Seasonal and life-stage variation in the reproductive ecology of a marine apex predator, the tiger shark *Galeocerdo cuvier*, at a protected female-dominated site

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ABSTRACT: Advancing our knowledge of the reproductive biology and mating systems of free-ranging sharks is inherently challenging. The large size and mobility of the specimens are just a few of the problems that make such studies complicated, and in some respects, impractical. The tiger shark *Galeocerdo cuvier* is a large, roving, apex predator found in many oceans throughout the world. Although their nomadic nature is primarily linked to large-scale migrations, these sharks can also display site fidelity. One site where this is known to occur is at Tiger Beach, Bahamas. Unique to the waters of this area is the consistent sighting of large females. While the sex-specific use of the area remains unknown, the shallow, warm environment could represent a critical habitat for reproductive events. To investigate the reproductive biology of tiger sharks at Tiger Beach, 65 individuals were opportunistically sampled between 2011 and 2014. Reproductive status of captured females (n = 59) was assessed with ultrasonography and by measuring circulating sex steroid hormones (progesterone, testosterone and estradiol). Our results indicate that Tiger Beach is a high-use site for female tiger sharks of mixed life stages. The results also suggest that Tiger Beach may function as a refuge habitat, allowing females to reach maturity free from male mating harassment, as well as functioning as a gestation ground where gravid females can benefit from year-round calm warm waters, which may reduce the gestation period and accelerate embryo development.

KEY WORDS: Tiger shark · Reproduction · Ultrasound · Blood hormones
Many shark species display predictable and seasonal long-distance migrations that are often associated with age and/or size-based segregation between and within the sexes (e.g. Weng et al. 2005, Domeier & Nasby-Lucas 2012, Carlson et al. 2014). Although certain adult migrations have been linked to reproductive events (e.g. mating, parturition; Chapman et al. 2015), empirical data to support these movements in sharks are rare and have only been observed in relatively lower trophic level species. For example, the only direct evidence of shark mating-site fidelity comes from nurse sharks *Ginglymostoma cirratum*, whereby this species has been observed to enter warm, shallow areas of the Dry Tortugas National Park (Florida, USA) in early summer to mate (Carrier et al. 2003). In addition, the only direct evidence of parturition site fidelity in sharks comes from parental genotypes of lemon sharks *Negaprion brevirostris* in Bimini, Bahamas, and blacktip reef sharks *Carcharhinus melanopterus* in Moorea, French Polynesia (e.g. Mourier & Planes 2013, Feldheim et al. 2014). These studies determined that adult females return to their respective nursery sites to give birth. While there have been observations of pregnant females aggregating in gestation grounds with elevated water temperatures (e.g. Bansemer & Bennett 2009, Nosal et al. 2014), few studies have found direct evidence of habitats used by large predatory female sharks to gestate or give birth. Given the conservation importance of designating such areas, further studies on the reproductive biology of these species are warranted (e.g. Awruch et al. 2014).

The tiger shark *Galeocerdo cuvier* is a large (up to 5.5 m in length), highly migratory, apex predator found worldwide in temperate and tropical seas (Compagno 2005). Although primarily a wide-ranging oceanic species, it also occupies a variety of other habitats, including coral reefs, pelagic environments, and oceanic atolls (Heithaus et al. 2007, Meyer et al. 2009, Fitzpatrick et al. 2012, Hammerschlag et al. 2012, 2015, Hazin et al. 2013, Papastamatiou et al. 2013, Werry et al. 2014). In the western central Atlantic, there is a shallow-water area on Little Bahama Bank nicknamed ‘Tiger Beach’ by dive tourism operators due to the high abundance of tiger sharks found there (Gallagher & Hammerschlag 2011). Satellite tracking data from this location have revealed that females exhibit periods of prolonged residency (up to 180 d), and the majority of sharks found here are generally at or above the size of sexual maturity (>300 cm total length; Branstetter et al. 1987, Whitney & Crow 2007). As such, it is possible that the predominately female use of this site may be related to a reproductive aspect of this species’ life history, such as mating or gestating. This is of particular significance since the removal (i.e. fishing pressure), or protection (i.e. sanctuary) of reproductively active female sharks can have a disproportionate effect on population size (discussed in Shiffman et al. 2014).

