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Using Plasma Vitellogenin in Loggerhead Sea Turtles to Assess Reproductive Maturation and Estrogen-Like Contaminant Exposure

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Abstract: Vitellogenin (VTG), an egg yolk precursor, is abnormally produced by male and juvenile oviparous species after 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. The presence of VTG was screened in plasma from 404 loggerheads from the northwestern Atlantic Ocean using a freshwater turtle antibody in western blots. The concentrations of VTG were semiquantified using band intensities calibrated to results from a loggerhead antibody enzyme-linked immunoassay. The detection and concentrations of VTG were in (from highest to lowest): nesting females, in-water adult females, subadult females, smaller females, unknown sex, and males. Loggerheads from this region begin vitellogenesis at ≅77 cm straight carapace length. 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 compared among 43 VTG-negative juveniles. The nine VTG-abnormal turtles had average blood PCB concentrations 8.5% higher, but not significantly different, than 46 VTG-negative juveniles ($p = 0.453$). In turtles less than 77 cm, blood PCB concentrations were significantly, but weakly, correlated with semiquantified VTG concentrations (tau = 0.1, $p = 0.004$). Greater blood OC concentrations were found in adult females than in 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. *Environ Toxicol Chem* 2023;42:1309–1325.

Keywords: Endocrine disruptors; Endocrine-disrupting compounds; Persistent organic pollutants; Organic contaminants

INTRODUCTION

All sea turtle species are on the International Union for Conservation of Nature Red List (2022). Their imperiled population status warrants the examination of chemical pollutant threats,

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because contaminants may negatively impact development and reproduction, which could translate into fewer or abnormal offspring and hence population declines. Loggerhead sea turtles (*Caretta caretta*) are broadly distributed in subtropical regions of the Atlantic, Indian, and Pacific Oceans, with the majority of nests occurring along the western coasts of the Atlantic and Indian Oceans (Conant et al., 2009; US National Marine Fisheries Service and US Fish and Wildlife Servie [U.S. NMFS & U.S. FWS, 2008]). In the southeast United States, the focus of the present study, most nesting occurs across a span of 2400 km of beach from Alabama to North Carolina (U.S. NMFS & U.S. FWS, 2008). This stock is categorized as threatened by the US Endangered Species Act (U.S. NMFS & U.S. FWS, 2008), and when aggregated on nearshore immature foraging grounds along the east coast of the United States, they have a sex ratio is 2:1 female:male (Wibbels, 2003). Sea turtle sex is not determined genetically, but rather by a cascade of hormones triggered by very specific temperatures during embryonic development (Wibbels, 2003; Yntema, 1976). Sex of immature sea turtles cannot be identified from external morphology, and hence plasma testosterone concentrations or 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. It first circulates in the blood to be deposited as a major protein precursor for egg yolk in oocytes. It 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 (Bruno et al, 2021; Myre et al., 2016; Smelker et al., 2014; Vargas, 2000). In nesting female loggerheads from Hutchinson Island, Florida (USA), VTG peaked in June with relatively high concentrations (15.37 mg/ml) and decreased toward the end of the nesting season; it 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 (*C. mydas* and *C. mydas agassizii*) and loggerhead sea turtle blood (Bruno et al., 2021; Herbst et al., 2003; Sifuentes-Romero et al., 2006; Smelker et al., 2014). Using ELISAs, nesting female loggerheads were found to have greater VTG concentrations than immature loggerhead turtles captured along the southeast coast of the United States (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 & Palmer, 1995; Rey et al., 2006; Verderame et al., 2016). 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 on 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 United States, 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 & 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. (2008) 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. In contrast, in freshwater turtles (*Chrysemys picta*), reduced VTG concentrations were found in females from a lake near a Superfund site on Cape Cod, Massachusetts (USA), which is contaminated with a mixture of inorganic and organic contaminants, most notably trichloroethene and ethylene dibromide, compared with a reference lake (Kitana et al., 2006; Rie et al., 2004). The results of 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 in the Stockholm Convention, including organochlorines (OCs), polybrominated diphenyl ethers, and perfluorooctane sulfonate (Keller, 2013). We focused specifically on one class of POPs, the OCs, because they were measured previously in the blood and fat of the same turtles sampled in the present 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, which can be found by means of a less invasive sampling technique (Keller, 2013; Keller et al., 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 (Camacho et al., 2013; Keller et al., 2004c; van de Merwe et al., 2010), 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 & Palmer, 1995). In other reptiles, endocrine disruption by OC pesticides, such as DDT, dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyldichloroethane (DDD), dieldrin, and chlordanes, have been observed in South African crocodiles (*Crocodylus niloticus*) and Floridian American alligators (*Alligator mississippiensis*; Arukwe et al., 2016; Guillette et al., 2000; Heinz et al., 1991; Milnes et al., 2005). Exposure to OCs has been 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 (Plísková et al., 2005; Safe, 1995). 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 (Calò et al., 2010; Jung et al., 2005; Vega-López et al., 2006), but the effect becomes anti-estrogenic with longer exposure times (Calò et al., 2010). The 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 (see Akingbemi & Hardy, 2001; Hayes et al., 2002; Kime, 1999; Sheehan et al., 1999). 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., 2002). Because adult sea turtles have low concentrations of circulating estradiol (10–50 pg/ml; Owens & Morris, 1985), marked VTG response to a single injection of estradiol (Heck et al., 1997), exposure to a mixture of contaminants (Keller, 2013), and similar hormones and receptors as the endocrine-disrupted vertebrates mentioned above (Owens, 1997), sea turtles may be sensitive to environmental contaminants that have estrogen-like activity.

