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# INABILITY TO INDUCE TYMPANIC SQUAMOUS METAPLASIA USING ORGANOCHLORINE COMPOUNDS IN VITAMIN A-DEFICIENT RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*)

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**ABSTRACT:** Previously, we reported that wild eastern box turtles (*Terrapene carolina carolina*) with aural abscesses contained higher body burdens of organochlorine (OC) compounds than those without the lesion. This lesion in captive chelonians is associated with turtles that are fed diets deficient in vitamin A. To examine the pathophysiology of this lesion and evaluate the relationship between OC burdens and vitamin A metabolism, we maintained red-eared sliders (*Trachemys scripta elegans*) under different conditions of OC exposure and dietary vitamin A concentrations from August 2005 to February 2006. Dietary vitamin A concentration (0 or 5 international units/g in the diet) and OC exposure (no OC compound or the mixture of 2 mg/kg chlordane, 0.25 mg/kg aroclor, and 1 mg/kg lindane) did not affect histologic score based on degree of squamous metaplasia of the tympanic epithelium or levels of plasma or liver vitamin A among the study groups. The results of this study suggest that 6 mo of exposure to the selected OC compounds, or similar duration of reduced dietary vitamin A concentrations do not influence the formation of squamous metaplasia and aural abscesses in red-eared sliders. Further studies are required to determine whether the duration of the experiment was insufficient, the OC compounds selected were inappropriate, the dosing was incorrect, and whether there are other unknown mechanisms causing the reported association between OC exposure and aural abscesses seen in eastern box turtles.

**Key words:** Aural abscesses, eastern box turtle, hypovitaminosis A, organochlorine compounds, red-eared slider, squamous metaplasia, *Terrapene carolina carolina*, *Trachemys scripta elegans*.

## INTRODUCTION

The presence and health of wildlife within a habitat is often an indicator, or “bioindicator,” of the overall health of that ecosystem. Reptiles are long-lived, are generally top predators within their ecological niches, and can be sensitive to pesticides; therefore, they may be useful bioindicators of terrestrial and aquatic habitat contamination (Lambert, 1996). Bioaccumulation of persistent pesticides, such as organochlorine (OC) compounds, has been documented in land (Tangredi and Evans, 1997) and aquatic turtles (O'Hara et al., 1996; Kannan et al., 2000). Organochlorine compounds have

been reported to interfere with vitamin A homeostasis in birds (Grasman et al., 1996), laboratory rats (Poon et al., 1995), and humans (Coenraads et al., 1994), and they may act similarly in reptile species (Holladay et al., 2001). Although the effects of OC compounds in reptiles are unknown, a previous study reported higher hepatic OC concentrations in wild eastern box turtles (*Terrapene carolina carolina*) with aural abscesses than without abscesses ( $0.04 \pm 0.01 \mu\text{g/g} \Sigma \text{OC}$  in nonabscessed turtles versus  $0.25 \pm 0.09 \mu\text{g/g} \Sigma \text{OC}$  in abscessed turtles; Holladay et al., 2001). In addition, turtles with this lesion displayed a trend toward lower serum and hepatic vitamin A levels,

suggesting that OC compounds may disrupt vitamin A metabolism in chelonians as well as other taxa.

Hypovitaminosis A, usually resulting from inadequate nutrition in captive turtles, is considered a predisposing factor for aural abscess formation (Murray, 1996). Aural abscesses are a common disease of chelonians, and in particular captive box turtles (Murray, 1996). Vitamin A deficiency in aquatic turtles is correlated with squamous metaplasia of the ocular, pharyngeal, and respiratory epithelium (Frye, 1979, 1991) as well as the middle ear and auditory (Eustachian) tube, predisposing the aural cavity to opportunistic bacterial infection (Murray, 1996). These bacterial infections apparently create the proper environment for abscess development. This suggests that if OC compounds induce hypovitaminosis A, and hypovitaminosis A causes aural abscesses, then OC exposure would cause aural abscess development. In a previous study by Holladay et al. (2001), high OC levels were positively correlated with aural abscesses secondary to squamous metaplasia of the aural epithelium. These findings support the earlier suggestion of Tangredi and Evans (1997) of an environmental toxicant as a factor in the pathogenesis of ocular and respiratory disease in North American chelonians. Association, however, does not indicate causality, and the purpose of this study was to experimentally determine whether exposure to OC mixture resulted in the development of aural abscesses in red-eared sliders (*Trachemys scripta elegans*).