In the present study, multi-year and multi-season data were opportunistically collected in order to investigate if this female-dominated site is potentially linked to reproduction. Although lethal sampling has historically been considered the most effective approach for collecting reproductive information from elasmobranch fishes, non-lethal approaches such as ultrasonography and analysis of plasma sex steroid hormones have become feasible and/or practical sampling alternatives (for review see Hammerschlag & Sulikowski 2011). Here, we integrated physiological sampling of plasma sex steroid hormones (progesterone, testosterone and estradiol), photo-documentation of mating scars, and ultrasonography to determine the reproductive status of tiger sharks present at Tiger Beach and elucidate the potential function of this location.

**MATERIALS AND METHODS**

**Project phases**

Fieldwork was conducted at Tiger Beach (26.86°N, 79.04°W) over the course of 5 research expeditions: December 2011, July 2012, October 2013, May 2014, and November 2014. Data collection consisted of 2 sampling phases. In Phase I (December 2011 and July 2012), blood samples were collected from captured sharks (*n* = 20; 18 females, 2 males). In Phase II, ultrasonography was added to this sampling protocol and individuals were also inspected for mating scars (*n* = 45; 41 females, 4 males).
Capture and sampling

Tiger sharks were captured using standardized circle-hook drumlines, which is a passive fishing technique (as described in Gallagher et al. 2014). Briefly, the gear consisted of a submerged weight base tied to a line running to the surface by means of an attached, inflatable buoy float. A 23 m monofilament gangion line (~400 kg test) was attached to the submerged weight by a swivel, which terminated at a baited 16/0, 5-degree offset circle hook. This method permitted sharks to swim in a 23 m radius circle around the base when captured. An hour after first deployment, each drumline was sequentially checked for shark presence. Once hooked, each shark was slowly brought to the boat and restrained on a dive platform, partially submerged in the water. To facilitate respiration, an oxygen enriched water pump was immediately inserted into the shark’s mouth. This capture and handling method was selected to promote shark vitality and reduce stress levels during sampling (Gallagher et al. 2014). For each individual captured, sex was recorded and stretched total length (TL) was measured to the nearest cm over a straight line along the axis of the body.

Blood sampling

Blood (~20 ml) was collected from the caudal vein using 2 chilled heparinized 14 gauge needles attached to 2 separate 10 ml plastic syringes. The heparinized blood sample was stored on ice for up to 20 min before being centrifuged at 3500 rpm (410 × g) for 3 min. The plasma was then removed and stored frozen at −20°C for future hormonal analyses.

Plasma steroid hormone extraction

Each plasma sample was extracted for progesterone (P₄), testosterone (T), and 17β-estradiol (E₂) following protocols from Tsang & Callard (1987) and Sulikowski et al. (2004). A 500 µl aliquot of each plasma sample was extracted twice for each hormone with 10 volumes of ethyl ether (ACS grade), and the liquid phase was evaporated at 37°C in a heat block under a stream of nitrogen. To account for procedural loss, prior to extraction each sample was spiked with 1000 counts min⁻¹ of tritiated P₄, T, and E₂ obtained from Perkin Elmer. The extract was reconstituted in phosphate buffered saline with 0.1% gelatin (PBSG); mean recoveries of P₄, T and E₂ were 59, 84, and 69%, respectively.

