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Using plasma vitellogenin in loggerhead sea turtles to assess reproductive maturation and estrogen-like contaminant exposure

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Acknowledgments
Partial funding was provided for this project by the Morris Animal Foundation, the Disney Wildlife Conservation Fund, the Oak Foundation, the Duke University Marine Biomedical Center, and National Marine Fisheries Service Southeast Regional Office grant # NA97FL0375, NA07FL0499, and NA03NMF4720281. Sea turtle sampling permits were issued by NOAA Fisheries Endangered Species Act Section 10(a)(1)(A) #1245; Georgia Department of Natural Resources Scientific Collection Permit CN#1140; Florida Fish and Wildlife Conservation Commission Marine Turtle Permit #140; North Carolina Wildlife Resources Commission #98ST65 and #99ST70, United States Fish and Wildlife Service #TE-676379-2. The *T. scripta* antibody was generously provided by Dr. Kyle Selcer at Duquesne University. Plasma samples were collected with the help of Sheryan Epperly, Larisa Avens (NMFS), David Whitaker, Bruce Stender, Phil Maier, Al Segars, and Rusty Day (SC DNR). Testosterone was measured by Michelle Lee (College of Charleston). Additional technical assistance was provided by Kathy Moore (NOAA) and Erik Sotka (College of Charleston).

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Data Availability
Data collected for this research paper, including supporting information, will be publicly available

Author Contributions
Raquel N. Corniuk: data curation, formal analysis, visualization, writing orginal draft and review and editing, *Jennifer M. Lynch: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, supervision, validation, visualization, writing original draft and review and editing, Michael D. Arendt: data curation, funding acquisition, resources, review and editing, Joanne Braun-McNeill: data curation, investigation, resources, review and editing, David W. Owens: conceptualization, data curation, formal analysis, investigation, methodology, resources, supervision, review and editing, Roldán A. Valverde: data curation, formal analysis, methodology, resources, review and editing, John R. Kucklick: methodology, resources, supervision, validation, review and editing, Patricia D. McClellan-Green: funding acquisition, methodology, resources, supervision, review and editing

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/etc.5612.

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Abstract

Vitellogenin (VTG), an egg yolk precursor, is abnormally produced by male and juvenile oviparous species upon exposure to estrogens. Plasma VTG in loggerhead sea turtles (*Caretta caretta*) helped us understand their reproductive maturation and investigate it as a biomarker of contaminant exposure. VTG presence was screened in plasma from 404 loggerheads from the Northwestern Atlantic Ocean using a freshwater turtle antibody in Western blots. VTG concentrations were semi-quantified using band intensities calibrated to results from a loggerhead-antibody ELISA. VTG detection and concentrations were highest to lowest: nesting females, in-water adult females, subadult females, smaller females, unknown sex, and males. Loggerheads from this region begin vitellogenesis ≅77 cm SCL. We classified VTG expression as abnormal in nine male or juvenile turtles. Organochlorine contaminant (OC) concentrations were measured in blood and/or fat biopsies of some turtles. One abnormal VTG female had the second highest fat polychlorinated biphenyl (PCB) and 4,4'-dichlorodiphenyldichloroethylene concentrations among 43 VTG-negative juveniles. The nine VTG-abnormal turtles had blood PCB concentrations 8.5 % higher, but not significantly different, than 46 VTG-negative juveniles ($p=0.453$). In turtles <77 cm, blood PCB concentrations were significantly, but weakly, correlated with semi-quantified VTG concentrations ($tau=0.1$, $p=0.004$). Greater blood OC concentrations were found in adult females than males, which motivated the creation of a conceptual model of OC, VTG and hormone concentrations across a reproductive cycle. A decision tree is also provided incorporating

VTG as a sexing tool. Abnormal VTG expression cannot conclusively be linked to endocrine disruption caused by these OC concentrations. Studies should further investigate causes of abnormal VTG expression in wild sea turtles.

Introduction

All sea turtle species are listed on the International Union on the Conservation of Nature (IUCN) Red List (IUCN 2022). Their imperiled population status warrants the examination of chemical pollutant threats since contaminants may negatively impact development and reproduction which could translate into fewer or abnormal offspring, hence population declines. Loggerhead sea turtles (*Caretta caretta*) are broadly distributed in subtropical regions of the Atlantic, Indian, and Pacific Ocean with the majority of nests occurring along the western coasts of the Atlantic and Indian Ocean (Conant et al. 2009, U.S. NMFS & U.S. FWS 2008). In the southeast U.S., the focus of the current study, most nesting occurs across a span of 2,400 km of beach from Alabama to North Carolina (U.S. NMFS & U.S. FWS 2008). This stock is categorized as threatened by the U.S. Endangered Species Act (U.S. NMFS & U.S. FWS 2008), and when aggregated on nearshore immature foraging grounds along the U.S. East coast, they have a sex ratio is 2:1 female: male (Wibbels 2003). Sea turtle sex is not determined genetically, instead by a cascade of hormones triggered by very specific temperatures during embryonic development (Yntema 1976, Wibbels 2003). Sex of immature sea turtles cannot be identified from external morphology; hence a combination of plasma testosterone concentrations and laparoscopy must be used (Braun-McNeill et al 2007). Adult female loggerheads migrate to their breeding grounds after approximately 2.5 years

of foraging (Miller et al. 2003). During the foraging periods, they must gain enough fat stores to undergo vitellogenesis, the process of egg yolk formation (Miller et al. 2003).

Vitellogenin (VTG) is a protein normally expressed in livers of mature female egg-laying species in response to increased concentrations of estradiol. VTG circulates in the blood to be deposited as a major protein precursor for egg yolk in oocytes. VTG has been detected in the blood plasma of nesting females of olive ridley (*Lepidochelys olivacea*), loggerhead, leatherback (*Dermochelys coriacea*), and green (*Chelonia mydas* and *Chelonia agassizii*) sea turtles (Vargas 2000, Myre et al. 2016, Bruno et al. 2021, Smelker et al. 2014). In nesting female loggerheads from Hutchinson Island, Florida, VTG peaked in June with relatively high concentrations (15.37 mg/mL) and decreased towards the end of the nesting season while VTG was undetected in nonreproductive active subadult females (straight carapace length (SCL) 81.0 +/- 1.12 cm) (Myre et al. 2016). In recent years, enzyme-linked immunosorbent assays (ELISAs) have been developed with antibodies produced against estrogen-induced purified VTG from green (*Chelonia mydas* and *Chelonia mydas agassizii*) and loggerhead sea turtle blood (Sifuentes-Romero et al. 2006; Herbst et al. 2003; Smelker et al., 2014; Bruno et al. 2021). Using ELISAs, nesting female loggerheads had greater VTG concentrations than immature loggerhead turtles captured along the southeast U.S. coast (Smelker et al. 2014).

Juvenile and male turtles may abnormally produce VTG when they are exposed to compounds with estrogenic activity. In several reptile species, VTG induction has been observed after injection with estrogens (Palmer and Palmer 1995, Verderame et al. 2016, Rey et al. 2005). In sea turtles, juvenile male and female Kemp's ridley sea turtles

(*Lepidochelys kempii*) injected with 1 mg/kg estradiol produced high concentrations of VTG from 1 to 31 weeks after the injection (Heck et al. 1997) with a peak in production at day 50 (Vargas 2000). Their blood plasma continued to appear opaque and viscous until at least 11 weeks after the injection, suggesting extended VTG presence, even after serum estradiol had returned to undetectable concentration (Heck et al. 1997). This sustained response shows that juvenile sea turtles of both sexes are capable of responding to estradiol and that abnormal VTG presence in the bloodstream may be prolonged. Five loggerhead sea turtles, determined to be male by plasma testosterone concentrations along the southeast coast of the U.S., had detectable VTG in their blood for unknown reasons (Smelker et al 2014).

Because of the sensitive induction of an easily detected protein in the blood, VTG has been suggested as a reptilian biomarker for exposure to and physiological effects of a broad diversity of environmental contaminants that have estrogenic activity (Arukwe et al. 2016). In a laboratory study, male red-eared slider turtles (*Trachemys scripta*) produced VTG after injection with the well-known and globally contaminating, organochlorine insecticide, 2,4'-dichlorodiphenyltrichloroethane (2,4'-DDT) (Palmer and Palmer 1995). Field studies examining VTG as a biomarker in reptiles have revealed mixed results likely due to exposure to a wide diversity of environmental contaminants that have differing toxicological mechanisms. Premature male crocodiles from a South African farm that were exposed to a diversity of pharmaceuticals, including ethinyl estradiol, and pesticides in the breeding pond expressed the same amount of estrogen and VTG as females (Arukwe et al. 2016). In contrast, Tada et al. (2007) showed no significant difference in plasma VTG concentrations in male Reeves' pond turtles

(*Chinemys reevesii*) among four sites with varying degree of estrogenic sewage treatment plant effluent; however, they noted five males with elevated VTG concentrations at the three most contaminated sites. On the contrary, in freshwater turtles (*Chrysemys picta*), reduced VTG concentrations were found in females from a lake near a Superfund site on Cape Cod, Massachusetts, which is contaminated with a mixture of inorganic and organic contaminants, most notably trichloroethene and ethylene dibromide, compared to a reference lake (Rie et al. 2004, Kitana et al. 2006). These latter studies may be explained by certain contaminants impairing estrogen synthetase, thereby causing a decrease in estradiol and subsequently lesser VTG (Kime 1999, Rie et al. 2004). No study to date has attempted to use VTG as a biomarker for estrogenic-contaminant exposure or effects in sea turtles.

