentiate among an early preovulatory, late preovulatory, and a depleted ovary (postovulatory; Rostal et al. 1996). In the early stages (beginning of the nesting season), the ovaries were filled with vitellogenic follicles. In the late preovulatory stage, we were able to distinguish only when 1 clutch was left, because of the presence of fewer follicles, coelomic space, and atretic follicles in some cases. When the ovary was depleted, we observed atretic follicles and 5 or fewer vitellogenic follicles, meaning that the clutch just laid was the last clutch of the season. An accurate estimation of the number of vitellogenic follicles using ultrasound was not possible; however, we could estimate the following: 1) ovaries in early stage (Fig. 1a): turtles will lay at least 2 more clutches following the

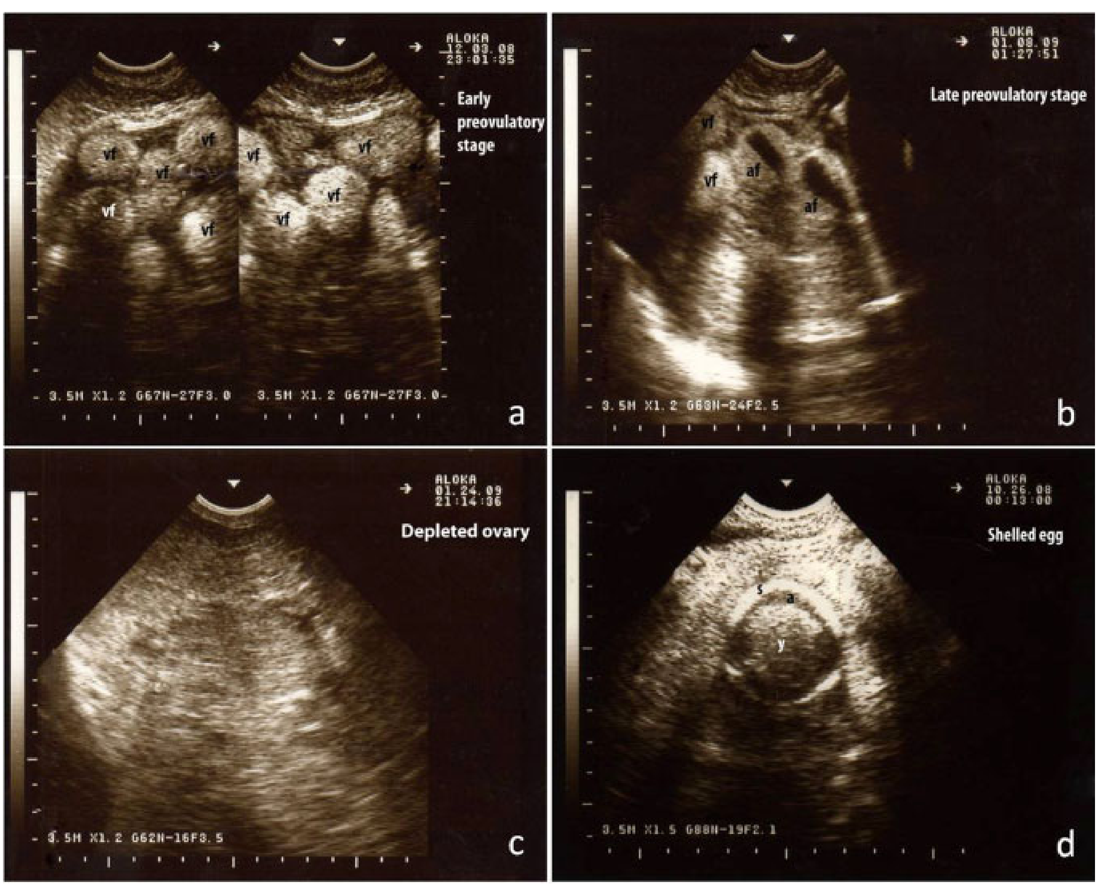

Figure 1. Ultrasonographic images of an ovary of a green turtle (*Chelonia mydas*) after a nesting event in Nombre de Jesús beach, Costa Rica. (a) The ovary was filled with vitellogenic follicles (vf). The turtle had at least 2 more clutches to lay during the nesting season. (b) Ovary in late preovulatory stage. The turtle had 1 more clutch to lay during the nesting season. In this stage we observed vitellogenic follicles (vf), atretic follicles (af), and some coelomic (empty) space. (c) The depleted ovary indicated that the turtle had completed the nesting season and would start migrating. We considered an ovary depleted when we identified fewer than 5 vitellogenic follicles remaining (none in this picture). (d) The presence of the shelled egg after oviposition indicates that a disturbance occurred during the nesting event causing the turtle to stop laying eggs and start covering the nest. s, shell; a, albumen; y, yolk.

present nesting event; 2) ovaries in late stage (Fig. 1b): turtles will lay 1 more clutch following the present nesting event; 3) ovaries depleted (Fig. 1c): the turtle will not lay more clutches during the season. In 4 cases, we found shelled eggs (Fig. 1d) still in the oviduct when the turtle was covering the nest. These encounters coincided with the presence of poachers or tourists around the turtles.

