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Nonlinear patterns in mercury bioaccumulation in American alligators are a function of predicted age



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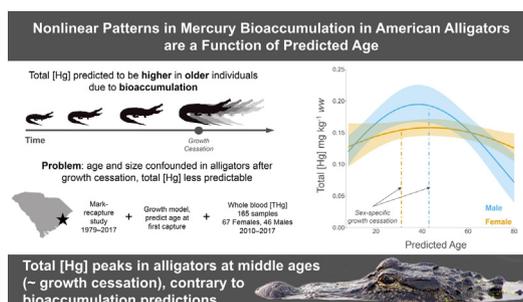
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HIGHLIGHTS

- Investigated mercury bioaccumulation patterns in two American alligator populations.
- Bioaccumulation predicts highest mercury concentrations in oldest individuals.
- Mercury concentrations peaked in middle age and body sizes, near growth cessation.
- Age is a better predictor of total mercury than size, but requires long-term study.
- Included suggestions for developing studies to inform meat consumption advisories.

GRAPHICAL ABSTRACT



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ABSTRACT

Mercury is a widespread, naturally occurring contaminant that biomagnifies in wetlands due to the methylation of this element by sulfate-reducing bacteria. Species that feed at the top trophic level within wetlands are predicted to have higher mercury loads compared to species feeding at lower trophic levels and are therefore often used for mercury biomonitoring. However, mechanisms for mercury bioaccumulation in sentinel species are often poorly understood, due to a lack of long-term studies or an inability to differentiate between confounding variables. We examined mercury bioaccumulation patterns in the whole blood of American alligators (*Alligator mississippiensis*) from a long-term mark-recapture study (1979–2017) in South Carolina, USA. Using a growth model and auxiliary information on predicted age at first capture, we differentiated between age- and size-related variation in mercury bioaccumulation, which are often confounded in alligators due to their determinate growth pattern. Contrary to predictions that the oldest or largest individuals were likely to have the highest mercury concentrations, our best-

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South Carolina
Determinate growth

supported model indicated a peak in mercury concentration at 30–40 years of age, depending on the sex, and lower concentrations in the youngest and oldest animals. To evaluate the robustness of our findings, we re-analyzed data from a previously published study of mercury in alligators sampled at Merritt Island National Wildlife Refuge in Florida. Unlike the South Carolina data, the data from Florida contained minimal auxiliary information regarding age, yet the best supported model similarly indicated a peaked rather than increasing relationship between mercury and body size, a less-precise indicator of age. These findings highlight how long-term monitoring can differentiate between confounding variables (e.g., age and size) to better elucidate complex relationships between contaminant exposure and demographic factors in sentinel species.

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1. Introduction

Elemental mercury (Hg) is a ubiquitous contaminant that enters the environment as a pollutant from anthropogenic activities (e.g., gold mining, waste incineration, coal-burning power plants) and through natural atmospheric deposition (Hower et al., 2010; Pirrone et al., 2010; Selin, 2009). Following deposition, sulfate-reducing bacteria commonly found in wetland sediments can readily convert Hg to bioavailable methylmercury compounds (e.g., CH_3Hg^+ , $(\text{CH}_3)_2\text{Hg}$; MeHg hereafter), which are potent neurotoxins that account for >95% of the Hg detected in biota (Bank et al., 2005; Compeau and Bartha, 1985; Wagemann et al., 1997). Adverse effects of Hg exposure are well-documented in humans and wildlife and include reduced neurological function and immunocompetence, increased embryonic deformities or mortality, and impaired reproductive output (Becker et al., 2017; Bergeron et al., 2011; Evers et al., 2008; Frederick and Jayasena, 2011; Grippo and Heath, 2003; Hopkins et al., 2013; Perrault et al., 2011). Hg bioaccumulates over an individual's lifespan and readily increases in concentration from lower to upper trophic levels (i.e., biomagnification) (Chumchal et al., 2011; Snodgrass et al., 2000). Long-lived apex predators are therefore often employed as sentinel species for Hg biomonitoring because their trophic position exposes them to both bioaccumulation and biomagnification (Sergio et al., 2008), and various demographic and behavioral traits (e.g., long lifespan, extended parental care, site fidelity) enhance their sensitivity to environmental stressors or disturbances (e.g., exposure to contaminants) (Benson et al., 2016; Duffy, 2002; Weaver et al., 1996).

Monitoring plans for Hg in sentinel species are most effective when designed to identify and differentiate between potentially confounding sources of variation in Hg concentrations (Smith et al., 2007). For example, studies that are limited in temporal scope or sample sizes across years may be poorly suited to reduce the uncertainty associated with a chronic environmental stressor such as Hg, which can vary annually though natural processes (e.g., atmospheric deposition, hydrology) and display long-term trends due to anthropogenic inputs (Pirrone et al., 2010; Zhang et al., 2016). Although monitoring for Hg may be most informative when longitudinal sampling of individuals is used, biological relationships between Hg and age or growth rates, as documented in several fish species (Lavigne et al., 2010; Sandheinrich and Drevnick, 2016), may be used as a surrogate for longitudinal sampling. Doing so, however, requires reliable indicators of age (e.g., otoliths, plumage patterns, body size), lest a limited understanding of growth patterns lead to spurious conclusions regarding mercury bioaccumulation as a function of putative age or a less-precise age class. Therefore, factors such as reliable indicators of age may warrant consideration when selecting sentinel species.

In the southeastern United States, American alligators (*Alligator mississippiensis*; hereafter alligators) are apex predators that exhibit strong top-down effects on prey community structure and function, and create habitat for other wetland species through

the creation of “alligator holes” (Bondavalli and Ulanowicz, 1999; Mazzotti and Brandt, 1994). The alligator is an effective sentinel species for Hg biomonitoring because alligators frequently occupy the top position within wetland food webs (Nifong and Silliman, 2013; Rosenblatt and Heithaus, 2011), are long-lived, and appear to exhibit long-term site fidelity (Lawson, 2019; Wilkinson et al., 2016). This suite of traits makes them amenable to long-term longitudinal sampling that is reflective of Hg in the surrounding environment (Milnes and Guillette, 2008). Recently, recreational harvest of alligator populations has been implemented throughout most of their range (inset, Fig. 1), prompting concerns for human exposure to Hg through the consumption of alligator meat (Ruckel, 1993; Smith et al., 2018). Therefore, the use of alligators as a sentinel species is relevant for both ecosystem and human health.

Despite the potential utility of alligators for biomonitoring, many studies have reported inconsistent findings with respect to Hg concentrations as they relate to demographic factors, such as sex or body size (Campbell et al., 2010; Heaton-Jones et al., 1997; Nilsen et al., 2017a; Rumbold et al., 2002; Yanocho et al., 1997). Recent studies suggest that alligators and other crocodylians exhibit determinate (i.e., asymptotic) rather than indeterminate growth, and continue to reproduce for many years following growth cessation in middle age (Campos et al., 2014; Taylor et al., 2016; Tucker et al., 2006; Wilkinson et al., 2016). For species with determinate growth, age and body size are confounded in individuals who are near or beyond the average size at growth cessation, in the absence of auxiliary mark-recapture data. In this context, an incorrect presumption of indeterminate growth, in which age could be inferred from body size alone, may obscure fine-scale relationships between age and Hg or other interacting variables (e.g., sex, metabolic requirements).

We investigated total mercury (THg) concentrations in whole blood of adult and subadult alligators from a population in South Carolina, USA, which is one of the longest-running crocodylian mark-recapture studies in the world (1979–present). Our goal was to investigate demographic, individual, and temporal variation in bioaccumulation patterns of THg, including previously-unexplored nonlinear effects. We use the term “bioaccumulation” here, as THg concentration in whole blood is an effective predictor of concentrations in Hg-bioaccumulating tissues (e.g., muscle, liver) for both alligators (Moore, 2001; Nilsen et al., 2017b) and other taxa (Bergeron et al., 2010; Cizdziel et al., 2003; Eagles-Smith et al., 2008). We predicted that THg bioaccumulation would differ among sexes, based on known differences in growth rates, movement, and habitat use patterns (Joanen and McNease, 1972, 1970; Lawson et al., 2018; Wilkinson et al., 2016). We also predicted that age-related changes in diet composition and metabolism would generate differences in THg bioaccumulation (Elliott et al., 2015 and references therein). Alligators exhibit positive allometry, in which changes in jaw structure, musculature, and bite force facilitate consumption of larger prey items throughout

