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RELATIONSHIPS AMONG AURAL ABSCESSES, ORGANOCHLORINE COMPOUNDS, AND VITAMIN A IN FREE-RANGING EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*)

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ABSTRACT: Aural abscesses are a common health problem in free-ranging eastern box turtles (*Terrapene carolina carolina*), and they have been associated with high body burdens of organochlorine (OC) compounds, which are known disruptors of vitamin A. The objective of this study was to determine if the presence of pathologic lesions in box turtles were correlated with increased and decreased levels of hepatic OC compounds and vitamin A, respectively. A graded scale for the pathologic changes observed in tissue samples collected from abscessed and nonabscessed box turtles over a 2-yr period (2003–04) was developed, and the levels of OC compounds and vitamin A in livers collected from the same turtles were determined through chemical analysis. Sixty-eight turtles (40 with aural abscesses and 28 without) were included in the study. Relationships between variables were analyzed using Spearman's Rank Correlation Test, where $P \leq 0.05$ was considered significant. Twenty-seven different OC compounds were identified. Mean ± standard deviation (SD) total OC compound level for all turtles was 0.35 ± 0.83 ppm (range 0–5.81 ppm), and mean ± SD vitamin A level was 72.8 ± 98.6 ppm (range 0–535.7 ppm). There was no correlation between pathologic score and total hepatic OC compound concentration ($r = -0.18$, $P = 0.16$). However, pathologic score was positively correlated with o,p′-DDT ($r = 0.25$, $P = 0.05$). Vitamin A was positively correlated with pathologic score ($r = 0.32$, $P = 0.01$), which was contrary to the expected result. There was no linear correlation between vitamin A and total hepatic OC compound concentration ($r = -0.04$, $P = 0.75$). However, a nonlinear regression provided a significant fit ($r^2 = 0.12$, $P = 0.02$), indicating an initial increase in vitamin A as the OC compound burden increased, followed by a decline as OC compound levels increased further. The hepatic OC compound residue concentrations in these box turtles were lower compared to levels found in freshwater aquatic turtles but similar to levels in other terrestrial reptile species. This study provides mixed results in support of a role of OC compounds, presumably of environmental origin, in the etiology of aural abscesses in free-ranging box turtles.

Key words: Aural abscess, eastern box turtle, organochlorine compounds, Spearman's Rank Correlation Test, *Terrapene carolina carolina*, vitamin A.

INTRODUCTION

Aural abscesses in eastern box turtles (*Terrapene carolina carolina*) are a common health problem of unknown etiology and significance to free-ranging populations (Brown and Sleeman, 2002). Holladay et al. (2001) reported that wild-caught box turtles with aural abscesses had significantly higher body burdens of organochlorine (OC) compounds when compared to turtles without aural abscesses. Furthermore, the turtles with aural abscesses also displayed a trend toward lower serum and hepatic vitamin A levels. Holladay et al. (2001) proposed that the OC compounds were acting as endocrine disruptors, inducing hypovitaminosis A, which resulted in squamous metaplasia of the tympanic cavity and abscess development. Support for this hypothesis includes histopathologic changes consistent with vitamin A deficiency, such as squamous metaplasia in the tympanic cavities

of turtles with aural abscesses (Brown et al., 2004), as well as the lack of support for a bacterial etiology (Feldman et al., 2006; Joyner et al., 2006). The objective of this study was to further elucidate potential cause-effect relationships among aural abscesses, OC compounds, and vitamin A levels in box turtles by determining if correlations existed between these variables.

MATERIALS AND METHODS

Sixty-eight turtles (40 with aural abscesses and 28 without) presented to the Wildlife Center of Virginia, Waynesboro, Virginia (38°02′N, 78°55′W), or the Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia, during 2003–04 were included in the study. All turtles were considered nonreleasable to the wild and were euthanized prior to sampling using an intravenous injection of pentobarbital sodium (Beuthanasia-D; Schering-Plough Animal Health, Union, New Jersey, USA) followed by an overdose of inhaled halothane (Halothane U.S.P.; Halocarbon Laboratories, River Edge, New Jersey, USA). Cases of aural abscesses were identified by observation of a unilateral or bilateral solid mass medial to the tympanum (Brown et al., 2004). Lesions were confirmed after euthanasia by surgical incision of the tympanic membrane and exposure of the inner caseous material. Turtles without abscesses consisted of animals that had been presented for traumatic injuries that did not involve the head (mostly due to automobile-related injuries) and had no evidence of swelling medial to the tympanic membrane. Nonabscess status was confirmed after euthanasia following surgical incision of the tympanic membrane and observation of grossly normal tympanic cavity epithelium of the middle ear. Sex of the turtles was determined by examination of the internal gonads. Turtles were weighed using a 1,000-g scale with a precision of ±5 g.