The OIP (mean $\pm$ SD) for this population was 12.0 $\pm$ 2.1 days ($n = 37$ individuals, 98 encounters). The OCF (mean $\pm$ SD) was 1.5 $\pm$ 0.8 ($n = 24$) and the ECF (mean $\pm$ SD) was 3.7 $\pm$ 1.8 ($n = 24$). The newly calculated ECF_U (mean $\pm$ SD) was 5.1 $\pm$ 1.3 ($n = 24$) (Table 1).

We also calculated the percentage of turtles seen with depleted and late staged ovaries during the months of August through February (Fig. 2). We observed 2 peaks where the greatest number of turtles left the breeding areas, October–November and January. Occasional observations in March, April, and June indicated the presence of very low numbers of nesting females on the nesting beaches.

Table 1. East Pacific green turtles (*Chelonia mydas*) with a complete record of their reproductive status through ultrasound; turtles were scanned every time we observed them on the beach (Nombre de Jesús, Costa Rica) in 2009. ECF_U is the effective clutch frequency (ECF) + the number of clutches remaining in the turtle calculated from ultrasonography.

Turtle	First seen	Last seen	ECF	No. clutches remaining[a]	ECF_U
1	28 Jul	27 Oct	8.55	0	8.55
2	3 Aug	31 Aug	3.32	2	5.32
3	11 Aug	1 Sep	2.74	2	4.74
4	15 Aug	27 Aug	2.00	2	4.00
5	18 Aug	14 Sep	3.24	2	5.24
6	22 Aug	23 Oct	6.15	0	6.15
7	22 Aug	2 Nov	6.98	1	7.98
8	25 Aug	6 Sep	2.00	2	4.00
9	28 Aug	9 Oct	4.49	0	4.49
10	2 Sep	14 Sep	2.00	2	4.00
11	9 Sep	12 Oct	3.74	0	3.74
12	10 Sep	13 Nov	6.31	0	6.31
13	10 Sep	18 Oct	4.15	2	6.15
14	15 Sep	26 Oct	4.40	2	6.40
15	17 Sep	22 Oct	3.91	2	5.91
16	19 Sep	25 Oct	3.99	2	5.99
17	10 Oct	22 Oct	2.00	2	4.00
18	10 Oct	23 Oct	2.08	2	4.08
19	11 Oct	25 Oct	2.16	2	4.16
20	12 Oct	18 Nov	4.07	0	4.07
21	15 Oct	25 Oct	1.83	2	3.83
22	15 Oct	6 Nov	2.83	2	4.83
23	17 Oct	30 Oct	2.08	2	4.08
24	18 Oct	11 Nov	2.99	2	4.99
Mean			3.67		5.13
SD			1.79		1.32

[a] Number 2 indicated that turtles had 2 or more clutches to lay during the season.

DISCUSSION

Our findings using regular tagging data and relocations on the beach indicated an ECF of 3.7 for this population. This is similar to the findings of Cornelius (1976) in Costa Rica and Alvarado-Díaz et al. (2003) in Mexico that suggested the East Pacific green turtles nest an average of 3 times per season. By adding the information obtained with the ultrasound to the ECF (calculating ECF_U), increased estimates 1.4 nests to 5.1 nests per season, which is closer to the average of 6 clutches per season reported by Bjorndal et al. (1999) for green turtles in the Caribbean. Clutch frequency in the Caribbean was previously reported to be 2.8 by Carr et al. (1978) but that information was not reliable because of incomplete beach surveillance and tag loss (Bjorndal et al. 1996).

The difference in the numbers of clutch frequency reported for different populations could be affected by loss of tags, beach surveillance, time of the year, and beach fidelity. This includes tagging or recapturing turtles a fraction of the night instead of all dark hours or not covering the complete nesting season. By using the information provided by the ultrasound on the reproductive stage of the turtles through the season, we could estimate a more accurate clutch frequency for the Costa Rican population. The accuracy of this type of information, such as clutch frequency and internesting intervals, could provide, for example, better knowledge of the number of females in a population, annual fecundity, and hatchling production (Miller 1997). The underestimation of these values could affect conservation strategies by overestimating the number of females in a population where number of females is estimated by number of nests. Alvarado-Díaz et al. (2003) calculated the number of females nesting per year based on OCF = 2.5 and compared it to the number of females nesting per year obtained by estimating it with ECF = 3.1. As a result, the calculated number of females in the population was lower with a greater clutch frequency given the same number of nests.