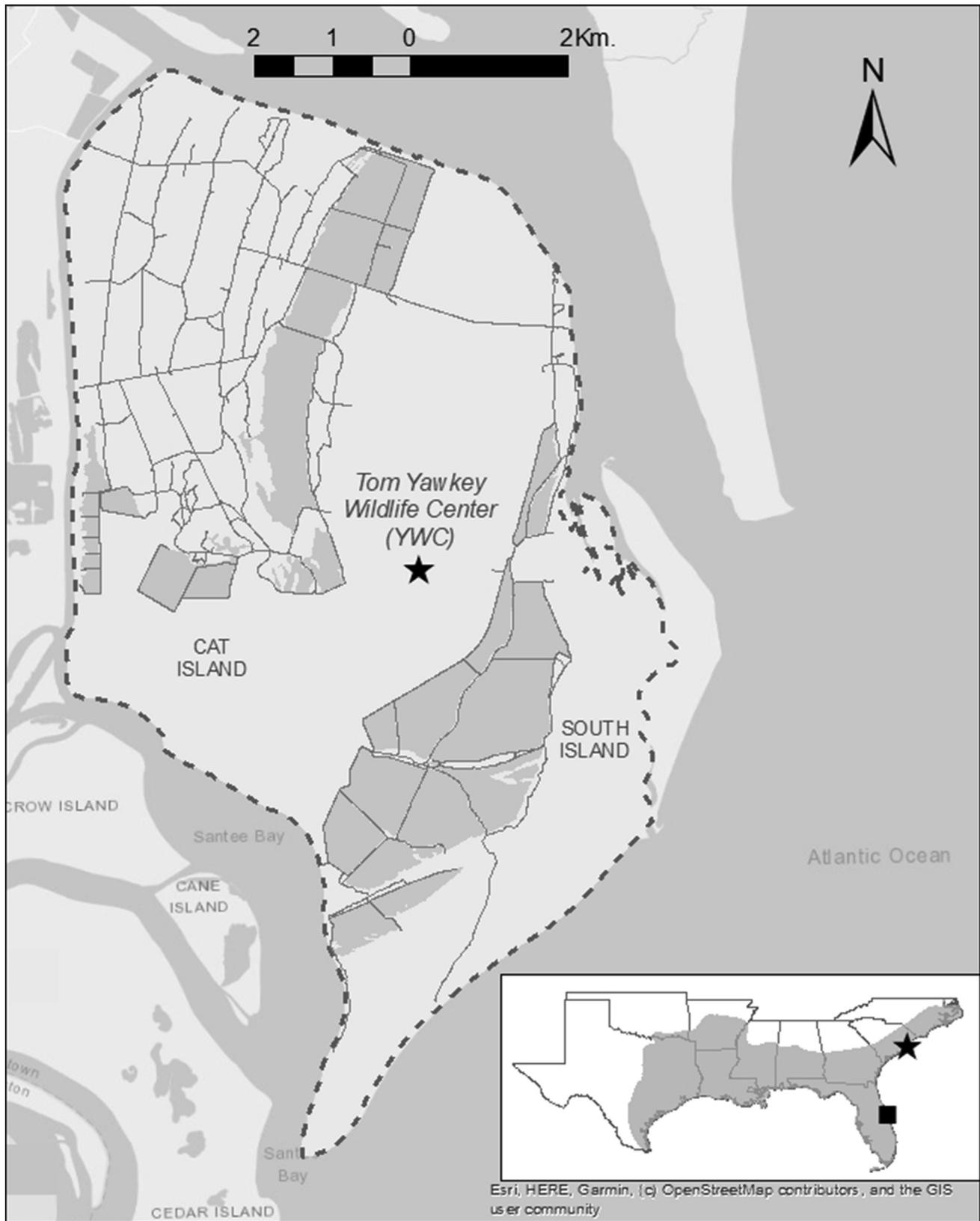


Fig. 1. A map of the Tom Yawkey Wildlife Center (YWC) in coastal South Carolina, USA, which has been closed to hunting for over 100 years. American alligator (*Alligator mississippiensis*) whole blood samples were collected on Cat and South Islands (denoted by the bold dashed line) within YWC from 2010 to 2017. YWC is comprised of 1012 ha impounded fresh and brackish water wetlands (dark gray areas within YWC), surrounded by a series of dikes and dirt roads (thin black lines). The inset (lower right) shows the alligator's distribution and our two study sites: YWC (black star) and the Merritt Island National Wildlife Refuge (MINWR; black square), described in detail in [Nilsen et al. \(2017a\)](#).

growth (Dodson, 1975; Erickson et al., 2003), which is supported by multiple stable isotope studies that examined relative trophic level across crocodylian size classes (Hanson et al., 2015; Nifong et al., 2015; Santos et al., 2018).

We were also interested in examining differences in bioaccumulation patterns of THg as a function of age or size, and predicted that determinate growth would produce nonlinear relationships. We used a growth formula derived from our study population (Wilkinson et al., 2016) to estimate predicted age for each individual based on its body size at initial capture. Additionally, we evaluated the applicability of our findings to other alligator studies that lacked auxiliary previous-capture information by conducting a post-hoc analysis on a previously published dataset (Nilsen et al., 2017a) from a shorter-term mark-recapture study, with uncertainty regarding the true age of individuals in the sampled population. Lastly, we examined our results in the context of how age or body size could relate to consumption risk in humans, as quantified by estimated THg muscle content based on whole blood content (Nilsen et al., 2017b).

2. Materials and methods

2.1. Study area

Our study focused on an alligator population on the north-central coast of South Carolina, USA. We captured alligators on the South and Cat Island portions of the 6033-ha Thomas A. Yawkey Wildlife Center (YWC; 33.217°N, -79.236°W), a state-operated wildlife management area that has been closed to alligator hunting since the early 1900s. YWC is surrounded by marine (salinity >26 parts per thousand; ppt) and brackish water habitats (5–25 ppt) (Fig. 1), in which the mean tidal range is 116 cm (<http://www.salt-watertides.com/cgi-local/seatlantic.cgi>). Our sampling area within YWC included tidal marsh (2524 ha), primarily comprised of smooth cordgrass (*Spartina alterniflora*) and black needle rush (*Juncus roemerianus*) and managed impounded wetlands (hereafter impoundments; 1012 ha). The impoundments contained both emergent vegetation, including smooth cordgrass, tall cordgrass (*S. cynosuroides*), and saltmarsh bulrush (*Scirpus robustus*), as well as submerged vegetation, such as widgeon grass (*Ruppia maritima*). Impoundment water levels were typically maintained at 60 cm water depth, with the exception of a spring draw-down period lasting approximately 5–6 weeks, to promote seed propagation. Water management practices and rainfall influenced impoundment water salinity, which ranged from 0 to 35 ppt.

2.2. Sample collection

We collected whole blood from alligators captured on YWC from 2010 to 2017 to examine THg bioaccumulation patterns (hereafter THg study); whole blood collection is a non-destructive technique that enables longitudinal sampling within individuals. These alligators were also part of a concurrent, long-term (1979–2017) mark-recapture study on YWC to evaluate alligator growth and demographic patterns. A portion of the individuals in the THg study had been previously encountered by the mark-recapture study (prior to THg study initiation in 2010); therefore, we used auxiliary capture information from these individuals to obtain predicted age conditioned on initial capture (Section 2.4.3.1). Alligators were captured on YWC intermittently using a combination of modified baited trip-snares (Murphy and Fendley, 1973), walk-through snares (Wilkinson, 1994), snare poles, snatch hooks (Cherkiss et al., 2004), and hand captures (for small alligators). For each individual, we determined the sex through cloacal examination (Chabreck, 1963) and recorded three standard mor-

phometric measurements (± 0.5 cm): total length (TL), snout-vent length (SVL; length measured from the snout to posterior margin of the cloaca), and tail girth (TG; tail circumference measured at the cloaca). Individuals were uniquely marked using a combination of toe clipping (1979–1993), tail and caudal scute notching (1979–2017) (Chabreck, 1963; Wilkinson, 1983), metal self-piercing tags applied to the webbing between toes (Conservation Tags 1005-1 (1979–1982) and 1005-681 (2009–2017), National Band & Tag Company) (Jennings et al., 1991), and passive integrated transponder (PIT) tags inserted subcutaneously above the right masseter (2009–2017) (GPT12, Biomark, Boise, ID) (Eversole et al., 2014). See Wilkinson et al., (2016) for a detailed description of capture, marking techniques, and calculation procedures for imputing values of SVL when SVL was not recorded. For the THg study, we targeted large subadults (Females: $63.032 \text{ cm} \leq \text{SVL} < 94.548$; Males: $63.398 \leq \text{SVL} < 95.098$) and adults (F: ≥ 94.548 ; M: ≥ 95.098) to increase the likelihood of encountering previously marked individuals. Though exceptions exist, alligators typically reach reproductive maturity at 180 cm TL (Joanen and McNease, 1980; Wilkinson, 1983). We established SVL cutoff values to distinguish between subadults and adults based on predicted SVL at 180 cm TL using sex-specific SVL:TL ratios measured from individuals with intact tails from our study population (Females: 0.517, Males: 0.520; Wilkinson et al., 2016).

In 2010 we began collecting whole blood from captured alligators for multiple ecotoxicology (including Hg), reproductive biology, and foraging ecology studies. Immediately following each alligator capture, we collected blood samples via the post-occipital venous sinus using a 6.4-cm sterile 20-gauge needle and a 30-mL syringe (Myburgh et al., 2014). Blood samples were transferred to three 10-mL lithium heparin Vacutainer tubes (BD, Franklin Lakes, NJ) and placed on wet ice in the field before being stored in a -20°C freezer until analysis. Following marking, measurements, and blood collection, all alligators were released at their capture sites. We acquired all necessary alligator sample collection permits from the South Carolina Department of Natural Resources, and the study was approved by the Institutional Animal Care and Use Committees at Clemson University (Permit nos. 2015007, 2016059) and the Medical University of South Carolina (Permit no. 3069).

2.3. Whole blood THg laboratory analysis

We used an automated Direct Mercury Analyzer (DMA-80, Milestone, Inc., Shelton, CT, USA; hereafter DMA) at the Savannah River Ecology Laboratory, University of Georgia (Aiken, SC, USA). The DMA uses thermal decomposition, gold amalgamation, and atomic absorption spectrometry to determine the mass fraction of THg in solid or liquid samples. We prioritized analyzing (1) longitudinal samples of whole blood from individuals that were captured multiple times within the THg study and (2) samples from individuals that were previously encountered by the mark-recapture study (prior to THg study initiation) so that we could obtain a more accurate predicted age. Blood samples were thawed at room temperature and placed on a Vortex homogenizer for 30 s, and 1 aliquot (100 μL) was transferred to a nickel weigh boat for analysis in the DMA. A portion of the blood samples contained extensive clots that we were unable to homogenize, therefore, we transferred each of the clotted whole blood samples to pre-weighed 15-mL polypropylene centrifuge tubes (VWR, Radnor PA) and freeze-dried them to a constant mass (± 0.1 mg) using a FreeZone lyophilizer (Labconco, Kansas City, MO, USA). We then manually homogenized the freeze-dried (hereafter solid) blood samples using a mortar and pestle, which were cleaned with 100% isopropyl alcohol between samples, before placing 0.01 g of each sample into the nickel weigh boats.