Radioimmunoassay

Steroid hormone concentrations were determined following a modified radioimmunoassay procedure from Sulikowski et al. (2004). Non-radiolabeled P₄, T and E₂ were obtained from Steraloids. The specifics of the non-radiolabeled stock concentrations, radio-labeled steroids, antibody characteristics, and titers can be found in Sulikowski et al. (2004). A Tri-Carb 2900TR liquid scintillation analyzer (Perkin Elmer) was used to determine radioactivity. The mean intra-assay coefficients of variation for P₄, T, and E₂ were 11, 10, and 6%, and the inter-assay coefficients of variation were 13, 10, and 10%, respectively. When hormone values for individual samples fell below detectable limits, those samples were concentrated and re-analyzed. When calculating mean (±SE) concentrations of each steroid hormone, any value that was non-detectable after concentration was assigned the lowest possible concentration that the assay would have been able to detect in the aliquot used (e.g. Prohaska et al. 2013).

Mating scars

The body of each captured shark from Phase II was examined for evidence of mating scars and bite wounds, which were identified as distinct, semi-circular marks along the flank or fins (Pratt & Carrier 2001). Mating scars also showed clear differences in coloration and contrast when compared to the normal pigmentation of the sharks’ skin; these changes ranged from minor (e.g. streak marks from single teeth, shallow punctures; Fig. 1A) to major (e.g. deep lacerations or bites exposing integument and dermis; Fig. 1B).

Ultrasonography

Ultrasonography was initiated during Phase II of the study, and was performed on the abdominal region of female tiger sharks (n = 41) to help assess reproductive stage. An Ibex Pro portable ultrasound (EI Medical Imaging) with a 60 mm curved linear array 2.5 to 5 MHz transducer (model 290470) capable of a 24 cm scan depth was used to obtain images of the reproductive tract of each female. Scanning was performed on the ventral surface from the pectoral to the pelvic fin in both a transverse and longitudinal orientation to obtain cross sectional and lengthwise images, respectively. Additionally, each
shark was imaged between the pectoral fins to view the ovary and follicles. Gain was auto-focused by tissue composition to optimize viewing for the near and far field for each individual. Depth of the scan ranged from 12 to 24 cm, depending on the image being obtained. Collected images and video loops were saved on the Ibex Pro at the time of sampling. Images and frozen video stills were then used to measure (via proprietary software pre-installed on the Ibex Pro ultrasound) pup diameter (cm) along the transverse axis (Fig. 2).

**Determination of reproductive stage**

After all data had been accumulated and reviewed, sharks were assigned into 1 of 3 distinct reproductive stages (immature, mature non-gravid, or mature gravid) using a 4-step process. First, ultrasound data were used to determine if Phase II sharks were gravid based on presence or absence of embryos (Step 1). For non-pregnant females, ultrasonography could not differentiate between immature and mature non-gravid sharks (Fig. 2). Thus, for sharks that were not pregnant, we used TL to separate mature non-gravid from immature individuals (Step 2). Based on a review of the primary literature, female tiger sharks in the study region generally mature at 300 cm or greater TL (e.g. Clark & von Schmidt 1965, Branstetter et al. 1987, Castro 2011). Thus, all Phase II sharks ≥300 cm TL and not pregnant (based on ultrasound) were categorized as mature non-gravid, and all those <300 cm TL were considered immature. Once the 3 reproductive stages were assigned for Phase II sharks, blood hormone values were compared between stages for significant differences (Step 3). Since hormone values and TL were the only available criteria with which to assess reproductive stage in Phase I sharks, ensuring that hormone profiles were linked to specific reproductive events was a critical component of the study (e.g. Hammerschlag & Sulikowski 2011). However, when the mean P₄, T, and E₂ concentrations were compared among reproductive stages (1-way ANOVA followed by Tukey’s HSD), hormone profiles between immature and mature gravid sharks were statis-
tically indistinguishable (P₄: F₂,38 = 0.82, p = 0.45; T: F₂,38 = 3.5, p = 0.04; E₂: F₂,38 = 11.9, p < 0.0001; Fig. 3). Because of the similarities in hormone concentrations between immature and mature gravid females, it was determined that blood hormone values alone could not accurately differentiate between these two reproductive stages in Phase I sharks (n = 18). Due to this unanticipated result, the final step required an additional quantitative method in order to differentiate between mature gravid and mature non-gravid individuals in Phase I sharks (Step 4). Using the collective empirical reproductive data (hormone concentrations and ultrasonography) from Phase II, a multiple logistic regression with forward selection (ΔAICc ≤ 3; Burnham & Anderson 2002) was generated to predict gravidity in mature (≥300 cm TL) tiger sharks sampled during Phase I. However, the interaction between all covariates in this study showed high colinearity (E₂ × T: r = 0.74; E₂ × P₄: r = 0.70; T × P₄: r = 0.77); therefore only the primary variables were analyzed. The resulting model fit (df = 1; coefficient of discrimination, D = 0.67; Tjur 2009) indicated that T and E₂ had significant predictive value in determining the probability of gravidity in mature sharks (Table 1). The relationship between the probability of gravidity and T and E₂ concentrations was represented by the solution to the log-odds (logit) function:

\[ p(\text{gravidity}) = \frac{e^{(4.321537 - 0.036736 \times E₂ - 0.009116 \times T)}}{1 + e^{(4.321537 - 0.036736 \times E₂ - 0.009116 \times T)}} \]

This model was then applied to concentrations of T and E₂ from tiger sharks in Phase I that had not been examined with ultrasonography, to determine probability of gravidity for mature individuals (>300 cm TL). An individual was considered to be gravid if the logit function returned a probability ≥0.75. Conversely, an individual was considered to be non-gravid if the logit function returned a probability <0.75. Phase I individuals under 300 cm TL were considered immature.

Using the reproductive classifications as determined above, seasonal differences in the frequency of reproductive stages of sharks captured at the study site were analyzed with chi-squared analyses. Data were statistically analyzed using R v.2.15.2. All data were tested for normality prior to parametric analysis, and all tests were considered significant at p ≤ 0.05.

**RESULTS**

In total, 65 tiger sharks were captured at Tiger Beach: 6 males (mean ± SD: 311 ± 53 cm TL) and 59 females (265 ± 100 cm TL; 41 in Phase II; 18 in Phase I), generating a sex ratio (M:F) of 1:9.8.

![Fig. 3. Mean (±SE) (A) progesterone, (B) testosterone, and (C) estradiol concentrations plotted by reproductive stage of Phase II female tiger sharks *Galeocerdo cuvier*. Lowercase letters denote statistically significant pairwise differences in hormone concentrations between stages (p < 0.05)](-)

Table 1. Model results used for determining the probability of gravidity in tiger sharks *Galeocerdo cuvier*. Akaike's information criterion (AICc) corrected for small sample sizes for each step lowered by 3. E₂: estradiol; T: testosterone

<table>
<thead>
<tr>
<th>Hormone</th>
<th>AICc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>42.316195</td>
</tr>
<tr>
<td>E₂</td>
<td>26.400483</td>
</tr>
<tr>
<td>E₂ × T</td>
<td>22.447960</td>
</tr>
</tbody>
</table>
Although low sample sizes precluded the use of statistical analysis, an increasing trend in pup diameter across sampling periods was observed in gravid females (Table 2). Using the fitted logit model, mature December Phase I individuals (n = 10) were all predicted as gravid, whereas the 1 mature shark in July was predicted as non-gravid (see Table S1 in the Supplement at www.int-res.com/articles/suppl/b024p175_supp.pdf). Based on the collected results, the females sampled in this study consisted of 19 immature (32%), 17 mature non-gravid (29%), and 23 mature gravid (39%) individuals (see Table S1 for full details).

A significant difference was found in the frequency of each reproductive stage observed by month ($\chi^2 = 32.9$, df = 14, n = 59, $p < 0.0001$; Fisher’s exact test $p < 0.0001$). When investigating the frequency of immature females observed each month, no significant difference between months was found ($\chi^2 = 8$, df = 4, n = 19, $p = 0.09$; Fig. 4). However, a significant difference in the observance of both non-gravid and gravid mature females by month was observed (non-gravid: $\chi^2 = 10.25$, df = 4, n = 17, $p = 0.04$; gravid: $\chi^2 = 23$, df = 4, n = 23, $p < 0.001$; Fig. 4), with a higher frequency of mature non-gravid females in May, October, and November, and a higher frequency of mature gravid females in October and December. Finally, mating scars were only observed on 7 individuals, 6 of which were non-gravid females.