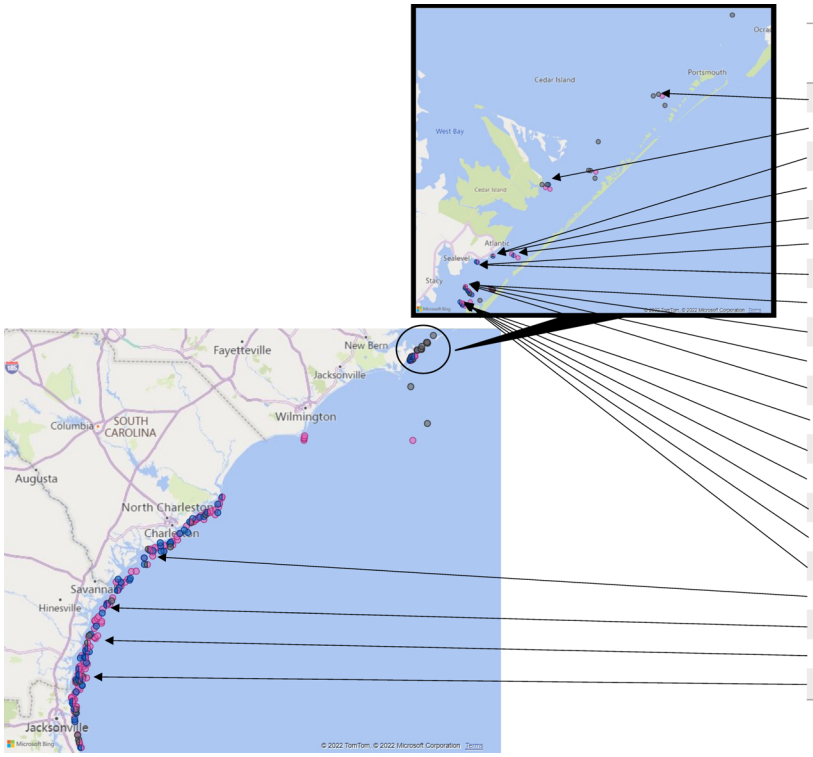
Another potential research use of VTG expression is as a sexing tool for sea turtles, used in concert with the testosterone sexing method. Understanding the sex ratio of sea turtle aggregations is important for modeling population growth rates, which is critical for assessing extinction risk for each species. However, sexing sea turtles is challenging because their lack of sex chromosomes makes laparoscopic examination of the gonads the only definitive sexing tool, but its use is limiting as it requires expertise to perform minor surgery (Braun-McNeill et al., 2007; Owens, 1997; Wibbels, 2003). 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; Jensen et al., 2018; Shertzer 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). In addition, 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., whether the turtle will 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 our study were to investigate the use of VTG in loggerhead sea turtles, with the aim of (1) further understanding reproductive maturation, (2) using it as a supplemental sexing tool, and (3) using it 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 with those of juvenile turtles that were not expressing VTG.

MATERIALS AND 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–November of 1998–2002) as bycatch in the pound net fishery (Epperly et al., 2007), from offshore waters of South Carolina, Georgia, and Florida (USA; summers of 2000 and



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 blood concentrations. The turtle ID, sex, and straight carapace length (SCL) are provided.

2002) in scientific trawling (Arendt et al., 2012); five of them were nesting females on Bald Head Island, North Carolina (USA; 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 (Supporting Information, Table S1).

Blood samples (≤2.2 ml/kg) were drawn within 15 min 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). 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 to 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 the remaining 372 turtles was 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 as female, and 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. Although 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 US nesting females (Frazier & Ehrhart, 1985; Miller, 1997; NMFS, 2008). Sexually mature and reproductively active females (>88 cm SCL min) 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.