Sea turtles accumulate many persistent organic pollutants (POPs) that are listed on the Stockholm Convention, including organochlorines (OCs), polybrominated diphenyl ethers, and perfluorooctane sulfonate (Keller 2013). Here, we focus specifically on one class of POPs, the OCs, because they were measured previously in the blood and fat of the same turtles sampled in this study (Keller et al. 2004b; Lynch unpublished data). The OCs include polychlorinated biphenyls (PCBs), DDT, chlordane, and chlordane metabolites. These OCs are lipophilic, so they preferentially accumulate in lipid-rich tissues like fat or eggs, but concentrations in fat are representative of concentrations in blood, a less invasive sampling technique (Keller 2013, Keller et al. 2004a, 2004b). Several correlative studies have suggested that sea turtles may be sensitive to sublethal effects of OCs, such as decreased hatchling body condition and blood chemistry indications of physiological and organ functions (Keller et al. 2004c, van

de Merwe et al. 2010, Camacho et al. 2013), but none have specifically addressed endocrine disruption.

Many OCs are known endocrine disruptors, but not all are estrogenic. As stated earlier, 2,4'-DDT caused VTG expression in red-eared slider turtles (Palmer and Palmer 1995). In other reptiles, endocrine disruption by OC pesticides, such as DDT, DDE, DDD, dieldrin, and chlordanes, have been observed in South African crocodiles (*Crocodylus niloticus*) and Floridian American alligators (*Alligator mississippiensis*) (Guillette et al. 2000, Heinz et al. 1991, Arukwe et al. 2016, Milnes et al. 2005). OC exposure was linked to developmental abnormalities (body size, reproductive tract anatomy, and spleen somatic index) and possibly population declines. The endocrine effects of PCBs are complicated in mechanism and outcome (Safe 1995; Plísková et al. 2005). Lower chlorinated PCB congeners are estrogenic as estrogen receptor (ER) agonists, whereas higher chlorinated congeners are anti-estrogenic through ER antagonism (Plísková et al. 2005). Beyond direct ER mechanisms, PCBs may produce anti-estrogenic effects through an aryl hydrocarbon receptor (AhR) mechanism (Safe 1995). In aquatic organisms, PCBs (specifically PCBs 126, 153, or a commercial PCB mixture) have resulted in VTG expression in female juvenile and male fish (Calo et al. 2010; Vega-Lopez et al. 2006; Jung et al. 2005), but the effect becomes anti-estrogenic with longer exposure times (Calo et al. 2010). PCBs and their hydroxylated metabolites mimicked estrogen to result in more red-eared slider turtle hatchlings becoming female, even though the eggs were incubated at male-producing temperatures (Bergeron et al. 1994). It is important to note that many environmental contaminants, beyond OCs, such as pharmaceuticals, industrial compounds, herbicides, pesticides, and heavy metals, can

alter hormone production in many ways and reproductive structures in organisms at very low concentrations (i.e., Sheehan et al. 1999, Kime 1999, Hayes et al. 2002, Akingbemi and Hardy 2001). Furthermore, mixtures of accumulated contaminants are known to produce estrogenic effects that are additive relative to each compound's potency for ER binding (Silva et al. 2001). Since adult sea turtles have low concentrations of circulating estradiol (10 - 50 pg/mL) (Owens and Morris 1985), marked VTG response to a single injection of estradiol (Heck et al. 1997), exposed to a mixture of contaminants (Keller 2013), and similar receptors and hormones in the species mentioned above, sea turtles may be sensitive to environmental contaminants that have estrogen-like activity.

Another potential research use of VTG expression is as a sexing tool for sea turtles, used in concert with the testosterone sexing method. Understanding the sex ratio of sea turtle aggregations is important for modeling population growth rates, which is critical for assessing extinction risk for each species. However, sexing sea turtles is challenging because their lack of sex chromosomes make laparoscopic examination of the gonads the only definitive sexing tool, but its use is limiting as it requires expertise to perform minor surgery (Owens 1997, Wibbels 2003, Braun-McNeill et al. 2007). Secondary sexual characteristics, including long tails and elongated and curved claws on males, do not lengthen until sea turtles reach sexual maturity (Casale et al. 2005). Decades ago, concentrations of plasma testosterone became a proven sexing technique for immature sea turtles (Owens 1997), but this method has minor limitations. Some turtles (usually less than 10%) fall within a range of testosterone concentrations that overlap between males and females, so the sex cannot be determined for these individuals. However, the use of mark-recapture and/or Bayesian models has allowed for

the accurate prediction of the sex of these 'unknown sex' turtles (Allen et al. 2015, Shertzer et al. 2018, Jensen et al. 2018). At low water temperatures (<16°C), male testosterone concentrations decline, which increases the chance of incorrectly identifying a male as a female (Braun-McNeill et al. 2007). Additionally, the testosterone sexing technique is not applicable for adult turtles because their sex can be determined with external visual examination and because large females either at or near maturity can have testosterone concentrations in the adult male range (Rostal et al. 1996), which increases the chance of incorrectly identifying a female as a male. However, assessing testosterone concentration in adult turtles is useful for determining reproductive status (e.g., will the turtle reproduce that season). Turtles producing VTG would most likely be female and this additional tool can help researchers sex more turtles, especially those in the subadult stage, and validate the conclusions made from testosterone concentrations.

The goals of this study were to investigate the use of VTG in loggerhead sea turtles to (1) further understand reproductive maturation, (2) for use as a supplemental sexing tool, and (3) as a biomarker of exposure to estrogen-like contaminants. We first determined the size at which female turtles begin vitellogenesis, or when females start naturally producing this protein. The expression of VTG in some subadult turtles assisted in categorizing them as female when testosterone concentrations and external morphology were inconclusive. Based on the determined size threshold, we used VTG expression to aid in identifying females that were otherwise categorized as unknowns and we identified the proportion of smaller, juvenile turtles that were abnormally expressing VTG prior to maturation. In an attempt to explain why these turtles were abnormally

expressing VTG, we compared their blood OC concentrations to juvenile turtles that were not expressing VTG.

Methods

Sample collection

Blood samples (n=416) were collected from loggerhead sea turtles that ranged from juveniles to adults. They were captured from inshore waters of Core Sound, North Carolina, USA (May to November of 1998 through 2002) as bycatch in the pound net fishery (Epperly et al. 2007), from offshore waters of South Carolina, Georgia, and Florida (summers of 2000 and 2002) in scientific trawling (Arendt et al. 2012) and five were nesting females on Bald Head Island, North Carolina (July 1998 and from June to July 1999) (Figure 1). Ten of these turtles were recaptured and resampled at least once, so a total of 404 individual loggerhead turtles were sampled (Table S1). Blood samples ($\leq$ 2.2 mL/kg) were drawn within 15 minutes after capture from the dorsocervical sinus using 21-gauge 1.5-inch double-ended needles and two to four 10 mL heparinized vacuum blood collection tubes (Becton, Dickinson, and Co., Franklin Lakes, NJ). A mixture of protease inhibitors was added to one tube of blood at 1.5 μg/mL leupeptin and 1.5 μg/mL aprotinin (final concentrations). A whole blood sample from each turtle was frozen at -20 °C until analysis for OC concentrations. Plasma, separated by centrifugation, from this tube was frozen at -80 °C until analysis for VTG and estradiol. Plasma from another heparinized blood tube for each turtle was stored at -80 °C for testosterone concentration analysis. Fat biopsies for contaminant analysis were surgically removed from the 44 juvenile loggerhead turtles from Core Sound, North Carolina described in Keller et al. (2004b). The turtles were tagged, measured, weighed, and

released near the capture location. All samples were analyzed for the following measurements from 1993- 2003.

Sex determination

A detailed flow chart describes how sea turtle sex assignments were made (Figure 2). A laparoscopy was performed on 44 juvenile turtles from Core Sound; the results were previously reported in Braun-McNeill et al. (2007). Sex of remaining 372 turtles were determined based on their plasma testosterone concentrations by radioimmunoassay, as described in Braun-McNeill et al. (2007). Loggerhead turtles with plasma testosterone concentration less than 200 pg/mL were classified female, while those with concentration above 300 pg/mL were categorized as males. These cutoffs were determined specifically for loggerhead sea turtles inhabiting the northwest Atlantic Ocean (Braun-McNeill et al. 2007). The sex was categorized as unknown if the testosterone concentrations were not analyzed, were between 200 and 300 pg/mL, or if the turtle was captured in water less than 16 °C because testosterone concentration becomes less reliable for predicting sex of immature turtles in cold waters (Braun-McNeill et al. 2007).