### DISCUSSION

In this study, the use of ultrasonography was found to be an effective tool to discern pregnancy in tiger sharks. This finding is similar to studies of other large sharks where ultrasonography was determined to be effective in assessing and monitoring reproductive status (e.g. Carrier et al. 2003, Daly et al. 2007). However, an unexpected result in our study was the difficulty in linking steroid hormone concentrations to specific reproductive periods, especially since these biomarkers have been correlated with morphological changes within the reproductive tracts of other elasmobranch species (e.g. Manire et al. 1995, Tricas et al. 2000, Sulikowski et al. 2004, Awruch et al. 2008, Prohaska et al. 2013). This was particularly problematic when differentiating between immature and mature gravid individuals regardless of the hormone analyzed. While ascertaining the mechanistic endocrine pathways of these hormones in tiger shark reproduction is beyond the scope of the current study, the apparent lack of correlation between hormonal signature and reproductive stage may be related to a sampling regime that failed to capture the transient periods in which these steroids were elevated. For example, hormone concentrations have been shown to vary widely between the different reproductive modes in elasmobranch species, between species exhibiting the same reproductive mode, and between individual species of the same genus over the course of their reproductive cycles (for a review see Hammerschlag & Sulikowski 2011, Sulikowski et al. 2011). In addition, when compared to other yolk sac viviparous species (aplacental viviparity), the hormonal values recorded in the current study are among the lowest documented (e.g. Prohaska et al. 2013, Awruch et al. 2014). Despite these anomalies, the patterns of estradiol and testosterone levels in the current study parallel those found in other adult female non-gravid yolk sac viviparous species (e.g. Tsang & Callard 1987, Awruch et al. 2014) in which
these hormones are associated with follicular growth leading to ovulation (Koob & Callard 1999, Awruch 2013). Although the role that testosterone plays in viviparous elasmobranchs remains unclear (Awruch 2013), previous research suggests that this hormone is a substrate for estradiol biosynthesis (Tsang & Callard 1982) and is correlated with follicular development (Koob et al. 1986). Given this, our findings suggest that the collective estradiol and testosterone concentrations can be used as a predictor of gravidity in sharks 300 cm TL and larger.

While pregnancy status could be empirically measured by ultrasound, the use of published length at maturity data for tiger sharks (300 cm TL; Clark & von Schmidt 1965, Simpfendorfer 1992, Castro 2011) was needed to delineate if a Phase I shark was immature or pregnant in the current study. Given the considerable variation in both tiger shark growth rates (e.g. Meyer et al. 2009) and size at maturity (260 to 347 cm TL; Whitney & Crow 2007, this study), we recognize that there is a certain level of uncertainty surrounding the designations as used in the current study. However, we believe our thorough review of the literature along with the data from Phase II sharks justifies our use of 300 cm as an appropriate length at maturity in the sampled sharks. In addition, the designation of 300 cm TL for maturity is approximately 65% of the maximum size for this species (Castro 2011), and is a conservative estimate that is more likely to designate mature individuals <300 cm TL as immature rather than vice versa.

In the present study, although the abundance of both non-gravid and gravid mature females fluctuated by month, immature females were consistently found in association with adults at Tiger Beach. These findings are particularly interesting in that our knowledge of habitat use in sharks suggests that most species exhibit a traditional triangle framework that is often associated with segregation (by size and sex) for the majority of the year (e.g. Springer 1967, Chapman et al. 2015). While the low sample size limits any definitive comparisons to this paradigm, our observation that immature females share the sampled habitat with both gravid and non-gravid adults suggests that Tiger Beach could function as an important site for female tiger sharks at different life history stages.