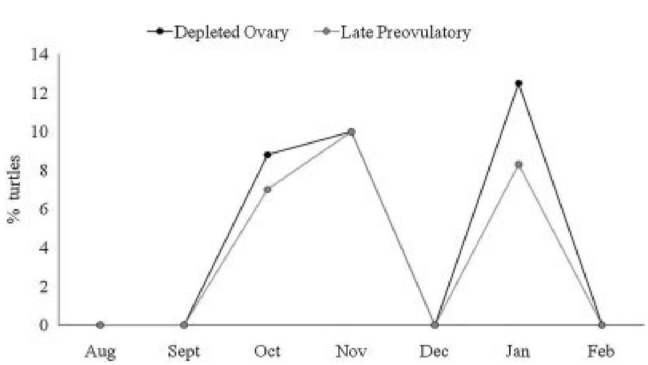

Figure 2. Proportion of East Pacific green turtles (*Chelonia mydas*) with depleted ovaries and late preovulatory staged ovaries by month in Costa Rica, 2008 and 2009. We calculated the proportions in relation to the number of turtles scanned by ultrasound every month.

Reproductive output may be affected by morphology or dietary resources, which would result in variations between populations within the same species (Broderick et al. 2003). Green turtles nesting in the Mediterranean (Broderick et al. 2003), in Florida (Johnson and Ehrhart 1996), and in Ascension Island (Broderick et al. 2006) nest an average of 3 times per season; in the Great Barrier Reef green turtles nest an average of 5 times a season (Chaloupka 2002). In Tortuguero, Atlantic Costa Rica, clutch frequency is 6 (Bjorndal et al. 1999). Demographic models reveal effective management options, and clutch frequency is used to build these models (Bjorndal et al. 1999, Chaloupka 2002, Broderick et al. 2006). Considering the existent of variability between populations, researchers must accurately estimate demographic parameters in order to provide reliable predictions of population viability. If such a key parameter as clutch frequency is incorrect, it could lead to a misinterpretation of the status of a population.

Occasional observations in March, April, and June may indicate that the nesting season for East Pacific Costa Rican green turtles extends all year round or at least 10 months as indicated by Cornelius (1976). We observed 2 peaks for migrating turtles (turtles with depleted ovaries) in the months of October–November and January, indicating that turtles may be arriving at the nesting beaches as early as June and turtles leaving the nesting grounds as late as April. However, in order to have a better understanding of the timing of the nesting season more observations throughout the year are necessary.

The use of ultrasound is a very successful methodology to understand the reproductive status of sea turtles. The scanner can be taken to the beach and the procedure does not require anesthesia or handling the animals and allows the acquisition of real-time images, which makes this methodology ideal for the study of wild animals (Rostal et al. 1990, Pease et al. 2010). The whole process can take between 5 min and 10 min. Complete visualization of the ovary is not possible through ultrasound (Robeck et al. 1990) and at the beginning of the nesting season each ovary could have from 50 follicles to more than 200 follicles (Rostal et al. 1990); therefore, estimating the number of eggs the turtle will lay during the season is impossible. However, this technique allowed us to identify the remaining reproductive output of every scanned turtle. At the beginning of the nesting season, the ovaries of most green turtles displayed large quantities of preovulatory vitellogenic follicles. Even though the follicles were different sizes (Etches and Petitte 1990), we had difficulty estimating the number of clutches the turtle would lay during the season at early stages of the cycle. As the season progressed and the follicles were ovulated, the ovaries started showing fewer follicles and the presence of atretic follicles. Atretic follicles have an anechoic line surrounded by an echoic yolk (Rostal 2007) and are the result of non-ovulated follicles during the nesting season. Sea turtles potentially reuse the energy from the stored yolk by reabsorbing the yolk from the oocyte (Rostal 2007). The presence of atretic follicles and fewer vitellogenic follicles at the end of the nesting cycle allowed the easy count of clutch cycles. These characteristics also occur in leatherback (*Dermochelys coriacea*) and olive Ridley (*Lepidochelys olivacea*) sea turtles showing that the reproductive system is common for different sea turtle species (Rostal et al. 1996, Rostal 2007) and that this technique can be applied to other sea turtles.

Our study illustrated that the use of ultrasound as a noninvasive technique was an effective way to accurately determine the reproductive status and reproductive effort of East Pacific green turtles. Given the need for more detailed demographic data to obtain a more complete understanding of the demographic status of sea turtle populations (Bjorndal et al. 2010), this technique should be used routinely in nesting beach studies.

MANAGEMENT IMPLICATIONS

Reproductive output is one of the most influential measures for population biology, conservation, and management of sea turtle populations. The information obtained from this study shows a greater reproductive output than previously described for the East Pacific green turtle. Greater individual output could represent a healthy population with good resources available to foraging adults, which would allow them to produce more offspring. However, greater seasonal output estimates could require modification of previous estimations in number of females in a population, decreasing numbers previously reported for this species. Given green turtles are in danger of extinction, especially the East Pacific population (International Union for Conservation of Nature [IUCN] 2010) largely because of egg poaching, accurate estimates of seasonal output are required to assess population viability.

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