We constructed an external 14-point calibration curve ranging from 0 to 200 ng using the solid Certified Reference Materials (CRM) for trace metals, PACS-2 marine sediment ($3.04 \pm 0.20 \text{ mg kg}^{-1} \text{ THg}$) and TORT-3 lobster hepatopancreas ($0.292 \pm 0.022 \text{ mg kg}^{-1} \text{ THg}$) from the Natural Resource Council of Canada (NRC-CNRC; Ontario, Canada). A detailed description of the calibration process is provided in Table S1. At the beginning of each day we performed a quality control check that included six instrumental blanks (empty slots within the DMA) interspersed with one PACS-3 and one TORT-2 sample to ensure proper machine functionality. Whole blood samples were analyzed in batches of approximately ten alongside one instrumental blank, two procedural blanks (empty nickel boats), one field blank (thawed Milli-Q Water from lithium-heparin vacutainers filled and frozen in 2011), one standard reference material, and one duplicate of a whole blood sample (Table S2). Instrumental and procedural blanks were used to quantify background THg concentrations within the instrument and weigh boats, whereas field blanks were used to correct for THg associated with the field sampling procedure. We calculated the limit of detection for our DMA analyses from $3 \times \text{SD}$ of all procedural blanks measured (Table S2) (Shrivastava and Gupta, 2011).

Blood samples were phase- (liquid versus solid) and matrix-matched to the reference materials within each run. For liquid samples, we used the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 955c levels 3 and 4, Toxic Metals in Caprine Blood, with reference values for total mercury at $17.8 \pm 1.6 \text{ ng g}^{-1}$ and $33.9 \pm 2.1 \text{ ng g}^{-1}$, respectively. For solid samples we used PACS-3, TORT-2, and a NIST SRM 955c level 4 vial that we freeze-dried using the same procedure for the blood samples. We prioritized analyzing liquid samples to replicate the methods of other recent alligator whole blood-based THg studies (Nilsen et al., 2017a,b, 2016) as closely as possible, and because our matrix-matched SRMs were certified for THg values in liquid phase. Additionally, we performed a cleaning procedure that included six machine blanks, two procedural blanks, one nickel boat with 0.1 g of all-purpose flour, and one quartz boat containing 0.1 g nitric acid between each analytical run.

2.4. Statistical analysis

2.4.1. Dry to wet weight conversion

To account for sample preparation differences in the solid phase samples (i.e., convert the solid phase DMA output to the same measurement unit as liquid phase samples), we used the following formulas from Lusk et al. (2005) to estimate the percent moisture content (M) for each solid phase sample (Eq. (1)). We then converted the sample's THg dried weight estimate (dw) to wet weight (ww) (Eq. (2)):

$$M = \frac{TM_w - TM_d}{TM_w} \times 100 \quad (1)$$

$$ww = dw \times \left(1 - \frac{M}{100}\right) \quad (2)$$

In Eq. (1), TM refers to the sample's total mass, which includes all of the material that was transferred to the centrifuge tube (i.e., not just the mass of the sample that was analyzed in the DMA), before (W subscript) and after (D) freeze-drying. Following the DMA analysis, we used Eq. (2) to convert the THg dry weight (dw) measured by the DMA in mg kg^{-1} , to wet weight (ww), so that the dried samples could be compared with samples that were only analyzed in their liquid phase (i.e., never freeze-dried). Moreover, to make our results comparable to other studies, we converted our whole blood THg measurements to estimated muscle THg concentration using a blood to muscle (both $\text{mg kg}^{-1} \text{ ww}$) conversion formula in Fig. 3 of Nilsen et al. (2017b):

$$\text{Muscle} = \frac{0.9475 \times \text{Blood} - 18.701}{1000} \quad (3)$$

2.4.2. Method duplicate comparison and phase adjustment

Twenty-three un-clotted whole blood samples were analyzed in both liquid and solid phases (hereafter method duplicates) alongside other phase-matched samples (Table S2) to determine potential THg losses from the freeze-drying (lyophilization) process (Litman et al., 1975; Ortiz et al., 2002). The method duplicates (17 females, 6 males) represented all study years except 2015 (Table S3). Using the converted wet weight (ww) THg measurements from the solid samples, we assessed differences in ww between paired method duplicates. We identified a single outlier, in which the converted THg ww measurement (i.e., the sample was run as a solid with a converted dw to ww) was extremely low. The difference between this method duplicate's liquid-run THg ww minus its converted solid-run THg ww was approximately 6x the mean difference between paired liquid and solid samples for all method duplicates. We concluded that the method duplicate outlier's solid-run THg value was an anomaly, as opposed to the liquid run sample value, because the liquid sample was run in duplicate during the liquid run and produced consistent THg values. As a result, the outlier's solid run THg value was excluded from all further analyses.

Following outlier removal, THg was significantly higher in liquid samples (mean: $0.142 \text{ mg kg}^{-1} \text{ ww} \pm 0.065 \text{ SD}$) compared to solids (0.136 ± 0.069) based on a two-sided paired Wilcoxon rank-sign test for small sample sizes ($p < 0.001$). The liquid samples averaged $0.006 \pm 0.009 \text{ mg kg}^{-1} \text{ ww}$ higher than the solids, though three solid method duplicates had higher THg measurements than their liquid counterparts. We used ANOVA to assess whether differences between liquid and solid phase samples could be attributed to any systematic factors (e.g., storage time, as indicated by Year). We transformed differences to satisfy normality assumptions for ANOVA by squaring the difference between paired method duplicates (liquid minus solid THg ww) and applying a Box-Cox transformation ($\lambda = 0.384$). We used a Shapiro-Wilk test to confirm that our transformed data showed no significant departure from a normal distribution, and we applied two one-way ANOVAs in which transformed differences were modeled as a function of Year or Sex, compared to a null model. Neither term was significant ($p > 0.05$), suggesting these effects played no systematic roles in differences between liquid vs. solid samples. Therefore, we added $+0.006 \text{ mg kg}^{-1}$ to all solid-run sample THg ww values, referred to hereafter as the "phase adjustment". We then averaged all within-run and method duplicates to obtain a single THg concentration value for each unique capture event. We conducted a redundant analysis in which we applied an alternative phase adjustment value that was derived from all method duplicates ($+0.007 \text{ mg kg}^{-1}$), including the outlier, to assess the sensitivity of our results to extreme values and methodological adjustments.

Lastly, for quality assurance purposes, we derived the percent recovery for each reference material sample. Percent recovery is the THg concentration for a sample produced by the DMA divided by the reference material's value, expressed as a percentage. We then created summary statistics by averaging the percent recovery for each reference material type and DMA run.

2.4.3. Linear regression

To ensure the data fit the assumptions of linear regression, we assessed it for outliers using boxplots, Cleveland dotcharts, and the $1.5 \times$ interquartile range (IQR) guideline (Tukey, 1977). Though the IQR procedure identified six potential outliers, these data points did not form a consistent pattern based on field, laboratory, or sample variables (e.g., sex, sample age, run), and their THg val-

ues were well within the range of values reported for alligators (Table 1 in Nilsen et al., 2017a). As the purported outliers were not suggestive of unusual specimens or protocol failure, we retained these values in subsequent analyses. We applied the Box-Cox procedure to produce transformed data values approximately normally distributed, which we confirmed through the Shapiro-Wilk test.

We examined a suite of covariates in a multi-model linear regression framework to evaluate our hypotheses regarding THg bioaccumulation in alligators. All covariates were continuous unless otherwise stated, with mean values and ranges reported in Table 1. We included both Year (categorical) and ordinal date (OD) (day of year) in our analyses to investigate seasonal and annual variation in THg deposition, which has been documented in other studies (Frederick et al., 2004; George and Batzer, 2008; Nilsen et al., 2017a). We included Sex (categorical) and Predicted Age (PA) (Section 2.4.3.1) to evaluate potential demographic differences between individuals, as well as SVL (i.e., body size) which serves as a proxy for age prior to growth cessation. Compared to smaller individuals, larger individuals are thought to feed more frequently on larger-bodied prey items that are more likely to have higher THg; similarly, if THg intake exceeds offloading, then we expect THg to positively vary with age. We also included body mass index (BMI) (described in 2.4.3.1), as individuals with higher THg loads are more likely to have reduced neuromuscular function, which could affect foraging behaviors and thereby body condition (Grippe and Heath, 2003; Nilsen et al., 2017a). In our models, BMI only plays a predictive role and has no cause-and-effect implication for THg. We checked for multicollinearity between our continuous covariates using linear regression and Pearson's correlation coefficients. The only correlation we detected was between SVL and PA ($r: 0.56$), so we did not construct any models that contained both of those terms. The continuous covariates contained no missing values and were z-standardized across years (mean = 0.0, SD = 1.0). Lastly, we also considered models that included Year or individual as a random effect, the latter to account for the nested structure in our dataset (i.e., repeated samples from individuals).

All statistical analyses were performed in R Version 3.6.0 (R Core Development Team, 2019).