Upon confirmation of death, the head was removed and immediately placed in 10% buffered neutral formalin. The liver was also harvested, wrapped in aluminum foil, and frozen at −20 C until analysis. Transverse sections of the formalin-fixed heads were made using a band saw at the level of the tympanum and decalcified for 1 wk in TBD-2 decalcifier (Thermo Shandon, Pittsburgh, Pennsylvania, USA). The tissue sections and tissue samples were then routinely processed and embedded in paraffin. Sections (7 μm) were cut from the embedded tissue and stained with hematoxylin and eosin. Tissue sections were examined without knowledge of their disease status, and the tympanic cavity epithelium was evaluated for pathologic changes. Pathologic changes evaluated included inflammation, hyperemia, keratinization, necrosis, hyperplasia, and dysplasia, and the severity of the lesions were scored using a previously described scoring system (Brown et al., 2004). Briefly, the presence and severity of changes for each diagnostic category were scored as minimum, moderate, or marked, and the scores for each of the six categories were added together. The final score for each turtle was based on the following aggregated scores: minimal pathologic change (1), moderate (2), marked (3), or severe pathologic change (4).

In addition, a 0.5-g liver sample from each turtle was placed into a 15-mm test tube for vitamin A analysis. Vitamin A acetate (100 μl at 2 ppm in ethanol) was added to each tube as an internal standard. Two milliliters of methanol were added, and the sample was homogenized using a Brinkmann Polytron Grinder (Brinkman Instruments, Westbury, New York, USA). The grinder probe was washed in 2 ml of methanol and added to the sample for a total of 4 ml of methanol. Three milliliters of cyclohexane were then added to the sample and vortexed for 30 sec. Tubes were centrifuged at 800 × G for 5 min to separate the layers. The cyclohexane layer was removed, placed into a 1-dram vial, and dried in vacuo. The cyclohexane extraction was repeated by placing the extracted cyclohexane solution into the same 1-dram vial and again drying it in vacuo. The sample was reconstituted with 100 μl of 20% ether in ethanol. Percent recovery of vitamin A was determined for each sample based on the recovery of the vitamin A acetate internal standard and was adjusted for vitamin A concentrations. A standard curve for vitamin A was made using freshly prepared standards at 10, 20, 40, 60, 80, 100, 140, 160, and 200 ppm with an r^2 of 0.999. A line from the linear regression for the standard curve of peak area (absorption) versus concentration of standard was generated, and the concentrations of the samples were calculated using this line.

Liver contaminants were also evaluated by gas chromatography (GC). Briefly, a matrix solid-phase dispersion (MSPD) procedure was used to extract pesticides from turtle livers. In MSPD, 0.5 g of finely chopped turtle liver was thoroughly mixed with 2 g of C18 Bondesil bulk sorbent (40 μm particle size; Varian, Harbor City, California, USA) using a mortar

and pestle. The sample-C18 mixture was transferred to a 2-g sorbent mass Bond Elut Florisil solid-phase extraction (SPE) cartridge containing 20-μm polyethylene frits (Varian, Harbor City, California, USA). Extraction cartridges were placed on the vacuum manifold with a stopcock for flow-rate control, and a vacuum pressure of about 2.5 mm Hg was applied as 4×5 ml MeCN was used to elute the analytes into 15-ml graduated, glass centrifuge tubes. A Supelco Visiprep 5-70030 12-port SPE vacuum manifold (Bellefonte, Pennsylvania, USA) was used for the elutions from the SPE Florisil columns, after which an Organomation Buchii model 111 N2 evaporator (Berlin, Massachusetts, USA) was used to concentrate the extracts to 1 ml by evaporation under a gentle stream of nitrogen prior to instrument analysis.