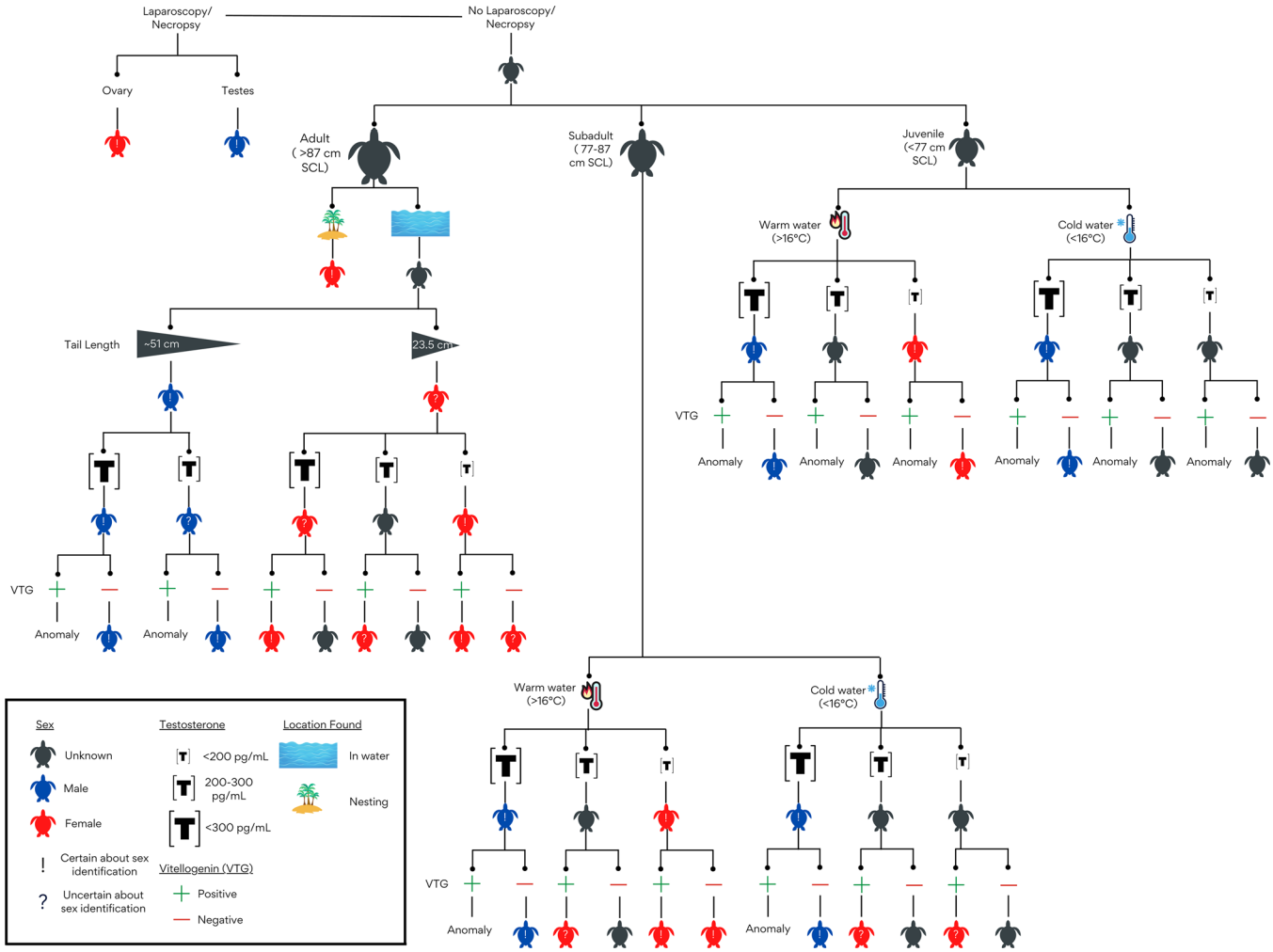


FIGURE 2: Flowchart of the process used for sexing sea turtles. The specific values provided in the chart for straight carapace length (SCL), tail length, water temperature, and testosterone concentrations are specific to only loggerhead sea turtles from the region of the present study and would need 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. VTG = vitellogenin.

VTG 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 & 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 markers were used on each blot, but the source and molecular weight 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% sodium dodecyl sulfate, 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. The proteins were transferred onto polyvinylidene difluoride 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 g/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++ and 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 the ECL++ Western Blotting Detection System (Amersham Biosciences). Detection was performed by autoradiography with four different time exposures (30 s, 1 min, 3.5 min, and between 6 and 10 min); exposure duration was recorded. If there was uncertainty as to whether VTG was present or absent, the sample was re-analyzed using 60–75 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) 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 and 2000 ng/ml. The unknown loggerhead plasma samples from nesting females were diluted 1:75 000 or 1:100 000. A rabbit anti-loggerhead VTG primary antibody was used, and the secondary antibody was goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad). An in-house plasma control sample from a VTG-positive olive ridley sea turtle was used as a positive control. Six wells/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/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 the RSDs were too high (10.3%–41.4%).

VTG semiquantified 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 (Ver 1.52k). Background corrections were performed following the 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 interblot 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 (Supporting Information, 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 semiquantitative, and reported VTG concentrations should not be directly compared with data outside of the present study. The limit of detection was determined first by using the turtle with the smallest band intensity in the following calculation (ng B5 VTG × [(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 multiplied by 3 to obtain a conservative limit of detection of 12.636 µg/ml.

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, recapture, large turtle, and/or VTG positive, and analyzed using similar techniques. Briefly, all blood samples (5 g each) were spiked with mass-labeled OC 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 semipreparative aminopropyl silane column or silica solid-phase extraction columns. Compounds of interest were measured using gas chromatography with either micro-electron capture detection or mass spectrometry. Limits of detection were approximately 1 ng/g wet mass for fat and 10 pg/g wet mass for blood. Calibration curves were prepared from National Institute of Standards and Technology 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.