2.4.3.1. Predicted age and body mass index calculations. In our YWC study population, individuals appear to exhibit determinate (i.e., asymptotic) rather than indeterminate growth (Wilkinson et al., 2016). We used the Baker et al. (1991) form of the Schnute (1981) growth formula to estimate predicted age at first capture for a given SVL using the sex-specific growth parameters for our study population as reported in Wilkinson et al. (2016). Note that the PA estimation formula (Eq. (4)) in Wilkinson et al. (2016) is incorrect; therefore, we used Eq. (5) in (Baker et al., 1991):

$$t_m = \tau_1 - \frac{1}{a} \times \ln \left(1 - \frac{y_m^b - y_1^b}{y_2^b - y_1^b} \{1 - \exp[-a(\tau_2 - \tau_1)]\} \right) \quad (4)$$

in which t_m and y_m denote the age and SVL of an individual at marking (i.e., first capture), respectively. The τ_1 and τ_2 terms are fixed values that indicate the minimum and maximum ages observed in a population (both sexes: 0–45), whereas y_1 (both sexes: 12.5 cm) and y_2 (females: 135.0 cm, males: 182.8 cm) denote the SVL at ages τ_1 and τ_2 , respectively. The a term is the fixed growth rate (females: 0.113 yr⁻¹, males: 0.098 yr⁻¹) and b (females: 0.721, males: 0.692) is the dimensionless shape parameter. We assigned the average age at cessation of growth (females: 31, males: 43) for individuals whose y_m was equal to or exceeded SVL at growth cessation (females: 131.4, males: 182.0) as estimated in Wilkinson et al. (2016). We then used the predicted age at first capture as a basis to estimate predicted age (PA) for all subsequent captures by counting forward in decimal years (to account for the actual date within a capture year) for each subsequent encounter.

We also evaluated the relationship between BMI as a predictor of THg. Animals were not weighed during the study; therefore, we opted to use the BMI estimator described by Nilsen et al. (2017a), which relies on the standard morphometric measurements on snout-vent length (SVL) and tail girth (TG) we collected.

Table 1
Summary of American alligator whole blood samples and covariate values for the Tom Yawkey Wildlife Center in South Carolina (2010–2017) and the Merritt Island National Wildlife Refuge in Florida (2007–2014). Whole blood sample summary totals are given in the first two fields, whereas the remaining fields are summarized with means \pm standard deviations and the range values given below in parentheses.

	Tom Yawkey Wildlife Center			Merritt Island National Wildlife Refuge ^a		
	Females	Males	Overall	Females	Males	Overall
Unique Individuals	67	46	113	72	97	169
# Maximum Blood Sampling Events						
1	40	36	76	66	84	150
2	16	8	24	6	12	18
3	9	2	11	0	1	1
4	2	0	2	0	0	0
Mean Days Between Blood Samples	957 \pm 706 (7–2256)	552 \pm 269 (285–1127)	864 \pm 653 (7–2256)	716 \pm 610 (106–1730)	683 \pm 629 (21–1877)	693 \pm 607 (21–1877)
Ordinal Date	157 \pm 41 (56–271)	139 \pm 64 (56–271)	150 \pm 51 (56–271)	177 \pm 103 (9–365)	181 \pm 114 (5–365)	180 \pm 109 (5–365)
Predicted Age ^b	31.36 \pm 12.96 (8.14–65.89)	23.07 \pm 13.10 (7.83–58.41)	28.45 \pm 13.56 (7.83–65.89)	19.00 \pm 5.85 (9.65–31.00)	20.60 \pm 6.93 (8.28–43.00)	19.95 \pm 6.54 (8.28–43.00)
Snout-Vent	127.264 \pm 11.577	141.686 \pm 30.054	132.333 \pm 21.172	114.746 \pm 12.003	145.238 \pm 20.919	132.654 \pm 23.273
Length (cm) ^c	(78.600–150.500)	(85.00–191.800)	(78.600–191.800)	(87.000–135.000)	(88.500–187.200)	(87.000–187.200)
Body Mass Index ^d	0.22 \pm 0.02 (0.15–0.26)	0.22 \pm 0.02 (0.16–0.26)	0.22 \pm 0.02 (0.15–0.26)	0.21 \pm 0.02 (0.15–0.25)	0.21 \pm 0.02 (0.14–0.26)	0.21 \pm 0.02 (0.14–0.26)

^a MINWR summary statistics and covariate means exclude four outlier samples identified by Nilsen et al. (2017a).

^b Predicted age derived using Eq. (4) (Eq. (5) in Baker et al., 1991) using sex-specific growth parameters from Wilkinson et al. (2016).

^c Snout-vent length (SVL) covariate values were comprised of field measurements (± 0.5 cm) and imputed values (± 0.001 cm) for cases in which SVL was not recorded in the field.

^d Body mass index derived using Eq. (5) (Nilsen et al., 2017a).

$$BMI = \frac{TG}{SVL * 2} \quad (5)$$

After assessing BMI covariate values, we opted to model BMI as a continuous covariate, rather than categorical as done by Nilsen et al. (2017a).

2.4.3.2. Model construction and selection. We began our model-selection process by constructing a set of models containing each of the four continuous covariates (BMI, OD, PA, SVL) modeled singly as a univariate effect or in combination with a quadratic effect. We also built two models that contained either Year or Sex as the sole fixed effect, two models that contained either Year or the individual alligator (Indiv) as a single random (intercept) effect, and an intercept-only (null) model. We also created interactive and additive models to investigate potentially biologically relevant relationships between Sex and OD, SVL, and PA covariates (*cov*), in which each relationship included five model structures: Sex + *cov*; Sex × *cov*; Sex + *cov* + *cov*²; Sex × *cov* + *cov*²; and Sex × *cov* + Sex × *cov*². We were particularly interested in the relationships between Sex and the specified covariates based on known sex-specific seasonal habitat use patterns, as well as documented maternal transfer of contaminants from nesting female alligators to egg yolks (Lawson et al., 2018; Nifong and Silliman, 2017; Roe et al., 2004). Interactive relationships between the Sex and OD terms allow mean THg concentrations prior to and following breeding and nesting activities to vary by Sex over the course of the year. Interactive relationships between Sex and age-indicator variables, SVL and PA, allow relationships between the covariates and THg to differ by Sex, which may be expected as male and female alligators in our study population differ in growth rates and age at sexual maturity (Wilkinson et al., 2016). Using the same five model structures employed for the covariate relationships with Sex, we evaluated the relationship between Year (fixed effect) and OD, as other studies have documented inter- and intra-annual variation in THg deposition (Nilsen et al., 2017a). Finally, any interactive or quadratic term appearing in a model was accompanied by its lower-order constituent effects as additive terms. Note that models containing random effects were fit with restricted maximum likelihood (REML) and deviance values are not directly comparable to non-REML fit models.

We used Akaike's information criterion adjusted for small sample size (AICc) to identify the most parsimonious models using the *MuMIn* package in R (Bartoń, 2018). Following the initial model construction phase (*n* = 33 models), we performed AICc model selection to identify potentially meaningful covariate combinations that we had not yet evaluated (e.g., ~Individual and BMI) based on covariate representation in the most competitive models (i.e., within 2 ΔAICc units of the best-supported) (Burnham and Anderson, 2002). However, exploration of additional models did not uncover any that were superior to those in the competitive model set; therefore, we report no model fitting results beyond those in the competitive set. For each competitive model, we considered the effect of a covariate informative if the 85% confidence intervals did not overlap zero (Arnold, 2010).

2.4.3.3. Post-hoc re-analysis of Merritt Island National Wildlife Refuge THg study. We conducted a post-hoc re-analysis of Nilsen et al.'s (2017a) data collected at Merritt Island National Wildlife Refuge (MINWR) in eastern-central Florida (inset, Fig. 1) from 2007 to 2014 (see Nilsen et al., 2017a for details on study site, sample collection, and laboratory methods). We were particularly interested in exploring effects that were not evaluated by Nilsen et al. (2017a), including predicted age and quadratic relationships for the covariate effects described in this paper. Like the YWC population, a mark-recapture study was initiated at MINWR in 2006, prior

to the Nilsen et al. (2017a) THg study in 2007. To estimate predicted age, we obtained additional data on SVL at first capture for the MINWR alligators (R.H. Lowers, *unpublished data*), and applied the growth model developed for our study population (Wilkinson et al., 2016) as described in Section 2.4.2, as no growth model currently exists for Florida alligators. We excluded four outliers that were removed in the original study, and applied a Box-Cox transformation ($\lambda = 0.02$) to the remaining MINWR data, which passed the Shapiro-Wilk test for normality upon transformation. We then followed the same procedure for covariate formatting (e.g., continuous vs. categorical) and standardization, model construction, and model selection as applied to the YWC data (Section 2.4.3.2). Note that BMI was modeled as a categorical covariate by Nilsen et al. (2017a), whereas here we treated it as continuous for comparison purposes. Lastly, we decided not to conduct a pooled analysis that included both YWC and MINWR individuals due to differences in mark-recapture study sampling period (OD in Table 1) and duration that would have caused confounding issues between site and the predicted age covariate.

3. Results

3.1. Quality assurance/quality control

The limit of detection (LOD) for our DMA analyses was 0.302 μg kg⁻¹ (means reported hereafter as ± SD unless otherwise defined), based on 57 procedural blanks (0.075 ± 0.101 μg kg⁻¹) (Table S2). However, three of the procedural blanks and both field blanks (*n* = 2; 0.688 ± 0.004 μg kg⁻¹) exceeded the LOD, therefore we blank-corrected our samples by subtracting the mean field blank THg estimate. The mean percent recovery was highest for SRM 955c level 3 (118% ± 9; range: 108, 137), followed by SRM 955c level 4 (117% ± 8; 104, 132), TORT-3 (101% ± 2; 99, 104), and PACS-2 (90% ± 6; 99, 104). The absolute difference between the mean Hg SRM value for each standard and its certified THg value was less than 2.5×certified THg SD for all standards. The overall mean recovery percentage across runs (*n* = 11) was biased high, and ranged from 99% ± 19 (run 11) to 127% ± 13 (run 4 in Table S2).