Sample extracts were then analyzed with an Agilent (Little Falls, Delaware, USA) 6890 gas chromatograph equipped with a 7683A autosampler, split/splitless injection inlet, and micro-electron capture detectors. The system was configured with two fused silica capillary columns, which connected to a guard column using a Y-press tight connector that led to one injector inlet (dual-column injection). The split/splitless injector was operated in the splitless mode at a temperature of 250 C. Ultrahigh purity helium was the carrier gas at a constant flow rate of 1 ml/min and an inlet pressure of 38.42 psi. The oven temperature was programmed as follows: 60 C for 1 min ramped at 10 C/min to 100 C and held for 1 min, and ramped again at 4 C/min to 275 C and held for 15 min. Chromatographic separations were performed using two columns. An RTX-5 (30 m×0.25 mm inner diameter [i.d.], 0.25 μm film thickness) fused silica capillary column (Restek, Bellefonte, Pennsylvania, USA) was the primary column.

Pesticide reference standards at 95% or higher purity were obtained from Restek (Bellefonte, Pennsylvania, USA) and Chem-Service (West Chester, Pennsylvania, USA). An A&D HR-120 analytical balance (Milpitas, California, USA) was used to weigh the samples, reagents, and standards. Stock solutions of 1,000 μg/ml were prepared in various solvents; spiking solutions and working standard pesticide mixtures were prepared in hexane. Confirmation of the identity of pesticides in turtle livers was performed by comparison with chromatographic separations on an RTX-35 (30 m×0.25 mm i.d., 0.25 μm film thickness) column from the same supplier. The micro-electron capture detectors were operated at 350 C with nitrogen makeup flow at 60 ml/min. Chemstation software was used for instrument control and data analysis. Additional confirmation of pesticides was performed using a Hewlett-Packard (Agilent, Wilmington, Delaware, USA) 5890 GC/MS. Pesticides identified in sample extracts were quantified using the external standard technique. The chromatographic response was linear (>0.9900) for all working standard concentration ranges. The limit of quantitation (LOQ) of each pesticide was taken as the highest spiking level that yielded a percent recovery within 70–130%. Organochlorine compounds tested for included aldrin, alpha-benzene hexachloride (BHC), beta-BHC, delta-BHC, gamma-BHC (Lindane), alpha-chlordane, gamma-chlordane, 4,4-DDD, 4,4-DDE, 4,4-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, methoxychlor, chlorpyrifos, o,p-DDD, o,p-DDE, o,p-DDT, diazinon, hexochloro-benzene (HCB), dicofol (Kelthane), metolachlor, and oxychlordane.

Spearman Rank Correlation coefficients were calculated as a measure of correlation between any two variables (pathologic score, hepatic vitamin A concentration, and hepatic OC compound concentration) for all turtles combined, for strata of turtles defined by sex (male vs female), as well as strata defined by weight (turtles with weight ≤254 g [first quartile], turtles with weight >254 g but ≤358 g [second quartile], turtles with weight >358 g but ≤406 g [third quartile], and turtles with weight >406 g [fourth quartile]). In addition, Spearman Rank Correlation coefficients were calculated as a measure of correlation between the individual OC compounds for all turtles combined and pathologic score as well as between individual OC compounds for all turtles and hepatic vitamin A. The relationship between hepatic vitamin A concentration and total hepatic OC compound concentration was determined by selecting the model that maximized the r^2 fit among candidate linear and nonlinear models (Sit and Poulin-Costello, 1994). All analyses were performed using SAS statistical package (SAS version 9.1.3, SAS, Cary, North Carolina, USA). Significance was set at $P \leq 0.05$.

RESULTS

Twenty-seven different OC compounds were identified (Table 1). The most frequently identified compounds included heptachlor epoxide, dieldrin, and oxychlordane. Mean±standard deviation (SD) total

TABLE 1. Organochlorine (OC) compounds detected in hepatic samples from 68 eastern box turtles from Virginia with and without aural abscesses sampled during 2003–04.