For adult turtles, sex was assigned based on external characteristics. While size is not the perfect indicator for age or sexual maturity, 88 cm SCL was selected as an average size cutoff for subadult to adult transition for loggerheads based on published size reports of U.S. nesting females (NMFS 2008, Miller 1997, Frazer and Ehrhart 1985). Sexually mature and reproductively active females (> 88 cm SCLmin) often have testosterone concentrations greater than 300 pg/mL, so the sexually dimorphic characteristic of longer tail lengths in males was used primarily to assign a sex to these

animals (Casale et al. 2005). When testosterone and tail length were inconclusive, turtles were categorized as unknown sex.

Vitellogenin measurements

VTG presence by Western blots

At the time of VTG analysis, between 2000 and 2003, an antibody for measuring VTG did not exist for any sea turtle species; therefore, a polyclonal antibody produced against VTG from a freshwater turtle, *Trachemys scripta*, was used (Selcer and Palmer 1995). The total protein concentration in plasma samples was determined by the Bradford method using bovine serum albumin (BSA) as a standard.

The immunoblotting procedure was modified from Selcer and Palmer (1995). Molecular weight (MW) markers were used on each blot, but the source and MW ladder range varied over the course of the project: 185-31 kDa, 190-31 kDa, 193-36 kDa, 230-33.5 kDa from Sigma-Aldrich, or 250-10 kDa from BioRad. Estrogen-induced *T. scripta* plasma was used as a control for successful antibody binding and nesting turtle (B5) plasma was used on each blot as a control for successful antibody binding to loggerhead VTG. Plasma samples were diluted in a sample buffer (5% 2-mercaptoethanol, 2.3% SDS, 62.4 mM Tris-HCl, pH 6.8, 0.02% bromophenol blue, 10% glycerol) in plastic vials and placed into boiling water for 4 min. Samples (50 μg protein) were separated on 5% polyacrylamide gels (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at 70 V for 3 h at 4 °C. The membranes were soaked in methanol, dried, and blocked overnight in phosphate-buffered saline (PBS) containing 3% BSA. The membranes were washed in PBS ++ (PBS containing 0.1% Tween 20 [volume fraction] and 0.1 % BSA [or 0.1 grams per 100 mL]) and incubated in the *T.*

scripta antibody (1:5000 dilution in PBS containing 0.1% Tween 20 and 5% BSA) for 2 h. The membranes were washed in PBS ++ then incubated in donkey anti-rabbit secondary antibody (1:10,000) in PBS containing 0.1% Tween 20 and 5% BSA for 1 h. The membranes were washed three times in PBS ++ and developed using ECL++ Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, England). Detection was performed by autoradiography with four different time exposures (30 s, 1 min, 3.5 min and between 6-10 min); exposure duration was recorded. If there was uncertainty as to whether VTG was present or absent, the sample were re-analyzed using 60 μg to 75 μg of sample protein.

VTG concentrations in nesting females by ELISA

In 2008, some of the loggerhead plasma samples previously analyzed by Western blot were analyzed by ELISA (Cayman Chemical kit, Ann Arbor, MI) in hopes of quantifying the VTG concentrations. An in-house ELISA developed with a loggerhead anti-VTG antibody was used according to the methods described in Smelker et al. (2014). Briefly, a 10-point calibration curve was made from purified loggerhead VTG, plated between 4 ng/mL to 2000 ng/mL. The unknown loggerhead plasma samples from nesting females were diluted 1:75000 or 1:100000. A rabbit anti-loggerhead VTG primary antibody was used, and the secondary antibody was goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Laboratories, Inc., Hercules, CA). An in-house plasma control sample from a VTG-positive olive ridley sea turtle was used as a positive control. Six wells per plate received this positive control, as adjacent duplicates located at three different sections of the plate. The duplicates were averaged to provide triplicate VTG concentration measurements per plate for this positive control. These triplicates were

used to calculate intra-assay relative standard deviation (RSD). Only one of the four ELISA plates had a passable RSD (1.1%), and this plate contained three nesting loggerheads and one non-nesting loggerhead turtle previously analyzed using Western blot. Data from the other three ELISA plates were not used because RSDs were too high (10.3% to 41.4%).

VTG semi-quantified concentrations in in-water turtles

Western blots containing VTG positive turtles were scanned into TIFF format and converted to 32-bit to preserve image quality. Analysis of the VTG band (218 kDa), in only VTG-positive lanes, was performed using gel functions in the National Institutes of Health ImageJ software (version 1.52k). Background corrections were performed following Heidebrecht et al. (2009) method of a rolling ball correction four-times the width of the band. The area under the curve was calculated to determine band intensity.

Each western blot included turtle ID B5, a VTG-positive nesting turtle, in the first lane as a positive control, and this served as an internal standard to reduce inter-blot variability. Band intensity of each VTG-positive sample (n=30 turtles) was normalized to the band intensity of turtle ID B5 on the same blot (band intensity ratio). A linear regression standard curve was created with band intensity ratios on the y-axis from three blots that contained three nesting (VTG positive) female plasma samples and one non-nesting, VTG-negative plasma sample. The X-axis was the ELISA-measured VTG concentration in each of those same turtles normalized to the ELISA-measured VTG concentration in turtle ID B5 (Figure S1, R^2= 0.94). Using this equation, VTG concentrations were calculated, using the band intensity ratio of each non-nesting VTG-positive turtle. Because the VTG concentrations were estimated from western blots, this

analysis is considered semi-quantitative and reported VTG concentrations should not be directly compared to data outside of this study. The limit of detection was determined first by using the turtle with the smallest band intensity in the following calculation (ng B5 VTG x ((intensity of the band in this turtle's lane/intensity of the band in the B5 lane)/slope of the calibration curve))/mL of plasma loaded into the well. Then, this number was then multiplied by 3 to obtain a conservative limit of detection of 12.636 µg/mL.

Organochlorine Contaminant (OC) Measurements

Concentrations of OCs, including PCBs and pesticides, were previously determined (Keller et al. 2004b) in fat biopsies and whole blood samples of 44 of the juvenile turtles examined for VTG. Subsequently, 31 additional loggerhead blood samples were selected based on criteria of interest, re-capture, large turtle, and/or VTG positive, and analyzed using similar techniques. Briefly, all blood samples (5 g each) were spiked with mass-labeled organochlorine internal standards prepared in *iso*-octane and extracted using method A described in Keller et al. (2004a), which utilized a liquid: liquid extraction technique with formic acid, hexane, and methyl-*tert*-butyl-ether. Extracts were cleaned using 5% deactivated alumina columns and either a semi-preparative aminopropyl silane column or silica solid-phase extraction columns. Compounds of interest were measured using gas chromatography (GC) with either micro electron capture detection or mass spectrometry. Limits of detection were approximately 1 ng/g wet mass for fat; 10 pg/g wet mass for blood. Calibration curves were prepared from National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 2261 (Chlorinated Pesticides in Hexane), 2262 (Chlorinated Biphenyl

Congeners in 2,2,4-trimethylpentane), 2274 (Chlorinated Biphenyl Congeners in Iso-octane II), and 2275 (Chlorinated Pesticides in Hexane II), and a PCB solution containing 15 additional congeners. Field and laboratory blanks and SRM 1589a (PCBs, Pesticides, PBDEs, and Dioxins/Furans in Human Serum) were used for quality control. Field blanks were created by pulling hexane-rinsed ultrapure water into Vacutainer tubes with double-ended needles of the same lot number used to collect turtle blood.

Statistics

All statistics were performed using R. The Nondetects and Data Analysis for Environmental Data (NADA) R package was used for data that fell below the limit of detection (Helsel 2005). Summary statistics were obtained using the Kaplan-Meier or regression on order statistical models. Differences in semi-quantified VTG concentrations and OC blood concentrations amongst groups of turtles binned by sex, size, and normal vs. abnormal VTG expression were determined by parametric or nonparametric tests depending on assumptions of normality and homogeneity of variance. A Fisher exact test was performed to determine the significance of the categorical data to aid in determining the threshold for natural production of VTG in females. Semi-quantitative VTG concentrations were correlated to SCL and to blood OC concentrations using R NADA Kendall's Tau.

Results

All raw data from individual turtles including capture information, testosterone concentrations, morphometrics, OC concentrations, VTG presence/absence, semi-quantified VTG, and final sex determinations can be found in Table S2.