## MATERIALS AND METHODS

Twenty-four red-eared sliders, approximately 1 yr of age, were obtained from a commercial dealer (Central Florida Reptile Farm, Cocoa, Florida, USA), and they were maintained at the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA) from July 2005 to February 2006. The mean  $\pm$  SD carapace

length of these turtles was  $12.5 \pm 3.2$  cm, and the mean  $\pm$  SD weight was  $225 \pm 120$  g. The first 6 wk were an acclimation period to the new environment and a balanced sinking food formulation. Approval of the Institutional Animal Care and Use Committee of the VMRCVM was obtained before purchasing of turtles.

Twelve 20-gal aquariums ( $77 \times 31 \times 32$  cm) were filled to the halfway mark with dechlorinated water. A sloped platform constructed of 2.5-cm polyvinyl chloride pipe and 0.5-cm plastic mesh was placed in each aquarium to allow the turtles to exit the water. Additionally, a filter (Fluval Internal Filter 1 Plus, 50 gph, Hagen Askoll, Vicenza, Italy) was placed in each aquarium to provide water filtration. The room was maintained within a constant temperature range from 23.8 C to 26.6 C. Lighting in the room was supplied by overhead lights as well as UV-B radiation lighting (SuperUV Reptile Daylight Lamp, Full Spectrum with UVB and UVA, 36 W, Energy Savers Unlimited Inc., Carson, California, USA) 40 cm above the water level. The photoperiod was maintained at 14 hr of light and 10 hr of darkness. Two turtles were arbitrarily placed in each aquarium, which corresponded to one of four treatment groups described below (a total of six turtles per treatment group). The aquariums were cleaned via siphoning of grossly visible organic matter daily. Complete water changes and cleaning of the aquarium accessories were performed weekly.

Two established omnivore diets (Hirikawa et al., 1984) were used (Land O'Lakes Purina Feed, LLC, St. Louis, Missouri, USA). Turtles were fed identical diets with the exception of vitamin A content. Groups 2 and 4 received a diet containing 5 international units (IU)/g vitamin A, whereas groups 1 and 3 were fed a diet devoid of vitamin A (groups summarized in Table 1). The 5 IU/g vitamin A level was based on recommended dietary levels for aquatic turtles of 2–8 IU/g diet (Donoghue, 2006). The diet was shipped as a powder, and then it was reconstituted in a ratio of 60:40 boiling water to powder. Human grade red food coloring was added to increase the contrast between the food and the surrounding environment. The food was poured into glass pans to achieve a thickness of 0.25 cm, and then it was refrigerated until set. These sheets of food were then cut into strips approximately  $0.5 \times 3$  cm.

Three OC compounds were selected as possible inducers of squamous metaplasia in this study. Chlordane and lindane have been detected in wild-caught eastern box turtles

TABLE 1. Mean  $\pm$  SD of chlordane, lindane, aroclor, and liver vitamin A (micrograms per gram) in red-eared sliders (*Trachemys scripta elegans*) experimentally treated with organochlorine (OC+/-) compounds, vitamin A-deficient diet (Vit. A +/-), or a combination.

	Chlordane	Lindane	Aroclor	Total OC	Vitamin A
Group 1 Vit. A -; OC +	5.00 $\pm$ 1.84	1.50 $\pm$ 0.74	1.01 $\pm$ 0.28	7.56 $\pm$ 2.63	9.34 $\pm$ 7.39
Group 2 Vit. A +; OC +	6.10 $\pm$ 3.48	1.80 $\pm$ 0.97	1.26 $\pm$ 0.61	9.18 $\pm$ 5.04	13.61 $\pm$ 5.05
Group 3 Vit. A -; OC -	ND <sup>a</sup>	ND	ND	ND	11.53 $\pm$ 8.16
Group 4 Vit. A +; OC -	ND	ND	ND	ND	12.85 $\pm$ 5.25
Wild-caught box turtles ( <i>Terrapene carolina</i> ) <sup>b</sup>					
No abscess	0.01	NR <sup>c</sup>	NR	0.04 $\pm$ 0.01	20.37 $\pm$ 6.18
Abscess	0.13 $\pm$ 0.10	NR	NR	0.25 $\pm$ 0.09	9.96 $\pm$ 2.88

<sup>a</sup> ND = not detected.