OC compound	No. of turtles in which compound was detected[a]	Mean for all turtles (n=68, ppm)	±SD	Maximum[b]
Aldrin	4	0.00101	0.00588	0.044
Benzene hexachloride (*a*)	2	0.00025	0.00157	0.012
Benzene hexachloride (*B*)	1	0.0004412	0.00364	0.03
BHC (*B*)	1	0.0004412	0.00364	0.03
Alpha chlordane	5	0.01118	0.06078	0.44
Gamma chlordane	4	0.00235	0.01231	0.091
o,p′-DDD	7	0.0000515	0.0001531	0.0005
p,p′-DDD	2	0.00237	0.01916	0.158
o,p′-DDE	3	0.0000221	0.0001034	0.0005
p,p′-DDE	7	0.04897	0.38312	3.16
o,p′-DDT	9	0.0000662	0.0001707	0.0005
p,p′-DDT	6	0.02198	0.17432	1.438
Diazinon	1	0.0005588	0.00461	0.038
Dieldrin	18	0.06431	0.19063	1.268
Endrin	5	0.01296	0.07531	0.63
Endrin aldehyde	1	0.0002941	0.00243	0.02
Endrin ketone	2	0.00107	0.00826	0.068
Endosulfan sulfate	7	0.00131	0.00461	0.026
Endosulfan 1	1	0.00381	0.03141	0.259
Endosulfan 2	3	0.0381	0.03141	0.012
Heptachlor	1	0.0000147	0.0001213	0.001
Heptachlor epoxide	35	0.08763	0.18029	0.96
Hexachlorobenzene	13	0.0003235	0.1597	0.016
Lindane	5	0.0003235	0.00199	0.016
Metolachlor	9	0.00551	0.01597	0.072
Methoxychlor	2	0.00143	0.00833	0.055
Oxychlordane	20	0.05699	0.13933	0.768
Total	**68**	**0.35343**	**0.83148**	**5.81**

[a] All 68 turtles were analyzed for all compounds.

[b] Values below the detection limit were considered at 0 ppm.

OC compound level for all turtles was 0.35±0.83 ppm (range 0–5.81 ppm), and mean±SD vitamin A level was 72.8±98.6 ppm (range 0–535.7 ppm). There was no significant correlation between pathologic score and total hepatic OC compound concentration (r=−0.18, P=0.16). However, pathologic score was positively correlated with o,p′-DDT (r=0.25, P=0.05). Nonsignificant positive correlations to pathologic score were also observed with o,p′-DDD (r=0.23, P=0.06) and metolachlor (r=0.23, P=0.07). Vitamin A was positively correlated with pathologic score (r=0.32, P=0.01) (Fig. 1). There was no linear correlation between vitamin A and total hepatic OC compound concentration (r=−0.04, P=0.75). However, a nonlinear regression did provide a significant fit (r^2=0.12, P=0.02), represented by the linear model ln(Vitamin A)= 4.377+0.0729*ln(Total OC)−0.3226*Total OC (functional form: Vitamin A= [79.6030]*[Total $OC^{0.0729}$]*[$0.7243^{\text{Total OC}}$]), indicating an initial increase in vitamin A as the OC compound concentration increased, followed by a decline as OC compound levels increased further (Fig. 2). There was no evidence of any significant correlations between variables for turtles stratified by sex (i.e., weight with total OC compound level, vitamin A with total OC compound level, pathologic score with total OC compound level, vitamin A with weight, and pathologic score with weight for male or female