Statistical analysis

All statistical analyses 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 semiquantified VTG concentrations and OC blood concentrations among groups of turtles binned by sex, size, and normal versus abnormal VTG expression were determined by parametric or nonparametric tests depending on assumptions of normality and homogeneity of variance. A Fisher's 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. Semiquantitative 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, semiquantified VTG, and final sex determinations can be found in the Supporting Information, 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. Our results showed that 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 (Supporting Information, Table S2). After using all tools available (Figure 2) and recognizing that some tools were missing for certain turtles, the final sex ratio was 241 female, 97 male, and 61 unknown sex, excluding the five nesting females.

The presence of VTG on the western blots helped assign the sex of five turtles. Two adult sized turtles (92.3 and 103.5 cm SCL) captured in-water had short tail lengths in the range of the other females (24.1 and 19.2 cm $Tail_{P\text{-}T}$; 7.8 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 and 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 semiquantified VTG concentrations were 360, 260, and 1240 μg/ml, respectively. This suggests that these three turtles were female.

VTG 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 the 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

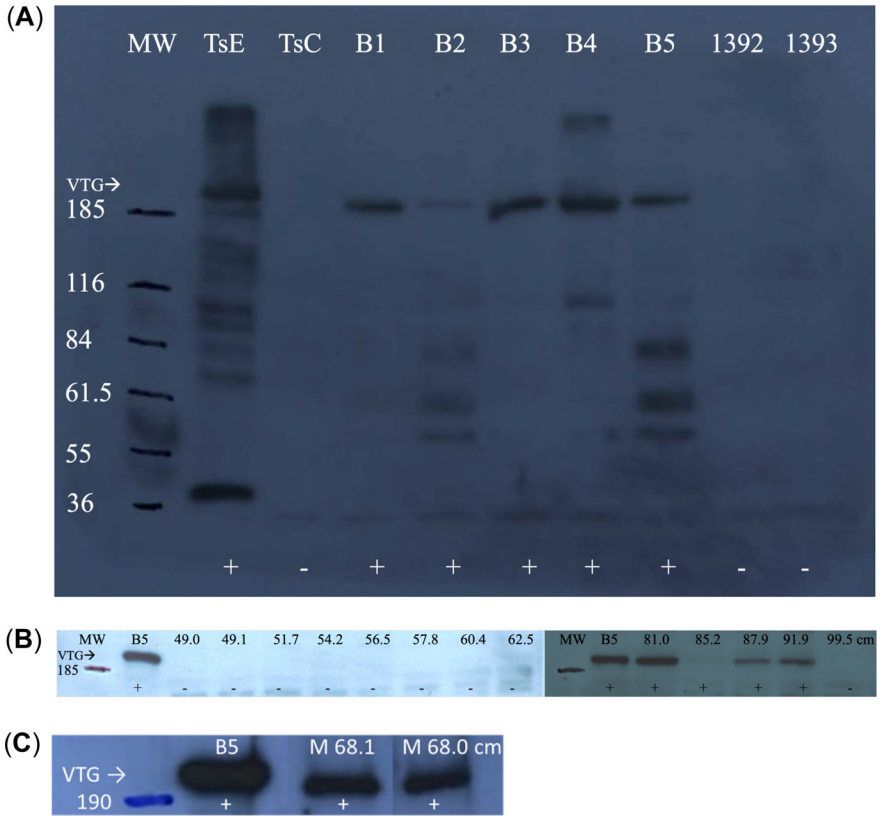


FIGURE 3: Western blots of plasma from (**A**) five nesting loggerhead sea turtles (labeled B1–B5) and two vitellogenin (VTG)-negative juvenile females (61 cm and 49 cm straight carapace length [SCL], respectively), (**B**) female loggerheads captured in water ranging in SCL, and (**C**) one male loggerhead (sex confirmed by laparoscopy) captured twice abnormally expressing VTG. A polyclonal antibody raised against VTG from a freshwater turtle, *Trachemys 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). The SCL in cm of each turtle is listed above each lane. Presence (+) or absence (–) of VTG is depicted under each lane.

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 (Figure 3A and Supporting Information, Figure S2), suggesting that, in its present form, this particular antibody is not specific to VTG alone; 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 less than 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, $R^2 = 0.937$; Supporting Information, Figure S1), indicating that the western blots were detecting the intended protein and were semiquantitative. Interblot variability was examined using plasma from five immature females in which the same plasma sample/female was replicated on multiple blots (RSD > 10%; Supporting Information, Figure S3). One likely reason for the large interblot 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. The VTG concentrations were averaged across blots for each turtle (Supporting Information, Table S3).

Differential expression of VTG 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 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, whereas 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 (50.6–94.7 cm), unknown sex <77 cm, and unknown sex >77 cm. This suggests that the threshold for normal VTG production is 77 cm SCL.