<sup>b</sup> Holladay et al. (2001) (chlordane and total OC levels extrapolated from figures).

<sup>c</sup> NR = not reported.

(Holladay et al., 2001). A commercial mixture of polychlorinated biphenyl (PCB) compounds (aroclor 1254) was selected based on previous research of PCB exposure in wild bird populations (Grasman et al., 1996) as well as controlled studies on laboratory amphibians (Jelaso et al., 2002). The OC and PCB mixture consisted of chlordane (72% technical grade chlordane, a commercial formulation of 43.2% octachloro-4,7-methanotetrahydroindane, 28.8% related compounds, and 28% petroleum distillate; donated by H. Holladay, Raleigh, North Carolina, USA), a commercial PCB mixture (aroclor 1254, lot no. NT01719, UltraScientific Analytical Solutions, North Kingstown, Rhode Island, USA), and lindane (99%  $\gamma$  isomer, Sigma, St. Louis, Missouri, USA). These three chemicals were mixed in corn oil to provide the following dosing concentrations: 2 mg/kg chlordane, 0.25 mg/kg aroclor, and 1 mg/kg lindane. All turtles were weighed once weekly, and a dose was calculated based on these weights for the turtles in groups 1 and 2. Each calculated dose of OC was placed in an incision made in a 0.15-g piece of hot dog. These pieces were hand-fed by forceps to the appropriate turtles within groups 1 and 2. The turtles of groups 3 and 4 (not receiving OC dosages) were fed similar-sized pieces of placebo hot dog and corn oil on the same schedule.

After 6 mo of exposure, each turtle was euthanized by an intravenous overdose (1.5 ml) of pentobarbital sodium (Beuthanasia-D, Schering-Plough Animal Health, Union, New Jersey, USA) injected into the subcarapacial sinus. Once euthanized, blood was immediately collected in a nonheparinized glass tube from each animal via jugular exsanguinations with a scalpel blade. The glass vial containing the whole blood sample was

placed in an ice bath. The turtles were then decapitated, and an incision using a no. 10 scalpel was made in each tympanic membrane to aid fixation of the middle ear. The whole head was then placed in 10% neutral buffered formalin for 72 hr for histopathologic investigation. The plastron was then removed, and the entire liver was collected and weighed. Two 0.5-g fresh liver samples from each turtle were wrapped in aluminum foil, and the samples were stored at -20 C for vitamin A, chlordane, lindane, and aroclor analysis. Blood was centrifuged (3,000 $\times$ G at 4 C), and the serum was collected and frozen at -20 C for additional vitamin A analysis.

Frozen serum and one of the liver samples were submitted to the Toxicology Laboratory, VMRCVM, and analyzed for retinol (vitamin A, alcohol) by high-performance liquid chromatography as described previously (Holladay et al., 2001). The remaining frozen liver samples were submitted to the Pesticide Residue Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA. The preserved turtle heads were trimmed and decalcified (formic decalcifying solution, US Biotex Corporation, Laboratory Products Division, Webbville, Kentucky, USA) for 8 days. The tissues were then routinely processed and embedded in paraffin. Sections were cut at 7  $\mu$ m and stained with hematoxylin and eosin. Tissue sections were examined without knowledge of their pathologic status, and the tympanic epithelia were evaluated for pathologic changes consistent with hypovitaminosis A. The severity of the changes was scored using a previously described scoring system (Brown et al., 2004) that consisted of four possible aggregated scores: minimal pathologic change (1), moderate (2), marked (3), or severe pathologic change (4).

All results are expressed as means  $\pm$  SD. Comparisons between the study groups OC burdens were performed using Student's *t*-test. A one-way analysis of variance (SPSS Statistical Package, SPSS Inc., Chicago, Illinois, USA) using the Tukey post hoc test was used to test the equality of the means of tympanic epithelial pathologic scores, liver vitamin A levels, and serum vitamin A levels among each treatment group. Significance was set at  $P < 0.1$ .