A potential explanation for the high percent recovery for SRM 955c level 3 (Table S5), particularly in run 4, is that it was the final run in which we used our single vial of this standard. While we followed NIST's recommendation (NIST, 2016) that a vial not be used if less than one-third of the original blood volume remained, due to potential evaporative losses that could increase the THg concentration, it is possible that evaporative losses occurred before the volume threshold was reached. We also note that the mean percent recovery is also higher than the certified range for SRM 955c level 4 (Table S5), after accounting for phase differences. However, the certified values for SRM 955 level 4 are in *ww*, whereas the mean sample value we calculated in Table S4 includes eight samples that were run as solids (runs 8–11 in Table S2), meaning that they were not phase-matched—which is why we also included phase-matched standards (TORT-3, PACS-2) for all of the solid runs. When the non-phase matched samples are excluded, the SRM 955c level 4 mean value drops to 111% ± 5 (Table S5).

3.2. Tom Yawkey Wildlife Center

3.2.1. Summary statistics

We analyzed 218 whole blood samples for THg (Table S2), which included 30 within-run and 23 method duplicates, associated with 165 unique capture events from 113 individual alligators (67 females [F], 46 males [M]) captured at YWC from 2010 to 2017 (Table 1). Based on SVL cutoff values for age class, adults comprised

96% of our capture events ($n = 159$; F: 105, M: 54) compared to subadults ($n = 6$; F: 2, M: 4). Our sample included 37 individuals (27 F, 10 M) that were recaptured during the THg study period, with a mean of 864 ± 653 days between recapture events (F: 957 ± 706 , M: 552 ± 269). Similarly, 38 individuals (27 F, 11 M) were initially encountered by the YWC long-term mark-recapture study prior to their first blood-sampling event for this study. Based on sample summary statistics (Table 1), females in our sample population appeared to be older and smaller than males, with a mean predicted age of 31.36 ± 12.96 years (range: 8.14–65.89) and SVL ranging from 78.6 to 150.5 cm (mean: 127.3 ± 11.6), whereas males averaged 23.07 ± 13.10 (range: 7.83–58.41) years of age, and SVL ranged from 85.0 to 191.8 cm (mean: 141.7 ± 30.1). Mean BMI (0.22 ± 0.02) did not differ between sexes, and only two females and one male were categorized as having “Low” BMI (i.e., BMI < 0.18 as specified by Nilsen et al., 2017a). Lastly, we generally captured females later in the year (mean ordinal date: 157 ± 41) than males (139 ± 64), though the range for ordinal date of capture was the same for both (56–271). The preponderance of females captured later in the year is an artifact of a research focus on alligator nesting ecology at YWC from 2009 to 2017 (P.M. Wilkinson, unpublished data). Over this period, both sexes were captured for general mark-capture purposes each year during April and May, while females tended to be captured during June and July (nesting season).

After converting the solid samples from dw to ww (mean percent moisture: 85.32 ± 3.37 SD), adding the phase adjustment ($+0.006 \text{ mg kg}^{-1}$) to the converted ww (solid samples only), averaging within-run and method duplicates, and subtracting the blank correction ($-0.000686 \text{ mg kg}^{-1}$) to all samples, THg whole blood averaged $0.15 \pm 0.05 \text{ mg kg}^{-1} ww$ for our study population (F: 0.15 ± 0.05 , M: 0.16 ± 0.07). All mercury values are hereafter reported in THg $\text{mg kg}^{-1} ww$ unless otherwise stated. Estimated muscle THg averaged 0.13 ± 0.05 (F: 0.13 ± 0.04 , M: 0.13 ± 0.06), and ranged from 0.02 to 0.32.

3.2.2. Model selection results

Of the 33 regression models we constructed (Table 2), two were considered competitive ($\Delta\text{AICc} < 2.0$) (Burnham and Anderson, 2002) and both indicated a quadratic relationship between THg and PA (Table 2). Our best-supported model contained 0.38 of the model weight (w_i) and included an interaction of Sex with both PA and PA^2 (Fig. 2a). The relationship between predicted age and THg in whole blood of alligators was quadratic, peaking at approximately 40 years in both males and females; the curvature and maximum points differed, however, between sexes (Fig. 2a). Based on 85% CIs, we found none of the effects uninformative: PA ($\beta = 0.15 \pm 0.08$ SE; 85% CI: 0.03, 0.27), PA^2 (-0.15 ± 0.07 ; $-0.26, -0.05$), Sex \times PA (0.26 ± 0.13 ; 0.07, 0.46), and Sex \times PA^2 (-0.33 ± 0.14 ; $-0.54, -0.12$). Our second best-supported model ($\Delta\text{AICc} = 0.64$; $w_i = 0.27$) also contained PA (0.19 ± 0.05 SE; 0.11, 0.27) and PA^2 (-0.20 ± 0.05 ; $-0.28, -0.12$) terms that were informative, but lacked an effect of Sex in either additive or interactive form (Table 1, Fig. 2b). Lastly, our redundant analysis that used the adjustment value derived from all method duplicate differences (including the outlier) produced identical model rankings and therefore is not discussed further.

3.3. Merritt Island National Wildlife Refuge

3.3.1. Summary statistics

Our post-hoc re-analysis of Nilsen et al.'s (2017a) data included THg measurements associated with 189 unique capture events from 169 individual alligators (72 females [F], 97 males [M]) captured at MINWR from 2007 to 2014 (Table 1). Like YWC, adults comprised most of the capture events ($n = 177$; F: 70, M: 107),

Table 2

Linear regression models representing hypotheses about total mercury (THg) bioaccumulation patterns in whole blood of American alligators captured on the Tom Yawkey Wildlife Center coastal South Carolina from 2010 to 2017. Only models within 15 ΔAICc units of the best-supported model are listed here, full list in Supplementary Material (Table S6).

Model ^a	Number of parameters	Deviance ^b	ΔAICc	w_i
Sex \times PA + Sex \times PA^2	7	4.45	0.00	0.38
PA + PA^2	4	4.64	0.64	0.27
Sex + PA + PA^2	5	4.62	2.08	0.13
Sex \times PA + PA^2	6	4.60	3.18	0.08
Sex \times SVL + Sex \times SVL^2	7	4.55	3.86	0.05
~Indiv.	3	*	4.43	0.04
Sex \times SVL + SVL^2	6	4.70	6.83	0.01
BMI	3	4.95	9.20	0.00
SVL + SVL^2	4	4.89	9.29	0.00
OD	3	4.96	9.31	0.00
SVL	3	4.96	9.43	0.00
Intercept	2	5.03	9.48	0.00
BMI + BMI^2	4	4.93	10.64	0.00
PA	3	5.01	11.21	0.00
Sex + SVL + SVL^2	5	4.89	11.23	0.00
OD + OD^2	4	4.95	11.28	0.00
Sex \times SVL	5	4.89	11.34	0.00
Sex + OD	4	4.96	11.39	0.00
Sex + SVL	4	4.96	11.39	0.00
Sex	3	5.02	11.54	0.00
Sex + PA	4	5.01	13.31	0.00
Sex + OD + OD^2	5	4.95	13.41	0.00
Sex \times OD	5	4.96	13.46	0.00
Sex \times PA	5	5.00	14.82	0.00

^a Model selection notation (following Burnham and Anderson, 2002) presents models according to the highest-order effects contained, with all lower-order constituent effects included as additive effects; a superscript ² denotes a quadratic effect, a + sign indicates an additive effect between two variables, a \times denotes an interaction, and a ~ indicates a random effect. Year (categorical) = annual variation, PA = predicted age of the individual at sampling based on estimated predicted age at first capture using the Wilkinson et al. (2016) growth formula for our study population; SVL = snout-vent length in cm at capture; Indiv. = individual alligator modeled as a random effect; BMI = body mass index at capture (Nilsen et al., 2017a); OD = ordinal date or day of year. The continuous covariates contained no missing values and were z-standardized across years (mean = 0.0, SD = 1.0).

^b Models containing random effects were fit with restricted maximum likelihood (REML) and deviance values are not directly comparable to non-REML fit models.

compared to subadults ($n = 12$; F: 8, M: 4). The MINWR data included 19 individuals (6 F, 13 M) that were recaptured during the study, with a mean of 693 ± 607 days between recapture events (F: 716 ± 610 , M: 683 ± 629). Additionally, 18 individuals (4 F, 14 M) were previously encountered by the MINWR mark-recapture study prior to the first blood-sampling event for Nilsen et al. (2017a).

Based on the estimated mean predicted age derived from the first-capture event information (R.H. Lowers, unpublished data) and the Wilkinson et al. (2016) growth model, individuals in the MINWR study averaged 19.95 ± 6.54 years of age (F: 19.00 ± 5.85 , M: 20.60 ± 6.93). The MINWR study sampled individuals over a broader range of ordinal dates (MINWR: 5–365, YWC: 56–271), but individuals were of similar body condition (BMI) and size (SVL) compared to the YWC population (Table 1). Additional MINWR mean covariate values and sex-specific comparisons, previously published by Nilsen et al. (2017a), are listed in Table 1.

MINWR THg whole blood averaged 0.18 ± 0.09 (F: 0.18 ± 0.09 , M: 0.19 ± 0.09 ; Nilsen et al., 2017a), whereas estimated muscle THg averaged 0.15 ± 0.09 (F: 0.15 ± 0.09 , M: 0.16 ± 0.09), and ranged from 0.02 to 0.52.