Semiquantified plasma VTG concentrations from VTG-positive turtles captured in-water ranged from 4.21 to 4340 μg/ml. The VTG concentrations were significantly different among turtle sex and size groups, as described next (Figure 4B). Male semiquantified VTG concentrations (2.0% detected, <12.6 μg/ml median; <12.6–1760 μg/ml) were similar to those of females <77 cm (3.7% detected, <12.6 μg/ml median; <12.6–1860 μg/ml) and were significantly lower than all other female groups. Nesting female semiquantified VTG concentrations (100% detected, 8980 μg/ml median; 932–15 400 μg/ml) were significantly greater than VTG concentrations in all other female turtle groups captured in-water. The semiquantified VTG concentrations significantly increased with SCL of the in-water female groups: from females <77 cm (values listed above), females between 77 and 87 cm (73% detected, 170 μg/ml median; <12.6–1830 μg/ml), to females >87 cm (90% detected, 963 μg/ml median; <12.6–4340 μg/ml). Within females, the semiquantified VTG concentration significantly correlated with the SCL (Supporting Information, 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 $Tail_{P\text{-}T}$ and 5.5 cm $Tail_{C\text{-}T}$). 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 VTG by individual loggerhead turtles

Nine juvenile turtles smaller than 77 cm SCL (total of 10 plasma samples due to one juvenile recapture) were expressing VTG, which we consider abnormal (Figures 3 and 4 and Supporting Information, Figure S4); VTG was detected in the plasma of 5 of the 216 female turtles and in 4 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 semiquantitative VTG concentrations of 1860 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 4 years; therefore, they were not clustered spatially or temporally (Figure 1).

One juvenile male was captured three times (turtle ID JMK 3-40, JMK 5-39, and JMK 5-37) throughout 2000 to 2002 in waters of North Carolina. On 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 semiquantified concentrations of 970 and 1760 μg/ml (Figure 3C). Sex was confirmed by laparoscopy during the first capture. This male was grouped with the other nine juveniles that were abnormally expressing VTG for assessing differences in plasma contaminant concentrations.

Assessment of VTG as a biomarker

Blood OC concentrations were compared among the nine juveniles that were abnormally producing VTG (total of 12

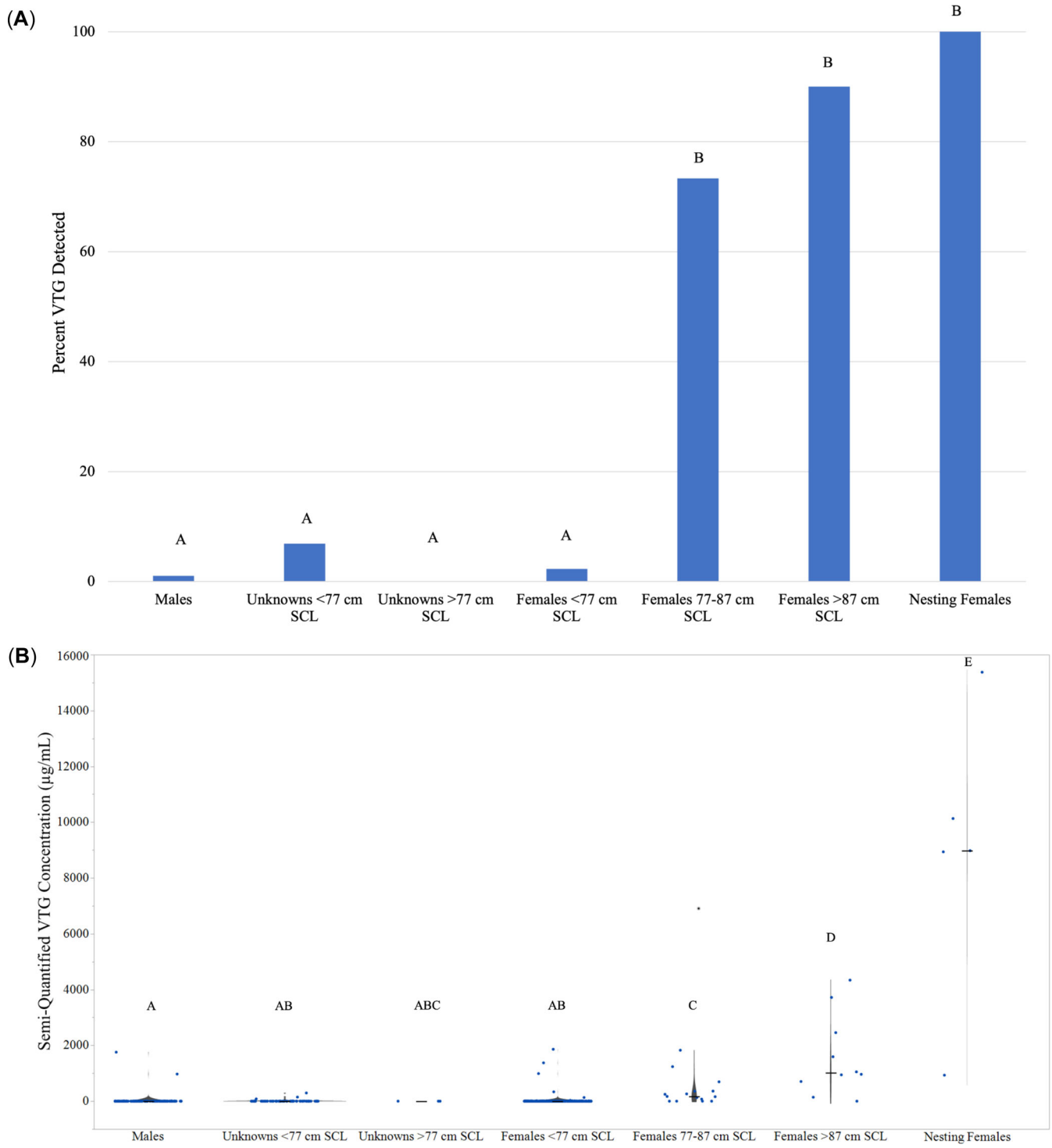


FIGURE 4: Vitellogenin (VTG) (**A**) detection via western blots and (**B**) semiquantified concentrations in loggerhead sea turtles: immature females <77 cm straight carapace length (SCL; $n = 216$) and 77–87 cm SCL ($n = 15$), mature females >87 cm SCL ($n = 10$), nesting females ($n = 5$), males ranging 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's exact test was performed in (**A**), and Wilcoxon pairwise comparisons were tested in (**B**).