Sex determinations

Determining the sex of the turtles was an iterative process; as additional information was collected on a particular turtle; the sex assignment became possible with more certainty (Figure 2). For example, without the consideration of SCL, tail length or VTG, plasma testosterone concentrations alone identified 100 of the non-nesting turtles as male and 236 as female, leaving 63 turtles unknown, but this was not the final sex ratio. Tail length for larger turtles helped to refine the sex assignments. From this study, males > 88 cm SCL displayed elongated tails of 51.0 cm ± 5.8 cm from the posterior tip of the plastron to the tip of the tail ($Tail_{P\text{-}T}$) and 8.6 cm ± 1.3 cm from the cloaca to the tip of the tail ($Tail_{C\text{-}T}$). Females > 88 cm SCL had an average tail length of 23.5 cm ± 3.67 cm and 5.8 cm ± 1.1 cm, respectively (Table S2). After using all tools available (Figure 2), and recognizing some tools were missing for certain turtles, the final sex ratio was 241 female, 97 male, and 61 unknown sex, excluding the 5 nesting females.

Presence of VTG on the Western blots helped assign the sex of five turtles. Two adult sized turtles (92.3 cm and 103.5 cm SCL) captured in-water had short tail lengths in the range of the other females (24.1 cm and 19.2 cm $Tail_{P\text{-}T}$; 7.8 cm and 4.5 cm $Tail_{C\text{-}T}$). Their testosterone concentrations were in the male range (>300 pg/mL), not surprising for adult females and causing sex assignment uncertainty. Their positive expression of VTG (turtle IDs 130 and 139) confirmed their initial female assignment based on external characteristics. The sex of three other turtles was initially classified as unknown because their plasma testosterone concentrations were either between 200 pg/mL to 300 pg/mL (IDs 2289, 4026) or inconclusive (ID 1385). All three turtles were positively expressing VTG. Their SCLs were (79.4, 81.5, and 77.3) cm; and semi-quantified VTG

concentrations were (360, 260, and 1240) µg/mL, respectively. This suggests that these three turtles were female.

Vitellogenin measurement controls

The *T. scripta* antibody detected a large protein in Western blots, approximately 218 kDa, in all five nesting female loggerhead turtles sampled (Figure 3a). This protein was of similar size to VTG identified at 213 kDa in a positive control, which was plasma from an estrogen-treated *T. scripta*, previously characterized by Selcer and Palmer (1995). The size and immunoreactivity of this loggerhead protein were consistent with VTG. A similar size protein was not detected in the negative control sample from a male *T. scripta* (Figure 3a). The antibody cross reacted with several other smaller molecular weight proteins in male or juvenile loggerhead plasma (Figures 3a and S2) suggesting that, in its present form, this particular antibody is not specific to only VTG; therefore, it should not be used in ELISAs for loggerhead plasma samples.

In the ELISA using the loggerhead antibody, the intra-assay variability in VTG concentrations was acceptable (RSD < 10%) for the olive ridley in-house plasma control sample on only one plate, the plate with plasma from nesting females (RSD = 1.1 %). The VTG concentrations in the nesting females normalized to turtle ID B5 measured by ELISA were strongly correlated with VTG band intensities from the Western blots (Spearman's rho= 0.9255, R^2=0.937; Figure S1), indicating that the western blots were detecting the intended protein and were semi-quantitative. Interblot variability was examined using plasma from five immature females in which the same plasma sample per female was replicated on multiple blots (RSD >10 %; Figure S3). One likely reason for the large inter-blot variation was oversaturation of B5, the internal standard. Another

reason is that the blots with the best bands were chosen, not considering the duration of time the X-ray film was exposed to the blot, which ranged from 30 s to 10 min. The variability tended to be lower in the turtles with lesser VTG concentrations and higher in turtles with greater VTG concentrations. VTG concentrations were averaged across blots for each turtle (Table S3).

Differential expression of vitellogenin in loggerhead turtles by sex and size

Presence or absence of VTG expression was determined by western blots in plasma collected from female turtles ranging from 45.7 cm to 97.5 cm SCL (Figure 3b provides an example). Seventy-three percent (73 %) of females between 77 and 87 cm SCL (n=15) expressed VTG, while 90 % of females captured in-water larger than 87 cm (n=10) (Figure 4a) expressed VTG. In contrast, only 2.3 % of females <77 cm SCL were VTG positive (n=216). The groups of female turtles >77 cm SCL had significantly greater percentages expressing VTG than the females <77 cm, males of all sizes (50.6 cm to 94.7 cm), unknown sex <77 cm, and unknown sex >77 cm. This suggests that the threshold for normal VTG production is 77 cm SCL.

Semi-quantified plasma VTG concentrations from VTG-positive turtles captured in-water ranged from 4.21 µg/mL to 4340 µg/mL. VTG concentrations were significantly different among turtle sex and size groups as described next (Figure 4b). Male semi-quantified VTG concentrations (2.0% detected, <12.6 µg/mL median; <12.6 µg/mL to 1760 µg/mL) were similar to females < 77 cm (3.7% detected, <12.6 µg/mL median; <12.6 µg/mL to 1860 µg/mL) and were significantly lower than all other female groups. Nesting female semi-quantified VTG concentrations (100% detected, 8980 µg/mL median; 932 µg/mL to 15400 µg/mL) were significantly greater than VTG concentrations

in all other female turtle groups captured in water. The semi-quantified VTG concentrations significantly increased with SCL of the in-water female groups: from females <77 cm (values listed above), females between 77 cm and 87 cm (73% detected, 170 µg/mL median; <12.6 µg/mL to 1830 µg/mL), to females >87 cm (90% detected, 963 µg/mL median; <12.6 µg/mL to 4340 µg/mL). Within females, the semi-quantified VTG concentration significantly correlated with SCL (Figure S4). Only one large female (SCL= 95.5 cm) above the mature-size threshold, 88 cm SCL, was not expressing VTG. This turtle was captured in waters offshore of Florida on July 31, 2000, with a female plasma testosterone concentration of 150 pg/ml and a short tail length (23.0 cm TailP-T and 5.5 cm TailC-T). In an attempt to explain the VTG expression in these turtles, we tried to measure estradiol concentrations in the same plasma samples, but the ELISA results were inaccurate and too variable.

Abnormal expression of vitellogenin by individual loggerhead turtles

Nine juvenile turtles smaller than 77 cm SCL (total of 10 plasma samples due to one juvenile re-capture) were expressing VTG, which we consider abnormal (Figures 3, 4, S4). VTG was detected in the plasma of five of the 216 female turtles and in four of the 61 turtles of unknown sex. One juvenile female turtle was captured three times: July 16, 1998 (turtle ID JMJ 11, SCL= 51.0 cm), June 2, 2000 (turtle ID JMK 3-6, SCL= 52.3 cm), and August 11, 2000 (turtle ID JMK 3-43, SCL= 53.7 cm). During the first capture, this turtle was not expressing VTG. However, during the two subsequent recaptures VTG was expressed with semi-quantitative VTG concentrations of 1860 µg/mL and 130 µg/mL. These nine VTG-expressing turtles were categorized well within the juvenile size range and not of breeding size (Casale et al. 2005, Smelker et al. 2014). Based on their

small size and the maturation threshold established above (>77 cm subadult females begin to express VTG), the presence of VTG in these turtles may be considered abnormal. They were captured in geographically distinct areas from each other and over four years; therefore, they were not clustered spatially or temporally (Figure 1).

One juvenile male was captured three times (JMK 3-40, JMK 5-39, JMK 5-37) throughout 2000 to 2002 in offshore waters of North Carolina. Upon the first capture (August 9, 2000) he was not expressing VTG at 63.4 cm SCL. During the second and third captures (October 21 and November 1, 2002) with SCLs of 69.0 cm and 69.1 cm, respectively, this male was expressing VTG at semi-quantified concentrations of 970 µg/mL and 1760 µg/mL (Figure 3c). Sex was confirmed by laparoscopy during the first capture. This male was grouped with the other 9 juveniles that were abnormally expressing VTG for assessing differences in plasma contaminant concentrations.

Assessment of vitellogenin as a biomarker

Blood OC concentrations were compared between the 9 juveniles that were abnormally producing VTG (total of 12 blood samples measured for OCs) and 53 juveniles that were considered normal because they were not expressing VTG (Table S4). The mean total PCB concentrations in the blood of the abnormal turtles were 8.5% higher, but not statistically significantly higher, than the normal juveniles ($p= 0.453$) (Figure S5). Of the many individual PCB congeners that were detected, none were significantly different between the normal and abnormal turtles, except for PCB 138. PCB 138 concentrations were significantly higher in the abnormal compared to the normal turtles ($p=0.03$). On the other hand, OC pesticides, trans-nonachlor, cis-nonachlor, and total chlordanes, were significantly lower in the abnormal juveniles

(p=0.02, 0.007, and 0.01, respectively). Blood concentrations of total DDTs and the grand total of OCs were not significantly different between abnormal and normal juveniles.

Blood OC concentrations were further assessed for correlations with semi-quantitative VTG concentrations in these same juvenile turtles (Table S4; Figure 5). Five significant correlations were observed ($p<0.05$), but all were very weak (tau < |0.15|). Total PCB, dieldrin, 4,4'-DDE, and total DDT concentrations were significantly ($p<0.05$) and positively correlated with VTG concentrations, whereas total chlordanes were significantly (p=0.009) and negatively correlated with VTG concentrations.