3.3.2. Model selection results

The mean for THg in whole blood for the MINWR alligators (Overall: 0.18 ± 0.09 , F: 0.18 ± 0.09 , M: 0.19 ± 0.09 ; Nilsen et al. 2017a) appeared similar to the YWC study population. The model

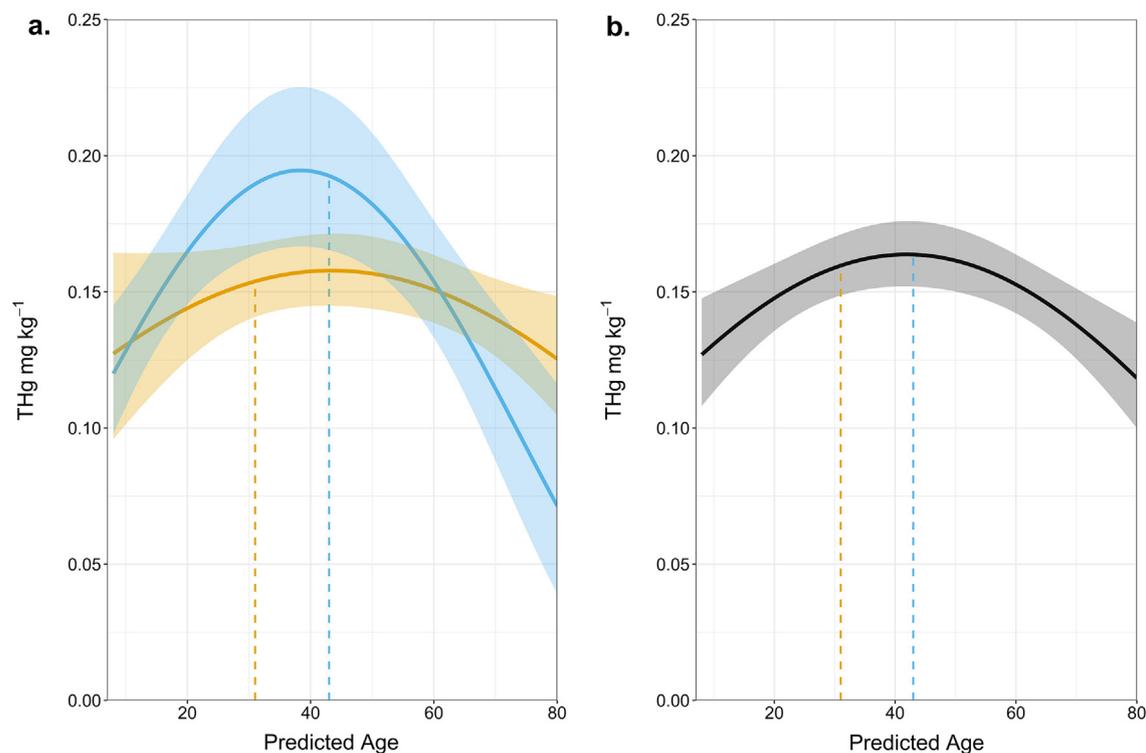


Fig. 2. Predicted total mercury (THg) mg kg^{-1} ww in American alligator whole blood samples from the Tom Yawkey Wildlife Center, South Carolina, USA (2010–2017). Predicted values are represented by the solid lines and the shaded areas represent the 95% confidence intervals. Panel a. shows the predictions from the best-supported linear regression model in our model set (Table 2), which contained Sex \times Predicted Age (PA) and Sex \times PA² covariate terms. Females are represented by the orange lines and the males by blue. The vertical dashed lines represent the sex-specific age at cessation of growth Wilkinson et al. (2016). Panel b. depicts predicted THg from the second best-supported model (Table 2) that only contained PA and PA² terms, with no sex interaction. For both models, the PA estimates were based on the estimated predicted age at first capture, including potential encounters prior to this study (1979–2009), using the growth formula for our study population by Wilkinson et al. (2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Linear regression models representing hypotheses about total mercury (THg) bioaccumulation patterns in the whole blood of American alligators captured on the Merritt Island National Wildlife Refuge in eastern Florida from 2007 to 2014. Only models within 15 ΔAICc units of the best-supported model are listed here, full list in Supplementary Material (Table S7).

Model ^a	Number of parameters	Deviance	ΔAICc	w_i
SVL + SVL ²	4	34.06	0.00	0.55
Sex + SVL + SVL ²	5	33.94	1.46	0.26
Sex \times SVL + Sex \times SVL ²	7	33.54	3.52	0.09
Sex \times SVL + SVL ²	6	33.94	3.59	0.09
Sex \times PA + PA ²	6	35.99	14.68	0.00

^a Model selection notation (following Burnham and Anderson, 2002) presents models according to the highest-order effects contained, with all lower-order constituent effects included as additive effects; (Year) denotes annual variation (categorical), a superscript² denotes a quadratic effect, a + sign indicates an additive effect between two variables, and a \times denotes an interaction. PA = predicted age of the individual at sampling based on estimated predicted age at first capture using the Wilkinson et al. (2016) growth formula for our study population; and SVL = snout-vent length in cm at capture. The continuous covariates contained no missing values and were z-standardized across years (mean = 0.0, SD = 1.0).

selection process identified two competitive models of 33 total in which $\Delta\text{AICc} \leq 2.0$ (Table 3) (Burnham and Anderson, 2002). Both competitive models involved a quadratic relationship between SVL and THg in whole blood of alligators, peaking at approximately 145 cm (Fig. 3). Despite the similarities in covariate structure, the most parsimonious model received more than twice the model weight ($w_i = 0.55$, Table 3) than the next best-supported model ($w_i = 0.26$). The best-supported model contained effects of SVL ($\beta = 2.03 \pm 0.35$ SE; 85% CI: 1.52, 2.54) and SVL² (-1.95 ± 0.35 ; $-2.46, -1.44$) that were both highly informative. The second best-supported ($\Delta\text{AICc} = 1.47$, Table 3) contained the SVL (2.01 ± 0.36 ; 1.50, 2.53) and SVL² (-1.91 ± 0.36 ; $-2.43, -1.40$) effects, but it also contained an additive Sex effect judged not to be informative (-0.07 ± 0.08 ; $-0.19, 0.05$) based on the Arnold (2010) criterion.

4. Discussion

4.1. Total mercury concentrations in whole blood

Our study is among the most comprehensive assessments of bioaccumulation patterns of total mercury (THg) in crocodylians to date and is the first to differentiate between size- and age-driven sources of variation in THg in adult alligators. Due to the temporal breadth of the YWC study (2010–2017), we analyzed whole blood samples of varying age and quality that required multiple processing methods and analytical adjustments. Previous studies have reported mixed results of storage time on THg concentrations in whole blood. Varian-Ramos et al. (2011) analyzed frozen samples of whole blood at different time points over a

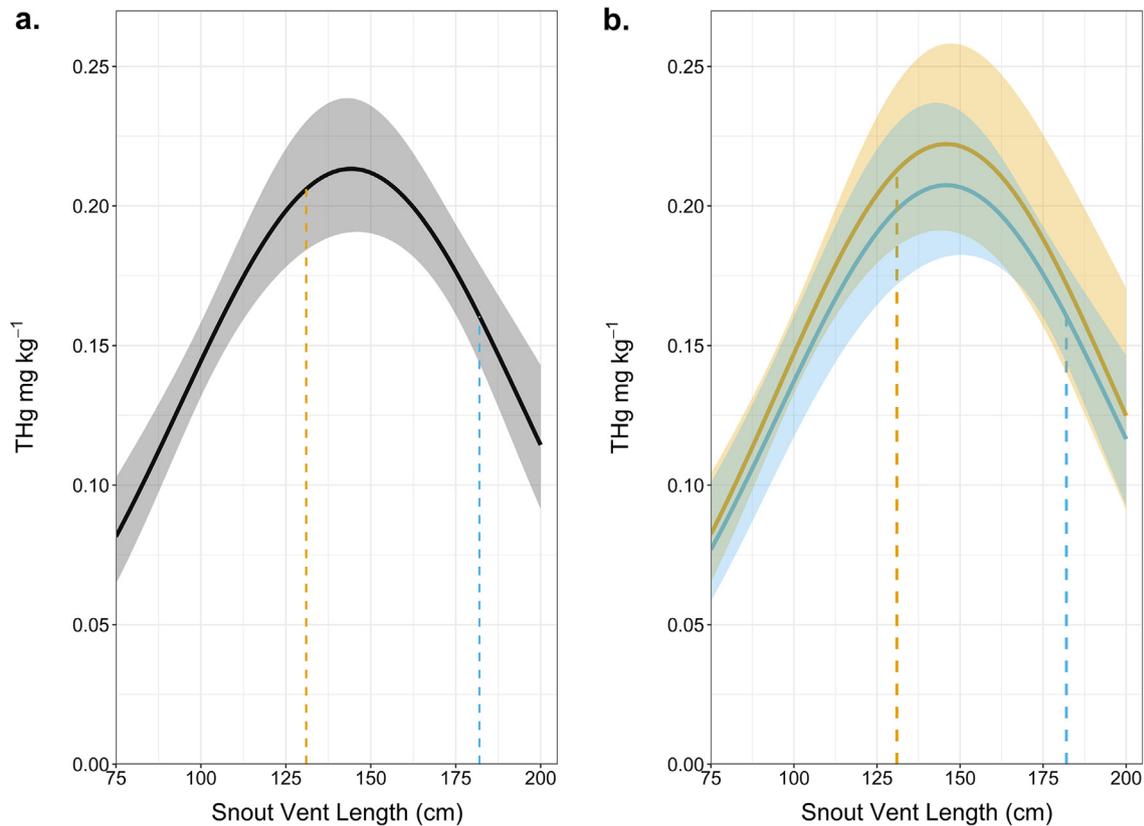


Fig. 3. Predicted total mercury (THg) mg kg^{-1} ww in American alligator whole blood samples from Merritt Island National Wildlife Refuge, Florida, USA (2007–2014). Predicted values are represented by the solid lines and the shaded areas represent the 95% confidence intervals, whereas the vertical dashed lines in panels represent the sex-specific SVL at growth cessation (females in orange, males in blue) derived by Wilkinson et al. (2016). Panel a. shows predictions from the best-supported linear regression model for MINWR (Table 2), which contained additive effects of snout-vent length (SVL) and SVL^2 covariate terms. Panel b. depicts predicted THg from the second best-supported model (Table 3), that contained additive effects of Sex, SVL, and SVL^2 . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