blood samples measured for OCs) and 53 juveniles that were considered normal because they were not expressing VTG (Supporting Information, 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 ($p = 0.453$; Supporting Information, Figure S5). Of the many individual PCB congeners that were detected, none were significantly different between the normal and abnormal

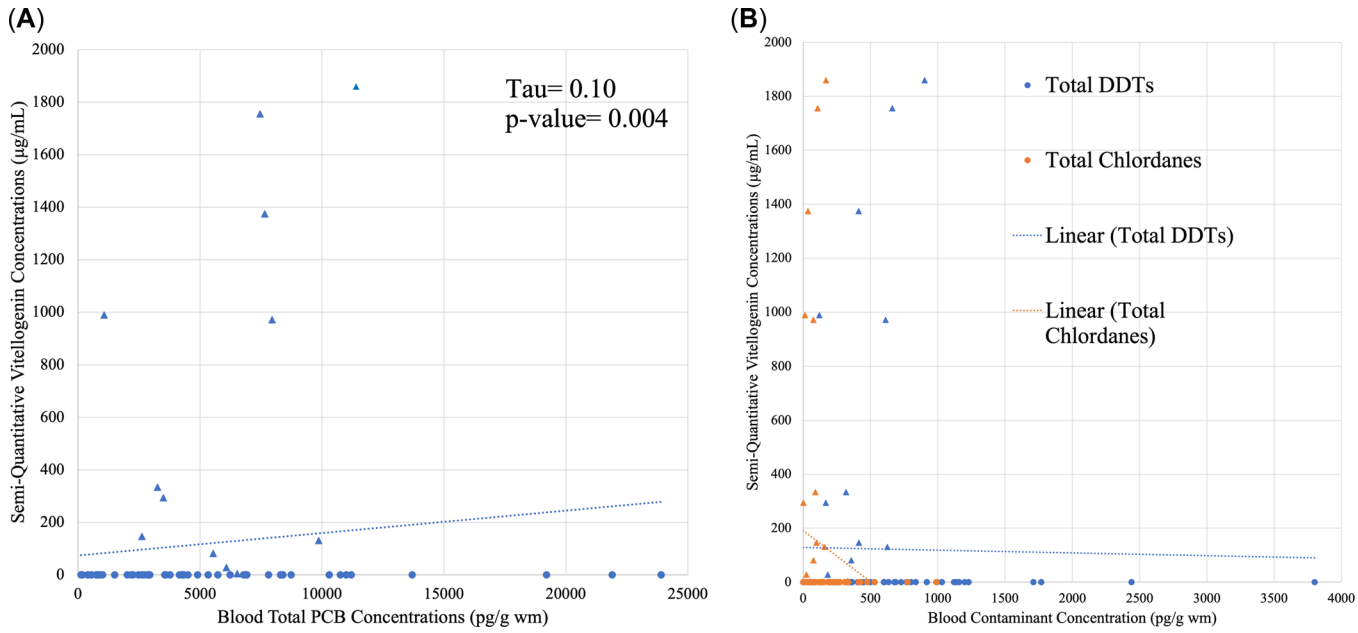


FIGURE 5: Correlations of semiquantitative vitellogenin (VTG) concentrations (µg/ml) in loggerhead sea turtles <77 cm straight carapace length with (**A**) blood total polychlorinated biphenyl (PCB) concentrations and (**B**) blood total dichlorodiphenyltrichloroethane and total chlordane concentrations (pg/g wet mass [wm]). 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 nondetects.

turtles, except for PCB 138. Polychlorinated biphenyl 138 concentrations were significantly higher in the abnormal compared with the normal turtles ($p = 0.03$). On the other hand, the 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 semiquantitative VTG concentrations in these same juvenile turtles (Supporting Information, Table S4 and 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 a wet mass basis among the juvenile turtles analyzed (Figure 6 and Supporting Information, Table S5).

Adult sex differences in OC concentrations

Because our study analyzed an additional set of blood samples from adult (>88 cm SCL) turtles for OC concentrations, sex differences could be examined (Supporting Information, 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 were not significantly different.