Of the 44 fat biopsies taken and analyzed for OC concentrations (Keller et al. 2004b), all but one was from normal, VTG-negative juveniles. The smallest female abnormally expressing VTG (SCL= 53.7 cm) had the second highest fat concentrations of 4,'4 -DDE and total PCBs on wet mass basis among the juvenile turtles analyzed (Figure 6, Table S5).

Adult sex differences in organochlorine concentrations

Because this study analyzed an additional set of blood samples from adult (>88 cm SCL) turtles for OC concentrations, sex differences could be examined (Table S6), albeit with a small sample size (n=3 females; n=3 males). Adult females had significantly higher PCB 138 and total PCBs ($p<0.05$). Other compounds, including dieldrin, total chlordanes, total DDTs, and total OCs were several fold greater in concentration in females than males, but not significantly different.

Discussion

This assessment of plasma VTG provides an improved understanding of the reproductive cycles of female loggerhead sea turtles. All five nesting females were expressing VTG and had the highest concentration of VTG compared to other groups of turtles. These findings are similar to the results of Smelker et al. (2014), in which nesting loggerheads had significantly higher VTG concentrations than turtles captured in water. They also corroborate the findings that VTG concentrations in green turtles are high and may even increase throughout the nesting season (Bruno et al. 2021). Presence of high circulating concentrations of VTG while on the nesting beach suggests that vitellogenesis of oocytes may still be occurring or that excess VTG has not yet been eliminated from the body. The latter is more likely because ample evidence shows that sexually mature females finish yolking all clutches of eggs before migrating to the mating and nesting areas (Miller 1997).

Not all breeding-size females caught offshore were expressing VTG, a finding that is also consistent with Smelker et al (2014). One female (turtle ID 2057, SCL= 95.5 cm) that was captured July 31, 2000 in waters offshore of Florida was not expressing VTG; this may be explained in many ways. Loggerhead turtles take at least a year off between nesting seasons (Miller 1997), and thus there may be rare times when VTG drops so low that it is undetectable by our methods. Vargas (2000) observed a cyclical pattern of VTG expression in nesting Kemp's ridley sea turtles with the lowest VTG concentrations occurring during the summer months of the nesting season. Since the nesting season of loggerheads in Florida is between April and September, it is possible that this female may have laid her final clutch of eggs early in the nesting season (Smelker et al. 2014). In addition, testosterone concentrations in nesting females are

known to drop stepwise with each clutch laid (Owens 1997). Concentrations as high as 300 pg/ml at the beginning of the nesting season decrease to less than 20 pg/ml after the last clutch is laid (Owens 1997). The testosterone concentration in this turtle, ID 2057, was low (15 pg/ml), which suggests that she either finished laying eggs for the season or that she did not lay that summer. Secondly, it is possible that this large and presumably old female is past her reproductive age. It is unknown if female sea turtles experience reproductive senescence and stop nesting at a certain age or size, although females that are greater than 95.5 cm SCL are seen on nesting beaches (Hawkes et al. 2005). Another possible explanation for the lack of VTG may be that this turtle was incapable of undergoing vitellogenesis due to a genetic or environmental cause. Her blood concentrations of OC contaminants were the fourth highest of all the turtles examined in this study, with the two highest being sick females and the third, a juvenile male. Adult females of many species, especially marine mammals, have lower concentrations than juveniles or adult males due to the maternal transfer of contaminants to eggs, milk, or tissues of offspring (Muñoz and Vermeiren 2020). If she had never produced eggs, it is possible that over a lifetime she would have accumulated OC levels greater than other turtles. It is unknown whether these levels could cause reproductive failure in sea turtles, but this should be the focus of future studies.

These data also helped describe reproductive maturation in loggerhead sea turtles. A better understanding of their basic biology will help researchers predict when individual turtles will join the reproductive age class. Based on the samples screened in this study, female loggerhead sea turtles along the southeast coast of the U.S. begin reproductive maturation around 77 cm SCL. From observations taken during necropsies,

77 cm SCL is the size threshold in which follicles begin to grow (David Owens, pers. observation). Although one nesting loggerhead was recorded at 74 cm SCL (Frazer and Ehrhart 1985), average nesting females along the U.S. Atlantic coast are 82 to 92 cm SCL (Smelker et al. 2014, Miller 1997; Frazer and Ehrhart 1985). This discrepancy indicates that females begin to produce VTG prior to active reproduction.

The ability to determine the sex of turtles is critical for sea turtle biology and conservation, but it has been a challenge (Figure 2). The sexes of juvenile turtles cannot be distinguished using external morphology; therefore, many studies rely on plasma testosterone to predict sex ratios (Owens 1997, Smelker et al. 2014). The findings of this study suggest that using VTG analysis in conjunction with testosterone and tail length measurements could help identify the sex of certain turtles that would otherwise be classified incorrectly or as unknown. VTG expression helped categorize five turtles as female that would otherwise be unknown or incorrectly identified as male. Two large turtles (turtle IDs 130 and 139) had testosterone concentrations much greater than 300 pg/mL, which can be typical for adult females (Owens 1997, Rostal et al. 1996). Turtles 2289 and 4026 had testosterone concentrations that fell between 200 and 300 pg/mL and turtle 1385 had inconclusive testosterone results. While presence of VTG can be misleading in abnormal males, all tail lengths and VTG expression in these turtles were consistent with those of females. Therefore, the use of VTG and tail length data in conjunction with plasma testosterone may increase the predictive ability of sexing these animals. Recently, Tezak et al. (2020) was able to obtain 90 % accuracy of sexing post-hatchling loggerhead sea turtles using anti-mullerian hormone (AMH). Future studies

should determine if AMH is a better sexing tool than testosterone and VTG for older life stages of loggerheads.

All OCs measured in these turtles are listed on the United Nation Stockholm Convention that seeks to protect human health and the environment from chemical contaminants that are persistent, bioaccumulative and toxic (Stockholm Convention 2019). Many OCs have been shown to disrupt the endocrine and reproductive systems of reptiles (Arukwe et al. 2016). A sexually dimorphic tail measurement was shown to be feminized in male snapping turtles collected from sites in the Great Lakes region that were more heavily contaminated with OC compounds (de Solla et al. 1998). Additionally, a population of American alligators inhabiting Lake Apopka, Florida, declined drastically following a pesticide spill that included DDT and dicofol (Guillette et al. 2000). The juvenile alligators from this lake showed signs of endocrine disruption, including decreased plasma testosterone concentrations and smaller phallus sizes (Guillette et al. 1996). These previous studies did not examine VTG. A more recent study found that male crocodiles from a South African farm located downstream of a sewage treatment plant were expressing the same amount of estrogen and VTG as females (Arukwe et al. 2016). This endocrine disruption, and reduced fertility and egg development observed at this farm, may be influenced by environmental contaminants, including 17α-ethynylestradiol, other pharmaceuticals, non-chlorinated and OC pesticides, detected in the farm waters (Arukwe et al. 2016). Therefore, OC contaminants could affect sea turtles in similar ways and more studies would help to understand the effects of pollutants on sea turtles.

Whether the OC concentrations in the sea turtle tissues resulted in abnormal VTG expression is inconclusive. On one hand, blood concentrations of certain OC compounds were significantly correlated with VTG concentrations. On the other hand, these relationships were so weak (tau < |0.15|) that they do not provide strong evidence of an estrogenic effect. Likewise, blood concentrations of PCB 138 were significantly greater in the abnormally VTG-expressing turtles than the normal group, suggesting that this relatively dominant PCB congener could be contributing to feminization effects. This result is challenging to corroborate with previous studies. PCBs are known to have complex endocrine actions, including crosstalk between the AhR and ER mechanisms (Safe 1995), and this particular PCB congener was not expected to have estrogenic activity based on human in vitro assays (Plíšková et al. 2005). This particular congener has not been tested for endocrine disruption in reptile or fish toxicology studies (Bergeron et al. 1994; Vega-Lopez et al. 2006; Jung et al. 2005; Calo et al. 2010). Furthermore, compounds with the greatest known estrogenic activity (e.g. 2,4'-DDD or 2,4'-DDT; EPA 2015) were not significantly different in concentration between the groups. Taken together these data suggest that the use of VTG as a biomarker of xenoestrogenic exposure in sea turtles remains inconclusive.