three-year period and detected an average 6% increase in THg concentrations. However, the increase in THg concentrations occurred instantaneously, rather than a progressive increase in THg over time, and storage time explained <11% of the increase in THg (Varian-Ramos et al., 2011). In contrast, Sommer et al. (2016), reported that multiple Hg species in whole blood remain stable for at least one year if stored below 23 °C. All YWC samples were analyzed in February and April 2018 (Table S2); therefore, including Year as a covariate in regression models could potentially capture temporal variation of Hg in the environment, sample age (freezer storage time), or both. Though none of our regression models that contained Year were competitive (Table 2, Fig. S1), we acknowledge that both freezer storage time and environmental factors may be confounded. South Carolina does not have a long-term monitoring network for environmental THg. It is theoretically possible, therefore, that THg may have increased over time in our stored samples (as observed in Varian-Ramos et al., 2011), while concomitantly environmental THg may have decreased. Such a phenomenon could produce a null effect of time similar to the pattern we observed.

The whole blood values of THg reported here for the YWC population in coastal South Carolina had an identical mean and standard deviation to a concurrent study in the same population (0.15 ± 0.05 SD mg kg^{-1} ww) (Nilsen et al., 2019), and similar to several sites in Florida, including MINWR (Nilsen et al., 2017a), Lake Lochloosa (0.20 ± 0.08), Lake Trafford (0.18 ± 0.07), and the St. Johns River (0.13 ± 0.06) (Nilsen et al., 2016). In contrast, THg in our samples appears to be considerably lower compared to samples from adult alligators in Florida occupying Water Conservation

Areas 2A (0.41 ± 0.22) and 3A (0.53 ± 0.42) near Everglades National Park (Nilsen et al., 2016), and compared to Par Pond at the Savannah River Site in South Carolina (0.32, converted from *dw* to *ww* using methodological adjustment described here, SD not reported) (Jagoe et al., 1998). Both Everglades and the Savannah River Site (approximately 233 km inland from YWC, Fig. 1) have an established history of Hg pollution from natural and anthropogenic sources (Brisbin et al., 1996; Frederick et al., 2004; Rumbold et al., 2008; Yanochko et al., 1997). Local Hg input may also explain why the findings of this study and Jagoe et al. (1998) contrast with South Carolina's increasing Hg gradient from the Blue Ridge/Piedmont physiographic region to the coastal plain (Guentzel, 2009), which is reflected in fish species and is primarily driven by the percentage of wetland area within each watershed (Glover et al., 2010).

4.2. Demographic factors in THg patterns

We detected three general patterns in THg concentrations in alligator whole blood in the YWC and MINWR populations (Tables 2 and 3): (1) mixed support for sex-specific differences in THg bioaccumulation; (2) consistent support for age indicators (PA and SVL), relative to other covariates; and (3) a quadratic relationship between age-indicators and THg. We discuss each in turn.

The model set for each population contained a competitive model that included sex-specific differences (Tables 2 and 3), though the Sex effect (covariate) was only informative for YWC and not MINWR. The relatively large sample sizes for both study populations may explain why we were able to detect sex-specific

effects (albeit, differentially informative), compared to many previous studies in alligators (Burger et al., 2000; Campbell et al., 2010; Rumbold et al., 2002; Yanochko et al., 1997) and other crocodylians that used smaller sample sizes (range: 10–78 samples) (Eggins et al., 2015; Schneider et al., 2012; Vieira et al., 2011). The best-supported YWC model included interactions between Sex and both PA and PA², producing a pattern in which male THg concentrations peaked at a higher value compared to females, but also declined at a faster rate (Fig. 2a). Whereas the second best-supported model for MINWR included an additive effect of Sex with SVL and SVL², in which sex-specific THg concentrations varied in parallel and peaked at middle sizes. Although the age indicator covariate's structural relationship to the Sex differed between the two populations, we posit that such differences are potentially an artifact of differences in the sampled populations. Compared to YWC, the maximum value of SVL for females in the MINWR study was curtailed (135 cm; YWC: 150). Our inability to find an informative sex-dependent pattern in the THg–SVL relationship for the MINWR population could be explained by the fact that the restricted data range in that population provided less support for estimating the sex parameter. In fact, the estimated peak for the female response curve occurred at 146 cm SVL (Fig. 3b), well above the maximum SVL observed for MINWR females (Table 1).

Similarly, the two best-supported models for YWC included predicted age, but the most parsimonious model for MINWR that contained predicted age was not competitive ($\Delta\text{AICc} = 14.68$) (Table 3). We suggest that the lack of support for the predicted age covariate at MINWR compared to YWC is likely due to three factors: (1) differences in mark-recapture study length duration (YWC: 39 years; MINWR: 9 years) which would limit the potential age ranges that could be observed; (2) the relatively limited number of individuals with auxiliary first capture data prior to the THg study for MINWR ($n = 18$) compared to YWC ($n = 38$); and (3) the use of a South Carolina-based growth model (Wilkinson et al., 2016) to derive predicted ages for MINWR alligators. Though latitudinal differences in temperature can create variation in the length of growing season for alligators, YWC growth rates are similar to those observed in coastal Louisiana, which has a similar latitude to that of MINWR (Jacobsen and Kushlan, 1989; Joanen and Mcnease, 1971; Wilkinson et al., 2016). While predicted age derived from a growth formula is a more direct indicator of “true” age, size (as quantified by SVL) may serve as an effective proxy in individuals that are still growing. In a post-hoc assessment, only 2% of the first capture observations in the MINWR dataset were of alligators at or above the mean sex-specific size at cessation of growth (F: 131.4 cm SVL, M: 182.0 cm SVL) (Wilkinson et al., 2016), compared to YWC (11%). As such, for cases like the MINWR dataset in which nearly all observations are from individuals that are still growing, SVL may be an effective proxy for age.

Both the YWC and MINWR model sets indicated strong support for quadratic patterns in THg bioaccumulation with age indicators (Tables 2 and 3). Due to the relatively short duration of both Hg studies (seven years) relative to the alligator's lifespan, we clarify that our inference in the relationship between THg and age indicators is restricted to comparisons at the population level, and not changes within individuals over their lifespan. Though we selected for samples from individuals that were captured on multiple occasions during the Hg study, we did not have sufficient data to conduct a traditional longitudinal analysis that included random intercepts and slopes (as a function of time) for each individual. In the YWC population, THg increased prior to the average onset of reproductive maturity at 15.8 years for females and 11.6 years for males (corresponding to 1.8 m TL) (Joaanen and McNease, 1980; Wilkinson et al., 2016), and THg subsequently peaked at 43 (female) and 38 (male) years of age (Fig. 2), before declining. The decreased THg that we observed in the oldest individuals

(Fig. 2) contrasts with studies in fish that have reported strictly linear, positive relationships between mercury and age (as determined by otolith analysis) (Chumchal and Hambricht, 2009; Lavigne et al., 2010; Malinowski, 2019). Multiple avian studies have failed to document age-related effects in adult individuals of known-age (Becker et al., 2002; Burger et al., 1994; Furness et al., 1990; Thompson et al., 1991).

We assert that the age-related decline in THg is biologically meaningful and not an artifact of our study design for several reasons. First, we determined that predicted age associated with each sample was not a function of capture year (i.e., we were not encountering older individuals in later study years). Therefore, more recent samples, for which storage time was shorter, were not characterized by lower THg values nor were they associated with older individuals. Second, it is unlikely that our results reflect a survivorship bias in our data, in which individuals with higher THg concentrations had higher mortality rates, leaving only individuals with lower THg available for encounter at the oldest ages. The maximum value we measured of THg in whole blood ($0.35 \text{ mg kg}^{-1} \text{ ww}$) is substantially lower compared to values observed in the Everglades ($1.33\text{--}1.56$) (Nilsen et al., 2016), and our maximum estimate of THg in muscle ($0.32 \text{ mg kg}^{-1} \text{ ww}$) is less than the value for fish consumption advised by the World Health Organization ($0.50 \text{ mg kg}^{-1} \text{ ww}$) (WHO, 1990). Lastly, there appears to be only a single reported case of potential mercury-induced mortality in a wild alligator (Brisbin et al., 1998), in which the individual had muscle THg concentrations ($3.48 \text{ mg kg}^{-1} \text{ ww}$) 27 times higher than the estimated YWC mean, and also surpassed all known lethality concentrations observed in dosing studies in other reptiles and amphibians (Grillitsch and Schiesari, 2010; Hall, 1980; Wolfe et al., 1998).