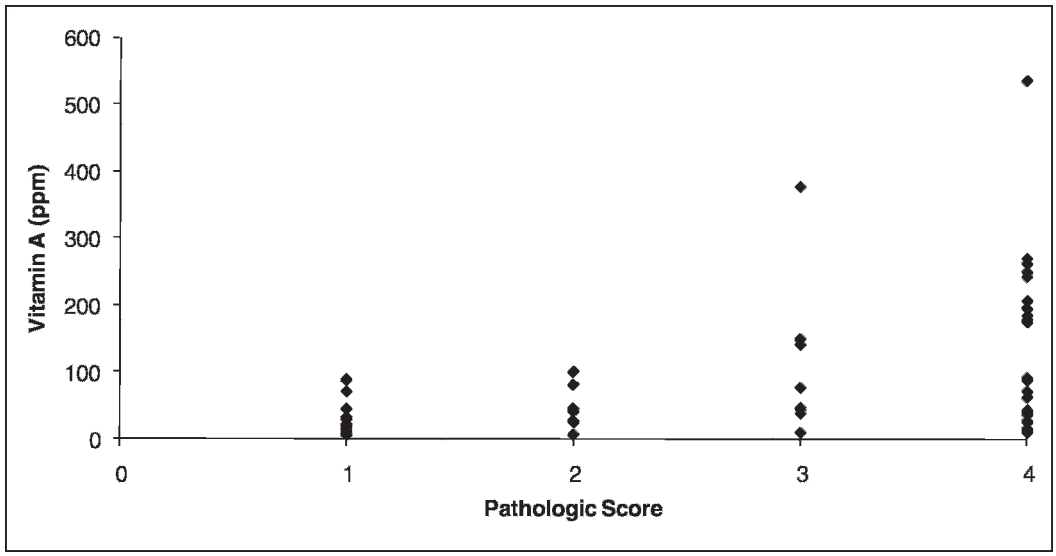

FIGURE 1. Bivariate scatter plot between aural abscess pathologic score (1 = minimal pathologic change, 2 = moderate, 3 = marked, and 4 = severe) and hepatic vitamin A concentration in 68 eastern box turtles from Virginia sampled during 2003–04. Significant positive correlation was found between the variables (Spearman's Rank Correlation Test, $r=0.32$, $P=0.01$).

turtles) or weight (vitamin A with total OC compound level, and pathologic score with total OC compound level for all four quartiles of weight).

DISCUSSION

Synthetic chemical contaminants have been observed to mimic hormones, act as

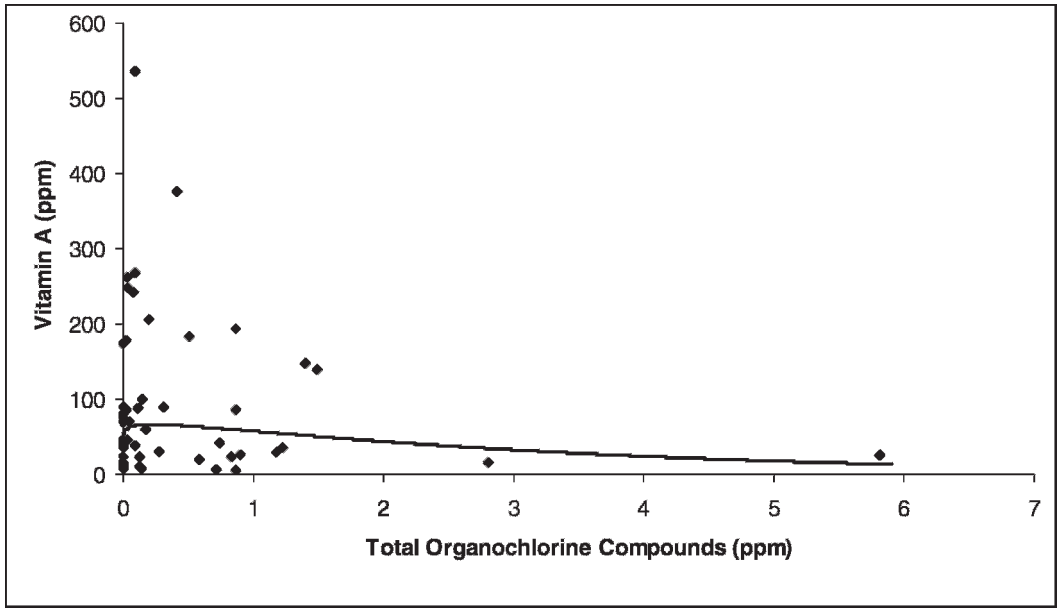

FIGURE 2. Bivariate scatter plot illustrating the nonlinear relationship ($r^2=0.12$, $P=0.02$) between hepatic organochlorine (OC) compound concentration and hepatic vitamin A concentration in 68 eastern box turtles with and without aural abscesses from Virginia sampled during 2003–04.

antihormones, or alter the synthesis and/or degradation of hormones. Contaminants can interact with various steroid hormone receptors, genetic transcription factors, and alter hormone-induced genetic responses. Endocrine-disrupting contaminants can bind to estrogen receptors, acting either as agonist or antagonists, as well as interact with the androgen, progesterone, or retinoic receptors (Sparling et al., 2000). Few studies have evaluated these effects in reptiles; however, the available studies suggest that reptiles exhibit a sensitivity to these compounds similar to that reported for birds and mammals, and they bioaccumulate contaminants to levels equal to, or greater than, that reported for birds and mammals (Sparling et al., 2000).