An estrogenic toxic equivalency factor (TEF) approach (e.g. Silva et al. 2001) was considered in the current study to address the additive estrogenic effects of the OC mixtures measured in the turtle tissues. The approach would have incorporated positive ER bioactivity factors of OC pesticides from the Environmental Protection Agency's Endocrine Disruptor Screening Program (EPA 2015) with positive and negative ER-binding factors for PCB congeners from Plíšková et al. (2005). After considerable debate,

we determined the approach was more risky than simply assessing OC concentrations against VTG expression. OC mixtures certainly interact with multiple receptors and biochemical processes; some of which induce estrogen responses, while others inhibit them (Plíšková et al. 2005; Safe 1995). In aquatic organisms, the exposure duration influences the direction of the effect (Calo et al. 2010). In fact, Safe (1998) warned that TEFs should only be used after they have been validated in animal models. The estrogenic and anti-estrogenic combined effects of PCB and OC pesticide mixtures have not been tested in reptiles. In fact, standardized human in vitro ER-binding screening tests have not even been performed to assess known concentrations of mixtures of PCBs and OC pesticides (Vinggaard et al. 2021). Because of the endocrine pathway complexities and large unknowns for reptiles, the TEF approach was abandoned in this study. Future studies are sorely needed to address the effects of environmentally relevant concentrations of contaminant mixtures in many animal models.

The adult females (>88 cm SCL) had greater concentrations of OCs in their blood than the adult males. This is contrary to results in marine mammals, in which males have dramatically greater concentrations than females, because of reproductive offloading mechanisms that males lack, allowing males to continually accumulate POPs (Yordy et al. 2010). Evidence of maternal offloading to sea turtle eggs is supported by significant correlations between maternal blood concentrations and deposited egg concentrations for green and leatherback turtles (van de Merwe et al. 2010, Stewart et al. 2011, Guirlet et al. 2010; Munoz and Vermeiren 2019). This has yet to be tested in loggerhead sea turtles. Few studies have tested whether maternal offloading leads to substantial differences between adult female and adult male POP concentrations, and the few results are

inconsistent. Ragland et al. (2011) observed one group of adult male loggerheads captured near Cape Canaveral, Florida, having greater blood POP concentrations than any other group of loggerhead turtles previously analyzed (Keller 2013). Barraza et al. (2020) did not detect a difference in blood POP concentrations between adult male and female green turtles in San Diego Bay, California. Likewise, Clukey et al. (2018) did not detect a difference in fat POP concentrations between adult male and female olive ridley sea turtles in the pelagic realm of the Pacific Ocean. More studies with larger sample sizes of turtles throughout the reproductive cycle of sea turtles would help explain the lack of sex difference.

Several factors may theoretically explain the greater OC concentrations in the blood of adult females than males. Temporal differences can be ruled out because they were captured in the same year. Possibly, the females had never nested, so they were still awaiting their first chance to offload OCs to their developing eggs. The females were 88.6 cm, 91.9 cm, and 95.5 cm SCL, so the smallest turtle is on the small end of reproductive maturity, and the uncertainty is great for assigning reproductive maturity of sea turtles using SCL (Miller 1997). Foraging location strongly influences the accumulated OC concentrations in sea turtles (Alava et al. 2011, Ragland et al. 2011, Keller 2013). It is possible the small sample size of these females (captured at 32.95 °N, 32.86 °N, and 30.30 °N) in comparison to males (captured at 32.94 °N, 32.46 °N, 32.46 °N) spent more time foraging further North along the U.S. Southeastern coast, where contaminants are greater than toward the South. However, their summer capture dates in June and July and latitudes do not support this explanation. Brunswick, Georgia (near 31 °N), deserves a brief discussion, as the coastal area around this city is heavily

contaminated by a higher-chlorinated pattern of PCBs due to Aroclor 1268 being released from a Superfund Site (Maruya et al. 1997; Kannan et al. 1998). The six adult turtles were not captured in close proximity to Brunswick, but one female was closer than the other five. None of the adults were in the top five contaminated sea turtles, but one of the top five PCB turtles was captured near Brunswick (turtle ID SC CC2050) and it had a soaring 34 % of its total PCBs as PCB 206, indicating that it had been foraging near Brunswick for enough time to take on the highly chlorinated PCB pattern of this region (Figure 1). In 1998, five diamondback terrapins from near Brunswick had 30% of its tissue PCB concentration consisting of nona-chlorinated biphenyls (like PCB 206; Kannan et al 1998). Aroclor 1268 contains 35% nona-chlorinated biphenyls (Kannan et al 1997). A final theoretical reason, and the most likely according to the authors' opinions, for the sex difference among adults is that blood concentrations of OCs can fluctuate drastically, (Keller et al. 2004b, Barrazza et al. 2020), especially with periods of foraging, migration, and reproductive behaviors (Keller 2013).

Considering the latter hypothesis above, we created a theoretical model of OC concentrations in the blood of adult male and female turtles based on behaviors, hormones, and VTG concentrations throughout a typical three-year female nesting season (Figure 7). Much of the detail on Figure 7 is not empirically known, rather hypothesized, and based on the best available information. This model suggests that it is possible for adult females during periods of strong vitellogenesis or intensive lipid mobilization to have greater OC concentrations than males. Hormone trends were taken from Owens (1997). OC concentrations in the blood are influenced by food intake, storage into or mobilization from fat reserves, and storage into follicles. The latter is missing for males,

making their theoretical model simpler. The average female loggerhead turtle consumes prey on her foraging ground for 2.5 years before migrating to breeding grounds just offshore of the nesting grounds and then lays two to four clutches of on average 112 eggs each per season with approximately 14 d between clutches (Miller et al. 2003). It is thought that during migration and nesting, sea turtles reduce or stop foraging (Perrault and Stacy 2018), so POP intake from food is cyclical. Leatherback turtles that remain an extra year on their foraging grounds produce eggs with greater concentrations of OCs than those that only forage for two years (Guirlet et al. 2010), suggesting that much of OCs transferred into the eggs may be from dietary intake instead of fat reserves (Munoz and Vermieren 2020). This may diminish differences seen between adult males and females. The cue for migration to the nesting grounds is not fully known, but plasma estrogen concentrations drop rapidly and plasma testosterone surges just before migration in females (Figure 7; Owens 1997). The fluctuations in plasma VTG concentrations across a reproductive cycle are not well known (Munoz and Vermieren 2020), but they should fluctuate with plasma estrogen concentration, which have been tested across reproductive cycles. VTG was detectable in post-mating *L. kempii* for seven months, which would be well after a complete nesting season (Rostal et al. 1998). Injections of estradiol lead to rapid and large VTG production that remained high for 31 weeks (Heck et al. 1997). The current study showed that at least one adult female had nondetectable VTG, so at some point during quiescence or senescence, VTG concentrations likely diminish. The OCs offloading mechanism in females requires the mobilization of OCs from fat reserves to the eggs through the blood, which happens during vitellogenesis, or follicle development, which takes place eight to ten months before breeding season

(Miller et al. 2003; Figure 7). Since the protein VTG transports lipids, especially triglycerides, from the blood to the developing oocytes to become egg yolk (Hamann et al. 2003), VTG also likely carries OCs from fat reserves and dietary intake (Munoz and Vermieren 2020). During periods of high VTG concentration in the blood of adult females or during periods of intensive lipid mobilization when females are nesting without eating, OC blood concentration may be expected to peak throughout one reproductive cycle. The females in this study may have been tested during this hypothesized period of peak blood OCs, making their concentrations greater than males. Once more than 300 eggs have been laid, females have offloaded a portion of their body burden of OCs that may become lesser than males (Figure 7).

The current data are inconclusive as to whether these environmentally relevant concentrations of contaminants could disrupt the loggerhead sea turtle endocrine system. The cause for abnormal VTG expression in ten juveniles, one being a male, is unknown but a number of reasons, both natural and human-influenced, could be speculated. Sea turtles naturally grow at different rates, so the smaller VTG-positive females may actually be much older than expected based on average size at age estimates. Exposure to exogenous natural estrogens, which were not measured, may also explain the VTG expression in these small turtles. It is possible, though unknown, that these animals had recently consumed prey items that had high concentrations of estrogens. However, as observed in fish, uptake of estrogens through the digestive tract may not be sufficient to induce VTG (Frederick et al. 2002). Lastly, exposure to estrogen-like contaminants may be another possible cause for the production of VTG in these turtles. Though not statistically significant, the average blood concentrations of PCBs were 8.5 % higher in

the nine VTG-expressing juveniles compared to the 46 normal juveniles. In addition, relatively high fat concentrations of total PCBs and 4,4'-DDE were found in the smallest abnormal female. These results do not eliminate the possibility that OC contaminants may have played a role in inducing VTG, but many other man-made estrogen-like contaminants (e.g., nonylphenol, bisphenol A) were not measured in these samples.