4.3. Mechanisms of age-related THg patterns in alligators

Traditionally, growth patterns in reptiles have been described as indeterminate (i.e., no growth cessation) (Charnov et al., 2001; Congdon et al., 2013; Kozłowski, 1996); however, there is increasing consensus that some species within reptile taxa exhibit determinate (i.e., asymptotic) growth, including lizards, turtles, and crocodylians (Campos et al., 2014; Congdon et al., 2001; Taylor et al., 2016; Tucker et al., 2006; Wilkinson et al., 2016; Woodward et al., 2011). While size could serve as an appropriate proxy for age in species with indeterminate growth, reliance on size as an indicator of age in determinate growth species is particularly problematic for individuals that are near or have growth cessation. In this context, it is not surprising that the majority of ecotoxicological studies in both alligators and other crocodylian species have either detected a weakly positive (Nilsen et al., 2017a; Schneider et al., 2012; but see Eggins et al., 2015) or non-existent relationship between size/age and mercury (Campbell et al., 2010; Rainwater et al., 2007; Rumbold et al., 2002), or reported an inconsistent relationship that differed in effect size depending on the tissue sampled or study site (Jagoe et al., 1998; Yanochko et al., 1997).

Concomitant with long-held assumptions of indeterminate growth, alligator studies to date have explored strictly linear (non-polynomial) relationships between mercury and age proxies, reflecting an assumption of mercury bioaccumulation throughout an individual's lifespan. Increases in mercury are to be expected for growing individuals and are supported by our results. During the growth phase, juveniles and young adults feed at lower trophic levels compared to adults (Hanson et al., 2015; Nifong et al., 2015; Santos et al., 2018) and are therefore expected to have lower THg than older, larger individuals. However, it is unclear how bioaccumulation patterns may change following growth cessation, given the dearth of studies that have evaluated known- or

minimum-age patterns in behaviors that could affect mercury bioaccumulation (e.g., diet, movement, etc.).

Our study indicates that THg begins to decline in individuals approximate to the age of expected growth cessation (Fig. 2a), and here we provide two general, non-exclusive hypotheses to explain this pattern: (1) alligators become more efficient at offloading mercury as growth cessation nears, or (2) older alligators are exposed to less mercury. Regarding the first point, alligators sequester mercury in keratinized tissues such as skin and claws (Burger et al., 2000; Jagoe et al., 1998), though it is unclear how the speed or efficiency of this process relates to age or sex, or how the loss of skin (sloughing) or claws (worn, broken) aids in THg excretion (Alibardi, 2003). Similarly, though female alligators can mobilize and deposit stored contaminants in developing eggs during vitellogenesis (maternal transfer) (Roe et al., 2004, F. Nilsen, *unpubl. data*), no crocodylian study has evaluated if maternal transfer significantly lowers body burdens of mercury following oviposition, including age-related variation. Multiple sea turtle studies have reported relatively low THg concentrations in eggs compared to maternal tissues (including whole blood), suggesting that THg depuration through vitellogenesis is relatively minimal (Perrault et al., 2011; Sakai et al., 1995). More importantly, the relationship we detected suggests that THg concentrations continue to increase for many years following the onset of sexual maturity at ca. 16 years of age (Fig. 2) (Joanen and McNease, 1980; Wilkinson et al., 2016), which is the opposite of what would be expected if maternal transfer was the primary mechanism responsible for THg bioaccumulation in females. Though further studies are needed, it is possible that both maternal transfer and sloughing of keratinized tissues could have interactive effects on other factors that regulate THg bioaccumulation.

Alternatively, reduced exposure to or less consumption of Hg following growth cessation would also produce the patterns we observed. Differences in exposure among age classes would require that the oldest and youngest adults/old subadults inhabit areas with lower mercury bioavailability compared to areas inhabited by middle-aged individuals. Although spatial segregation of adult size classes has been documented in Nile crocodiles (*Crocodylus niloticus*) (Hutton, 1989), we suggest this is unlikely for alligators in our study area where extensive population surveys and capture efforts in fixed locations have demonstrated considerable spatial overlap among adult size classes (Lawson, 2019). Additionally, large alligators (>273 cm TL) are generally the most cannibalistic and consume both juveniles and young adults (122–212 cm TL) (Rootes and Chabreck, 1993) further demonstrating a spatial overlap among crocodylian size/age classes.

Lower mercury concentrations in the oldest individuals could also reflect reduced mercury intake from either an age-related shift in diet, where food items were characterized by different mercury loads, or from an age-related change in the amount of food consumed. While age-related differences in diet between adult and juvenile alligators are well-established through stable isotope and stomach content analyses (Delany et al., 1999; Nifong et al., 2015; Santos et al., 2018), fine-scale, size-related variation within adults or longitudinal patterns within individuals remain relatively unexamined. In estuarine crocodiles (*C. porosus*), Hanson et al. (2015) detected a quadratic relationship between body size and trophic position (as indicated by $\delta^{15}\text{N}$), with medium-sized individuals foraging upon a larger proportion of prey items from higher trophic levels compared to the smallest and largest individuals. Moreover, Hanson et al. (2015) did not detect evidence of spatial segregation among size classes, suggesting that individuals were feeding in the same areas. Additionally, Rivera et al. (2016) reported that large adult Yacare caimans (*Caiman yacare*) had significantly lower THg content than seven common carnivorous fish prey species, despite being a top predator; though THg concentra-

tions in smaller size classes were not examined. However, as with THg, long-term, longitudinal studies are needed to provide a means by which to evaluate age-related diet patterns within adults and other age classes.

Reduced Hg intake as a consequence of reduced food consumption could also occur due to senescence. Though age-related declines in metabolism are well-documented across wildlife taxa for both sexes (Elliott et al., 2015 and references therein), such patterns have yet to be investigated in reptiles. The quadratic relationship we detected suggests that the cessation of growth (31 years for females, 43 years for males, vertical dashed lines in Fig. 2a) coincides with the onset of the decline in THg. In the YWC alligator population, mark-recapture data indicate that females continue to reproduce for at least twenty years following the cessation of growth (Wilkinson et al., 2016). Therefore, while maternal transfer of THg does not explain the patterns we observed in younger, smaller adults, this could act synergistically as a depuration mechanism in older females, particularly those that have ceased growing. We further acknowledge that variation in THg concentrations in early life stages not evaluated by this study (e.g., hatchlings) could affect individual growth rates or trajectories and bias estimates of predicted age. However, multiple studies focused on fish indicate strong support for biodilution, in which mercury accumulation is determined by individual growth rates, rather than initial mercury concentrations determining eventual growth rates; consequently, faster-growing individuals accumulate mercury at lower rates than slower-growing individuals (Lavigne et al., 2010; Sandheinrich and Drevnick, 2016). Though further studies are needed to determine if biodilution effects are present in alligator populations, in this context it is unlikely that variation in early-life Hg exposure biased our estimates of predicted age.

4.4. Implications for consumption advisories

We acknowledge that the use of predicted age to predict THg content in whole blood of alligators is potentially problematic, and additional study is warranted if this relationship is to be used to inform guidelines regarding consumption of alligator meat. The use of a predictor measured with error introduces an unknown degree of bias to regression estimates derived through ordinary least squares (Draper and Smith, 1998). Because we predicted age at first capture through application of the Baker et al. (1991) formula, we anticipate that estimates from our regression models are prone to bias. However, bias is mitigated if the spread of true age in the sample is large relative to the spread of error (Draper and Smith, 1998). Here, the prediction error for age is positively related to SVL at first capture (i.e., prediction error increases as individuals approach the average size at growth cessation). Therefore, bias in the regression estimates is least when the sample comprises mostly smaller animals that are still growing. More first capture observations in the YWC sample represented alligators that had reached the average size at growth cessation (11%) than in the MINWR sample (2%), but a majority of individuals (95%) in both samples were below the threshold of growth cessation when they were first captured. For this reason, we suggest that the general quadratic pattern of THg with age is robust; however, we suggest further study of the issue if the relationship between age/size and THg were to be used to inform consumption guidelines. Studies that included samples of known-age individuals would better identify the conditions under which reliance on predicted age introduces biases relating to THg bioaccumulation. Lastly, the mean whole blood THg concentrations (and their associated muscle THg estimates) were fairly similar for YWC and MINWR. Replicating our study in geographic areas in which alligators are expected to have a different or broader range of THg

concentrations in muscle or whole blood (e.g. Everglades) could assess whether the relationship between THg and predicted age we reported is robust to local variation in Hg deposition and bioavailability.

5. Conclusion

Mercury is a ubiquitous contaminant that is biomagnified within wetland food webs. We detected a previously undescribed pattern of THg in blood samples from two alligator populations, in which THg peaks at middle age approximately coinciding with the cessation of growth. Therefore, our data suggest that regulatory agencies interested in minimizing risk from consumption of alligator meat may consider developing additional studies to further examine this relationship. While this pattern contrasts with previous assumptions of increasing THg throughout an individual's lifetime, we posit that the observed reduction in THg following the cessation of growth may be due to age-related changes in metabolism or foraging behaviors. However, we acknowledge that further study is needed to rule out other causal factors such as age-related THg excretion, the influence of early Hg exposure on growth rates (e.g., biodilution), and individual variation in bioaccumulation tendencies. This study highlights the means by which long-term, longitudinal monitoring studies could be used to differentiate between potential confounding effects of time, age, and size in sentinel species, the latter two of which are particularly important for long-lived reptiles.

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Appendix A. Supplementary information and data availability

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.135103>. The data used in this study are publicly available in the US Geological Survey Science Data Catalog (YWC) at <https://doi.org/10.5066/P98XHBCY> and though Mendeley Data (MINWR) at <https://doi.org/10.17632/vycpgt6ycf.1>.

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