While tiger sharks are provisioned by dive tourists at Tiger Beach, the large-scale movement and migration patterns of sharks from this site and other neighboring areas (Hammerschlag et al. 2012, 2015), as well as the lower prevalence of male tiger sharks at Tiger Beach, suggests that dive tourism is not primarily responsible for tiger shark residency at this site. In addition, the environment at Tiger Beach is a shallow (average 5 m deep), warm (due to its proximity to the equator), mostly homogenous sand flat with irregular seagrass patches and infrequent small patches of coral. Given that this habitat is not typically associated with the primary prey of tiger sharks (e.g. Heithaus et al. 2002), it is possible that Tiger Beach may not serve as a foraging site for this species. Although it may not be linked to foraging, Tiger Beach is situated in the lee of Grand Bahama Island, which produces relatively stable and calm water conditions year-round. Given the reported 15 mo gestation period in tiger sharks (Whitney & Crow 2007), we suggest that gravid or recently mated female tiger sharks may be using the Tiger Beach area in part to benefit from the year-round calm warm water, which likely reduces the gestation period and accelerates embryo development. For example, while not directly measured in the current study, warm water use for gestation has been observed in several other species of gravid elasmobranchs (e.g Bansemer & Bennett 2009, Jirik & Lowe 2012, Nosal et al. 2014). Furthermore, the lack of males and dominance of females at Tiger Beach may reduce mating attempt harassment by males (Sims et al. 2001, Jacoby et al. 2010, Wearmouth et al. 2012). Indeed, copulation in sharks is often violent, and females can incur significant injury from mating and related attempts (Pratt & Carrier 2001). Moreover, male sharks will often attempt to copulate with immature females (Calich & Campana 2015). Due to the skewed sex ratio observed over in the current study, we suggest that females at different life-stages likely use the area as a refuge from males. This behavior has been observed in female nurse sharks, which have shown both avoidance and shallow water refuging from males (Pratt & Carrier 2001). While the mating and parturition behavior of tiger sharks visiting Tiger Beach is currently unknown, Lea et al. (2015) reported that mature male tiger sharks tagged off Bermuda migrated to Bahamian waters during the winter months, leading the authors to speculate that their presence in the area was linked with mating. This coincides with the period when the highest proportion of mature females as well as those individuals with mating scars were observed at Tiger Beach. Thus, it is possible that mating occurs in the Bahamas at or near Tiger Beach during the winter months. While the absence of neonate tiger sharks at Tiger Beach suggests parturition occurs elsewhere, timing based on a gestation of around 15 mo would mean pupping occurs in late spring and summer, which coincides
with the abundance of neonates found in the North Atlantic (Natanson et al. 1999, Castro 2011). However, additional research, including tracking pregnant females, is needed to further investigate these ideas.

The findings of the current study have conservation implications, since fishing aggregations of gravid females may pose threats to the viability and health of local and regional populations (discussed in Shiffman et al. 2014). Tiger Beach is within the boundaries of the Bahamas Exclusive Economic Zone (EEZ), where commercial longlining has been banned for decades and sharks are protected from all harvest due to the establishment of a shark sanctuary in 2011. Although the results of this study suggest that Tiger Beach is occupied by sharks of mixed life-history stages, it is not known how long or to what extent individuals utilize Tiger Beach or the protected Bahamian waters. If gravid females spend little time within the sanctuary and/or give birth outside the sanctuary, they may be vulnerable to exploitation when they leave the area. However, the relatively high abundance of tiger sharks in the Bahamas compared to the rest of the Caribbean (Ward-Paige et al. 2010) could be attributed in part to the protection of mature and/or gravid females in the Bahamas. As a popular dive tourism site, future research should also attempt to consider if and how tourism activities (e.g. noise, food, boats, divers) could impact shark health, reproduction, and offspring fitness (Gallagher et al. 2015). Given the unique nature of the findings presented here, further research is required in order to better understand the relationship between habitat use and the reproductive status of female tiger sharks at Tiger Beach and within Bahamian waters.

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