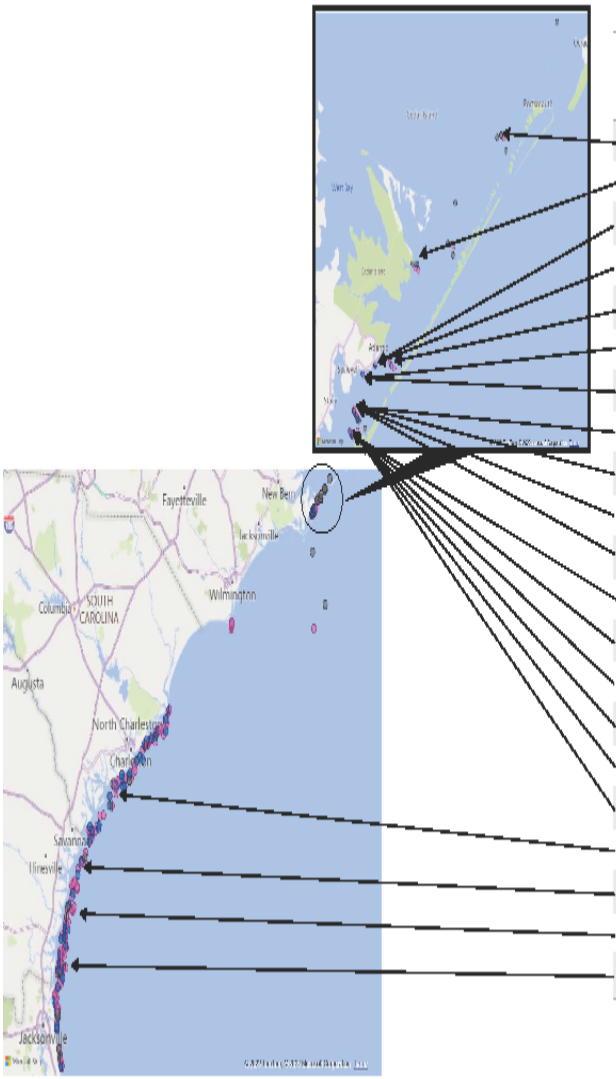
Only 2.3 % of the juveniles were expressing VTG, including one male. This may initially seem insignificant, but the loggerhead sea turtle is a threatened species, and this is a very small subsample of the population. From 1989 to 2006 the Florida population experienced a 43 % decline in nest density, rebounding only recently (Witherington et al. 2009). However, estimating sea turtle populations is challenging and often there are global overestimates, which can lead to mismanagement of these populations (Casale and Ceriani 2020). Therefore, endocrine disruption of 2.3 % of juveniles could be significant at the population level and this number could be increasing over the years. Another study performed by Zaccaroni et al. (2009) found VTG expression using western blot analysis in 75.4 % of the loggerhead juveniles (CCL<75 cm) in Italian waters; however, they did not examine environmental contaminants. It is currently unknown if abnormal expression of VTG causes reproductive problems in sea turtles as has been noted in fish (Cheek et al. 2001). Fish exposed to an estrogen-like contaminant (2,4'-DDT) for 8 weeks exhibited VTG induction and reduced fertility and hatching success. The relative contribution of contaminants as a threat to sea turtle populations still requires more research.

Conclusions

This study shows that most female loggerhead sea turtles from the southeast coast of the U.S. enter vitellogenesis at 77 cm SCL. The detection of VTG in these larger

females helps to describe the transition from juveniles to adults. Determining this threshold is important to use VTG as a biomarker for estrogen-like contaminant exposure. Ten turtles smaller than the threshold were abnormally producing VTG, including one juvenile male. These abnormal juveniles did not have significantly higher OC contaminants in their blood. Evaluating the OC contaminants in the fat, only one abnormal juvenile, an abnormal female, was analyzed, and it had the second highest total PCB and 4,4'-DDE concentrations. Therefore, it is inconclusive as to whether these environmentally relevant concentrations of OCs are disrupting the endocrine system of loggerhead sea turtles and further studies are required to examine the effectiveness of VTG as a biomarker for estrogen-like contaminants. The new empirical data provided here on testosterone, VTG, and OC concentrations provides an updated hypothetical model of these compounds through the reproductive cycle of loggerhead sea turtles.

Figure Captions



Turtle ID	Sex	SCL (cm)	Abnormal VTG (Y/N)	Top 5 PCB (Y/N)	Top 5 DDT (Y/N)	Top 5 Total Chlordanes (Y/N)
JMK 3-62	U	54.4	Y	N	N	N
JMK 3-66	U	55.3	Y	N	N	N
NC 1235	M	67.1	N	N	N	Y
NC 1057	M	71.3	N	Y	Y	Y
JMK 5-47	F	74.4	Y	N	N	N
JMK 5-39	M	68.0	Y	N	N	N
JMK 5-37	M	68.1	Y	N	N	N
NC 1328	F	64.7	N	Y	Y	Y
JMK 5-42	U	71.1	Y	N	N	N
JMK 3-26	F	53.7	N	N	Y	Y
JMK 3-1	F	63.6	Y	N	N	N
NC 1301	M	57.8	N	N	N	Y
JMK 3-37	F	66.5	N	N	Y	N
JMK 3-7	F	72.2	N	N	Y	N
JMK 3-33	M	66.3	N	Y	N	N
JMK 3-6	F	52.3	Y	Y	N	N
JMK 3-43	F	53.7	Y	N	N	N
SC CC0024	F	61.1	Y	N	N	N
SC CC4105	F	66.0	Y	N	N	N
SC CC2037	F	76.9	Y	N	N	N
SC CC2050	F	74.6	N	Y	N	N

Figure 1. Map of capture locations of all loggerhead turtles sampled (n=404). Circles are pink for females (F), blue for males (M), and gray for turtles of unknown sex (U) sex. The table on the right lists 12 sea turtles abnormally expressing vitellogenin (VTG) and/or the turtles with the top five highest polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), and total chlordane concentrations. The turtle ID, sex, and straight carapace length (SCL) are provided.

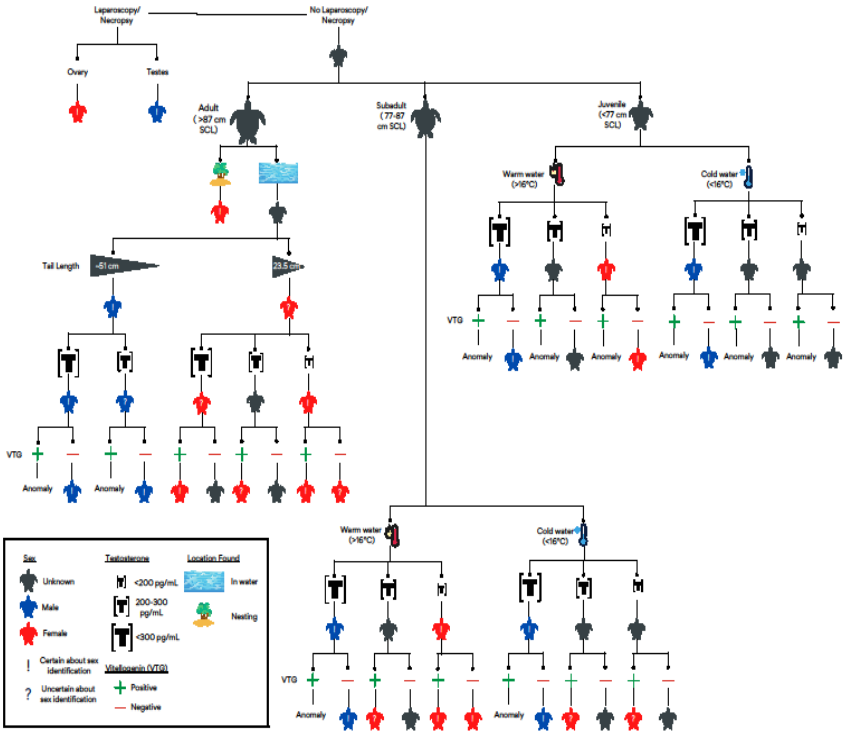


Figure 2. Flowchart of the process used for sexing sea turtles. The specific values provided in the chart for straight carapace length (SCL), tail length, water temperature, and testosterone concentrations are specific to only loggerhead sea turtles from the region of this study and would need to be adjusted for other species and locations. Each scenario ends with a male= blue, female= red, or unknown sex= gray with a level of classification certainty (!= certain,?= uncertain). The anomalies deviate from what is expected, so a sex classification is not provided.

Figure 3. Western blots of plasma from a) five nesting loggerhead sea turtles (labeled B1-B5) and two vitellogenin (VTG)-negative juvenile females (61 cm and 49 cm straight carapace length (SCL), respectively), b) female loggerheads captured in water ranging in SCL, and c) one male loggerhead (sex confirmed by laparoscopy) captured twice abnormally expressing VTG. A polyclonal antibody raised against VTG from a freshwater turtle, *T. scripta*, was used to visualize VTG. MW= molecular weight markers (apparent molecular weights shown along left in kDa); Ts E= estrogen-induced *T. scripta* (positive control); Ts C = control male *T. scripta* (negative control). SCL in cm of each

turtle is listed above each lane. Presence (+) or absence (-) of VTG is depicted under each lane.