No linear correlation between hepatic OC compound concentration and severity of aural abscess pathologic changes was found in our study. Consequently, this study does not support a causal relationship between aural abscesses in box turtles and OC compounds. The importance of the significant positive correlation between o,p′-DDT and pathologic score is uncertain, especially since it was not detected in all the turtles with aural abscesses. However, in studies with red-eared sliders (*Trachemys scripta*), o,p′-DDT was found to have higher estrogenic activity than DDT (Sparling et al., 2000). O,p′-DDT is a metabolite of DDT, and some OC compounds are known require metabolization before they are able to interfere with receptor binding (Heussen et al., 1993). Furthermore, even structurally related compounds may have different degrees of biologic activity (Guillette et al., 1995). Consequently, the relative contribution of o,p′-DDT to any endocrine-disruptive effect may be greater than some of the other detected compounds.

The positive correlation between vitamin A and pathologic score was contrary to the expected result, as we anticipated a significant negative correlation. Organochlorine compounds are known to deplete vitamin A by interaction with the plasma carrier protein, transthyretin, as well as alteration of its metabolism in the liver and other organs (Coenraads et al., 1994; Poon et al., 1995; Grasman et al., 1996). It is possible that this mechanism may result in an initial accumulation of vitamin A prior to depletion of stores, offering a potential explanation for the apparent paradoxical result. Increased vitamin A levels due to contaminants have been reported in other species (Heussen et al., 1993). In addition, this may provide an explanation for the significant nonlinear relationship between vitamin A concentration and total OC compounds level, whereby there was an initial increase in vitamin A as the OC compound burden increased, followed by a decline as OC compound levels increased further (Fig. 2). Furthermore, plasma constituents such as proteins or lipids can modify the accessibility of OC compounds to functioning cells, and thus the biologic activity of these compounds will be dictated by the amount that is free, or unbound, and available to the cells. Interaction of the compounds at the receptor level must also be taken into consideration, as many of the turtles had more than one compound identified (Sparling et al., 2000). Consequently, all these factors may confound the detection of evidence suggesting cause-effect relationships. Dose-dependent risk has been demonstrated for estradiol-induced sex reversal of turtle embryos, even at low doses (Sheehan et al., 1999); however, it is likely that the relationships (if any) between the variables analyzed in our study are complex and nonlinear.

Potential additional confounders include the fact that most of the turtles included in this study had been presented to a wildlife rehabilitation facility. While every effort was made to exclude animals that had received medications or that had been fed an artificial diet, it is possible that some turtles had unknown histories of

such treatment, illustrating the potential limitations of using animals from wildlife rehabilitation facilities. In addition, it can be difficult to obtain a sufficiently large enough sample to elucidate relationships between contaminants and health effects in free-ranging wildlife.

Other studies have also reported sex differences in OC burdens, as certain OC compounds are maternally transferred to eggs (Sparling et al., 2000; Rauschenberger et al., 2004), resulting in lower concentrations in adult females. Sex differences in the degree of changes in hepatic vitamin A concentrations in response to polychlorinated biphenyls have also been reported in Japanese quail (*Coturnix japonica*; Cecil et al., 1973). In addition, for snapping turtles (*Chelydra serpentina*), relationships have been found between body mass and OC compounds in the liver, indicating that these compounds are accumulated continuously with size and age (Sparling et al., 2000). However, differences between male and female turtles or by weight were not detected in this study.

The hepatic OC compound residue concentrations in these box turtles were lower compared to levels found in freshwater aquatic turtles but similar to levels in marine turtles and terrestrial reptile species (Sparling et al., 2000). The OC residues reported in our study add to the scant data available for box turtles. While the route of exposure for these box turtles is unknown, they are considered generalist omnivores (Dodd, 2002) and were likely exposed from consuming insects and gastropods (Sparling et al., 2000). In addition, the longevity of box turtles probably allows the accumulation of these persistent compounds over time. The levels and variety of OC compounds we found in our study illustrate the ubiquitous nature of environmental contamination with these persistent contaminants.

In summary, this study provides mixed results in support of a role of OC compounds, presumably of environmental origin, in the etiology of aural abscesses in free-ranging box turtles. Due to the inherent difficulties in performing field-based studies to determine causal relationships, we recommend laboratory-based experimental studies to further investigate any potential causal link between OC compounds and aural abscesses in box turtles. However, further epidemiologic studies, such as comparisons between geographic clustering of cases of aural abscesses in box turtles and possible clustering of OC-associated diseases in humans, may be warranted.

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