DISCUSSION

This assessment of plasma VTG provides an improved understanding of the reproductive cycles of female loggerhead

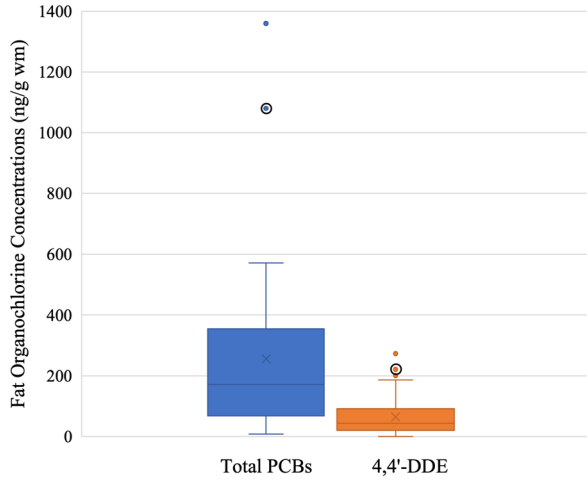


FIGURE 6: Box and whisker plot of the total polychlorinated biphenyl (PCB) and 4,4′-dichlorodiphenyldichloroethylene (4,4′-DDE) concentrations (ng/g wet mass) in the fat of immature loggerhead sea turtles ($n = 44$). Data points circled belong to an abnormal female expressing vitellogenin (straight carapace length = 53.7 cm). Data were previously published in Keller et al. (2004b).

sea turtles. All five nesting females were expressing VTG and had the highest concentration of VTG compared with 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). The 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 on July 31, 2000 in offshore waters 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. Because 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. Second, it is possible that this large and presumably old female is past her reproductive age. It is unknown whether 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 our 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 & Vermeiren, 2020). If she had never produced eggs, it is possible that over a lifetime she would have accumulated OC levels greater than those of 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 we screened, female loggerhead sea turtles along the southeast coast of the United States begin reproductive maturation at approximately 77 cm SCL. From observations taken during necropsies, 77 cm SCL is the size threshold in which follicles begin to grow (David Owens, personal observation). Although one nesting loggerhead was recorded at 74 cm SCL (Frazier & Ehrhart, 1985), average nesting females along the US Atlantic coast are 82–92 cm SCL (Frazier & Ehrhart, 1985; Miller, 1997; Smelker et al., 2014). 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 our 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. The VTG expression helped us to 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. Although 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 whether 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 (USA/Canada) that were more heavily contaminated with OC compounds (De Solla et al., 1998). In addition, 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, and nonchlorinated 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 us 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 the 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. Polychlorinated biphenyls are known to have complex endocrine actions, including cross-talk 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; Calò et al., 2010; Jung et al., 2005; Vega-López et al., 2006). Furthermore, compounds with the greatest known estrogenic activity (e.g., 2,4′-DDD or 2,4′-DDT; US Environmental Protection Agency [USEPA], 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 (see Silva et al., 2002) was considered in the present study to address the additive estrogenic effects of the OC mixtures measured in the turtle tissues. This approach would have incorporated positive ER bioactivity factors of OC pesticides from the USEPA's (2015) Endocrine Disruptor Screening Program with positive and negative ER-binding factors for PCB congeners from Plíšková et al. (2005). After considerable debate, we determined that the approach was more risky than simply assessing OC concentrations against VTG expression. The OC mixtures certainly interact with multiple receptors and biochemical processes, some of which induce estrogen responses, whereas others inhibit them (Plíšková et al., 2005; Safe, 1995). In aquatic organisms, the exposure duration influences the direction of the effect (Calò et al., 2010). Moreover, 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 our 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 (Guirlet et al., 2010; Munoz & Vermeiren, 2020; Stewart et al., 2011; van de Merwe et al., 2010). 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 (USA). 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, 91.9, 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; Keller, 2013; Ragland et al., 2011). It is possible that the small sample size of these females (captured at 32.95°N, 32.86°N, and 30.30°N) in comparison with males (captured at 32.94°N, 32.46°N, and 32.46°N) spent more time foraging further north along the US 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, because the coastal area around this city is heavily contaminated by a more highly chlorinated pattern of PCBs due to Aroclor 1268 being released from a Superfund site (Kannan et al., 1998; Maruya et al., 1997). 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., 1998). 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 (Barraza et al., 2020; Keller et al., 2004b), especially with periods of foraging, migration, and reproductive behaviors (Keller, 2013).

Considering the latter hypothesis, 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 3-year female nesting season (Figure 7). Much of the detail in Figure 7 is not empirically known, but 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). The 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/season, with approximately 14 days between clutches (Miller et al., 2003).

It is thought that during migration and nesting, sea turtles reduce or stop foraging (Perrault & 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 2 years (Guirlet et al., 2010), suggesting that much of the OCs transferred into the eggs may be from dietary intake instead of fat reserves (Muñoz & Vermeiren, 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 (Muñoz & Vermeiren, 2020), but they should fluctuate with plasma estrogen concentration, which have been tested across reproductive cycles. In postmating *L. kempii*, VTG was detectable for 7 months, which would be well after a complete nesting season (Rostal et al., 1998). Injections of estradiol led to rapid and large VTG production that remained high for 31 weeks (Heck et al., 1997). The present study showed that at least one adult female had nondetectable VTG, so at some point during quiescence or senescence, VTG concentrations likely diminish. The OC 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 8–10 months before breeding season (Miller et al., 2003; Figure 7). Because 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 (Muñoz & Vermeiren, 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 our study may have been tested during this hypothesized period of peak blood OCs, making their concentrations greater than that of 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 those of males (Figure 7).