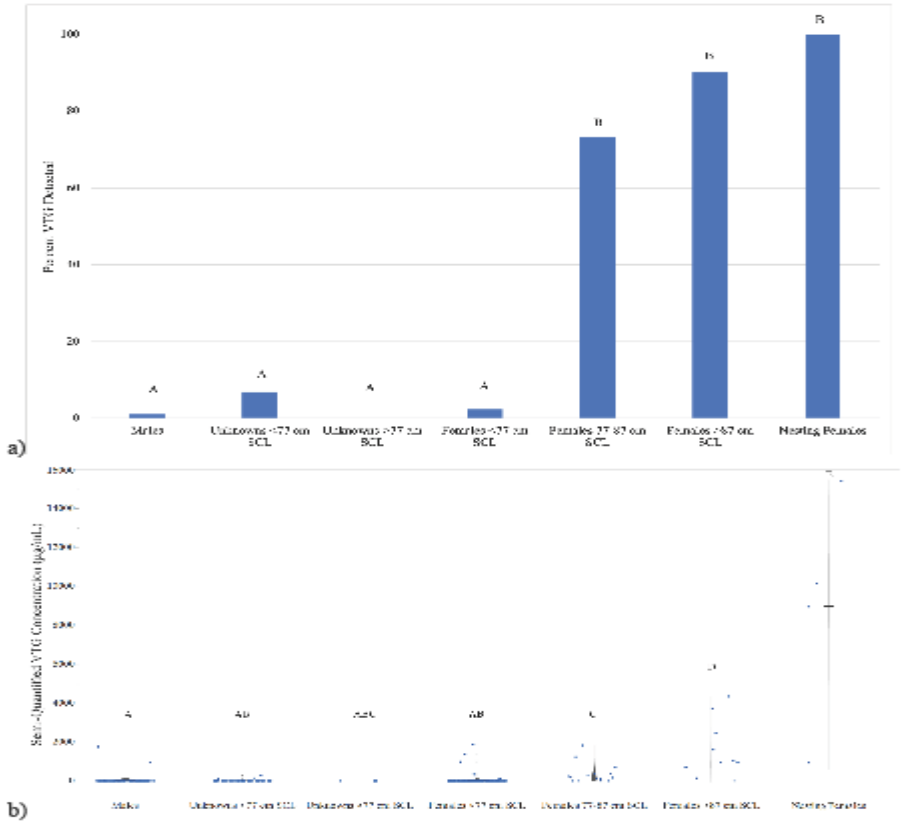


Figure 4. Vitellogenin (VTG) a) detection via western blots and b) semi-quantified concentrations in loggerhead sea turtles: immature females <77 cm straight carapace length (SCL, n= 216) and 77-87 cm SCL (n= 15), mature females >87 cm SCL (n= 10), nesting females (n= 5), males ranging from 50.6 cm to 94.7 cm SCL (n= 97), unknown sex <77 cm (n= 58), and unknown sex >77 cm (n= 3). Different letters within a plot indicate significant differences (p-value <0.05) among turtle groups. A Fisher exact test was performed in a, and Wilcoxon pairwise comparisons were tested in b.

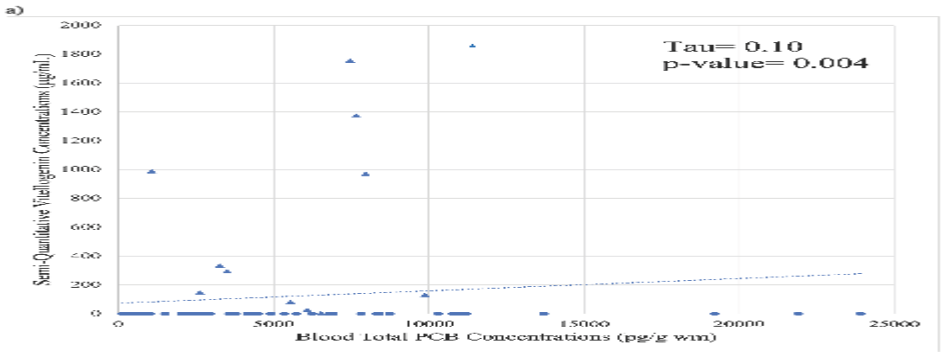


Figure 5. Correlations of semi-quantitative vitellogenin (VTG) concentrations (µg/mL) in loggerhead sea turtles <77 cm straight carapace length with a) blood total PCB concentrations and b) blood total DDT and total chlordane concentrations (pg/g wet mass). Raw data are plotted as a scatterplot and a linear trend line is shown for visualizing the direction of the slope. Circles represent sea turtles not expressing VTG and triangles represent those expressing VTG. The statistics shown are from R NADA Kendall Tau correlations that take into account the non-detects.

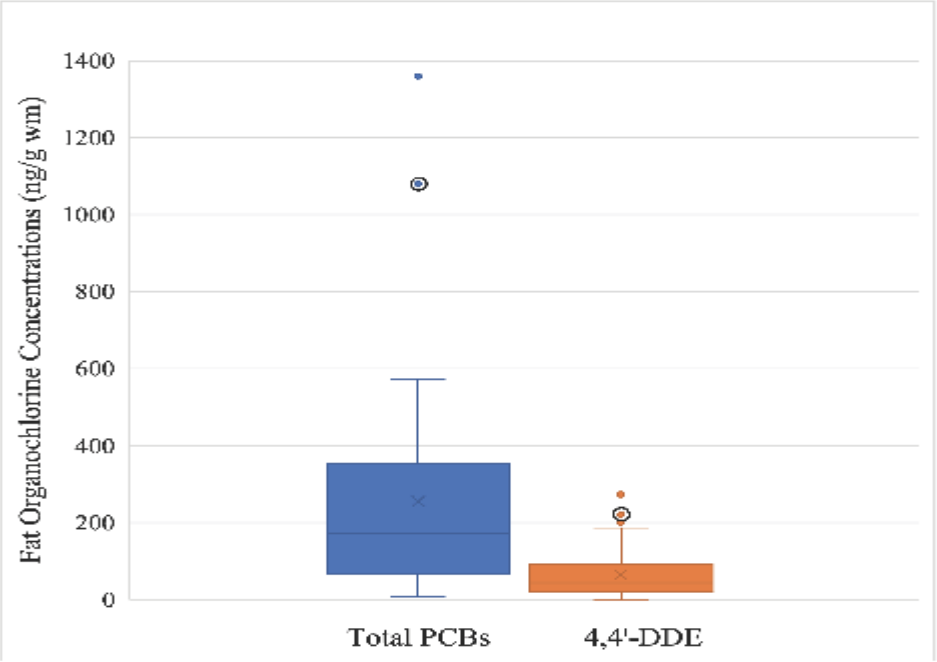


Figure 6. Box and Whisker plot of the total PCB and 4,4' DDE concentrations (ng/g wet mass) in the fat of immature loggerhead sea turtles (n= 44). Data points circled belong to an abnormal female expressing vitellogenin (SCL= 53.7 cm). Data was previously published in Keller et al. 2004b.

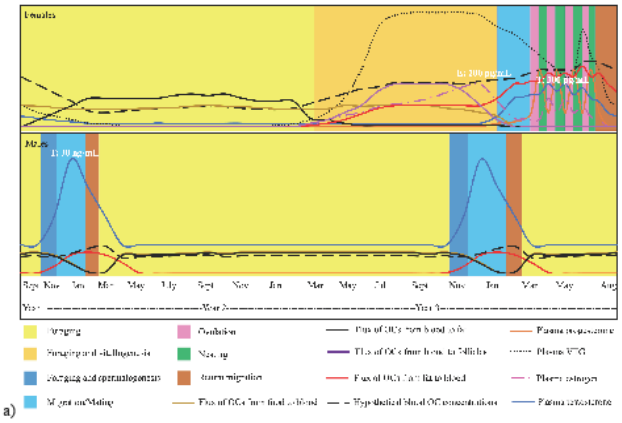


Figure 7. Theoretical models of (a) hormone, vitellogenin (VTG), and organochlorine contaminant (OC) concentrations for female and male loggerhead sea turtles through a typical three-year female reproductive cycle. Background colors represent behaviors, lines hypothesize hormone, VTG, and OC concentrations based on data from this study and other publications (behaviors, fat stores and follicle development: Wibbles et al. 1990, Miller 1997, Miller et al. 2003, Rostal et al. 1997, Owens 1997, Hamann et al. 2003; hormones: Owens 1997, Myre et al. 2016, VTG: Heck 1997, Ho 1987, Rostal et al. 1998, Vargas 2000, Hamann et al. 2003, Myre et al. 2016, Bruno et al. 2021; OCs: Rybitski et al. 1995, Alava et al. 2011, Ragland et al. 2011, Keller 2013). Flux refers to the estimated rate of change from one tissue to the next. (b) Graphic showing the pathways of OC tissue distribution with arrows that represent the flux (rate of change) from one tissue to the next and hypothesized OC concentrations in three tissues at the beginning of behavioral timeframes. Thicker arrows represent a faster rate of change. OC concentration ranges (ng/g) are represented by letters. Lower case letters are concentrations near the minimum of the range, capital letters are near the maximum of the range.

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