## RESULTS

None of the livers from the turtles in groups 3 and 4 (no OC exposure) contained any detectable chlordane, aroclor 1254, or lindane. The livers from all the turtles in groups 1 and 2 (OC exposure) contained all three of these compounds (Table 1). There were no significant differences detected among the mean levels of any of the three compounds between the two latter groups. No significant differences were observed in the tympanic epithelial pathologic scores, liver vitamin A levels, and serum vitamin A levels among any of the four study groups. Group 1 (OC exposure and vitamin A-deficient diet) did have the lowest numeric vitamin A levels, suggesting a possible emerging trend. Lymphocytic inflammation in the large ganglia at the base of the brain was noted in specific individuals of all groups with no association to treatments.

## DISCUSSION

The results of this study suggest that OC exposure may not contribute to the formation of squamous metaplasia of the tympanic epithelium and aural abscesses in red-eared sliders. In addition, 6 mo of OC exposure does not seem to alter vitamin A metabolism in red-eared sliders as has been observed in birds, laboratory rodents, and humans exposed to diverse OC compounds or OC compound mixtures (Coenraads et al., 1994; Poon et al., 1995; Grasman et al., 1996). The significance of the lymphocytic inflammation of

the large ganglia at the base of the brain is unknown. It may represent an underlying pathology unrelated to this specific study.

All of the turtles dosed with the OC mixture contained these compounds in their livers, and those not dosed with the mixture contained no detectable amounts of any of these chemicals. Based on the OC levels found in wild-caught eastern box turtles with aural abscesses (Table 1) by Holladay et al. (2001), it would seem that the levels of OC achieved were sufficient to cause similar pathology. However, it is possible the duration of exposure for which these levels were present was not sufficient to alter the metabolism of vitamin A or deplete vitamin A stores. Additionally, despite our efforts to select compounds that have been implicated in inducing hypovitaminosis A without using doses that cause overt toxicity, other OC compounds may be responsible for the observed effect.

The effects of hypovitaminosis A on proper epithelial development and maintenance is well documented in many species (Chopra et al., 1990; Frye, 1991; Cortes et al., 2005). A striking result of this study is that vitamin A levels were not reduced in turtles being fed diets void of vitamin A, compared with turtles fed diets containing vitamin A. The turtles were all greater than 6 mo of age, so any yolk stores of vitamin A should have been depleted. The only other food item offered to these groups other than the prepared diet was a small piece of hot dog and corn oil fed once weekly. The hot dogs and corn oil were not a significant source of vitamin A based on the product nutrition labeling. However, it was possible that the study did not progress long enough to produce hypovitaminosis A or that the assumption that OC compounds would alter vitamin A metabolism in red-eared sliders may be incorrect. The former is supported by the group of turtles ingesting both the OC compound and the vitamin A-deficient diet having the lowest numeric levels of vitamin A.

There may also be an unknown mechanism causing the reported association among OC body burdens, hypovitaminosis A, and aural abscesses. Organochlorine compounds and vitamin A are fat-soluble. It is possible that OC must be present within a body system for a certain period or at a certain level to alter lipoprotein function and thereby disrupt proper vitamin A metabolism. Another possible complicating factor could be species variation. Aquatic turtles tend to have a higher fat content to their livers, in addition to greater visceral and subcutaneous adipose stores than terrestrial species (Sleeman and Brown, pers. obs.). Increased adipose tissue and lipid stores in the liver could have resulted in a greater ability to store OC without clinical manifestations compared with box turtles. It may not be until the animal is required to use these fat stores, thereby liberating the OC compound, that a clinical problem would arise (Geluso et al., 1981; Pelletier et al., 2003). This scenario is more commonly seen with acute OC toxicities, but it cannot be ruled out with this study. Another possible species variation other than lipid distribution could be specific metabolic pathway differences between aquatic and terrestrial turtles. Consequently, red-eared sliders may not be an appropriate experimental model. These questions require further study to answer and to determine the role, if any, of OC compounds as endocrine disruptors in chelonians. Future studies are needed to examine a longer duration of OC dosage, alter the dose of an OC compound, change the OC compounds used, or replicate the experimental design with a terrestrial species of chelonian.

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