The present study 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 10 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, although 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. Although not statistically significant, the average blood concentrations of PCBs were 8.5% higher in the 9 VTG-expressing juveniles compared with 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 & 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

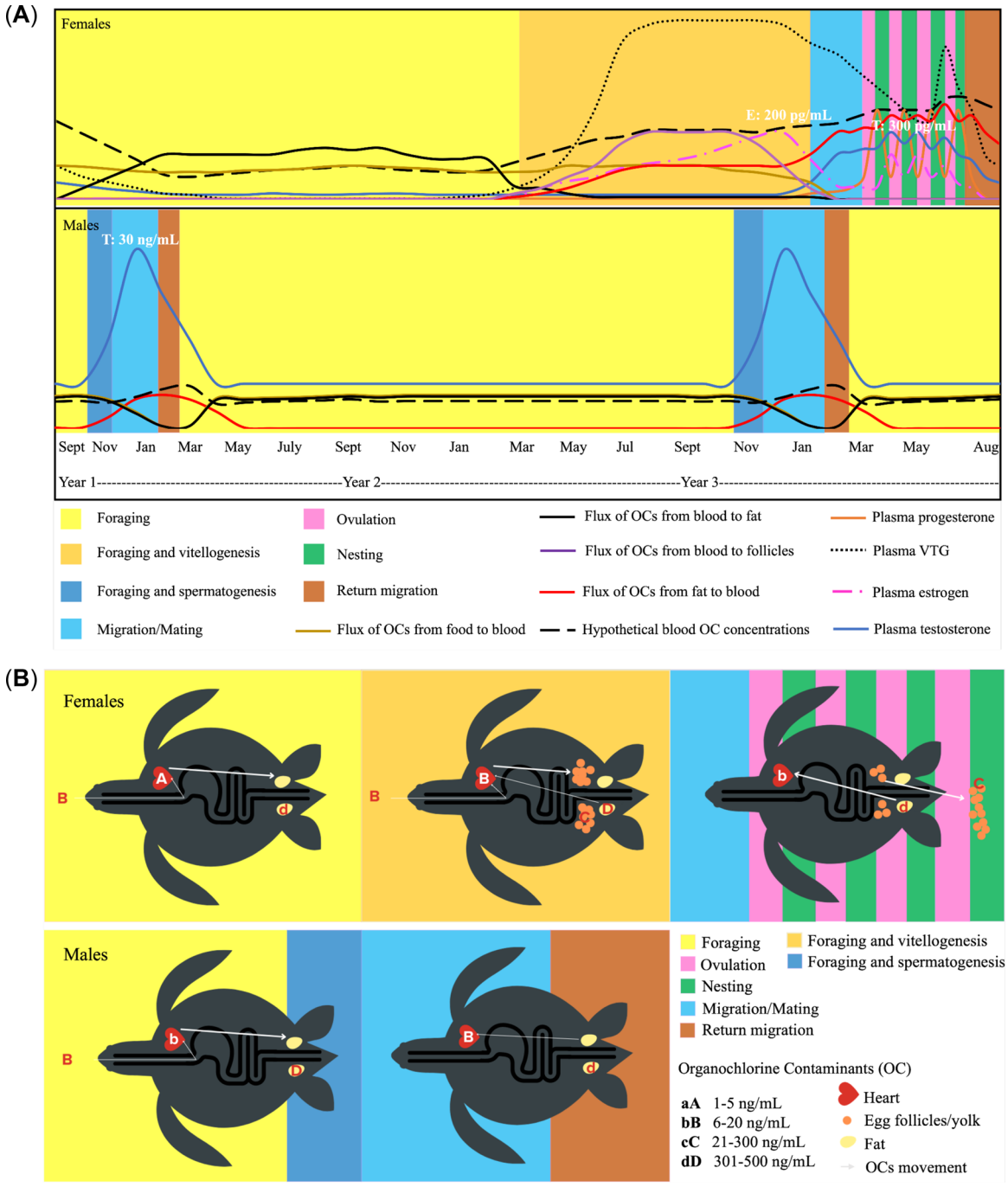


FIGURE 7: Theoretical models of (**A**) hormone, vitellogenin (VTG), and organochlorine contaminant (OC) concentrations for female and male loggerhead sea turtles through a typical 3-year female reproductive cycle. Background colors represent behaviors, lines hypothesize hormone, VTG, and OC concentrations based on data from the present study and other publications (behaviors, fat stores, and follicle development: Wibbels 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 et al., 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. The OC concentration ranges (ng/g) are represented by letters. Lower case letters are concentrations near the minimum of the range, and capital letters are near the maximum of the range.

Zaccaroni et al. (2009) found VTG expression using western blot analysis in 75.4% of the loggerhead juveniles (<75 cm curved carapace length) in Italian waters; however, they did not examine environmental contaminants. It is currently unknown whether 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

Our study shows that most female loggerhead sea turtles from the southeast coast of the United States 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. During evaluation of 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 we provide on testosterone, VTG, and OC concentrations form an updated hypothetical model of these compounds through the reproductive cycle of loggerhead sea turtles.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5612.

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Disclaimer—Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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Data Availability Statement—Data collected for our study, including the Supporting Information, are publicly available at https://doi.org/10.21395/mds2-7461.

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