

PATHOLOGY, PHYSIOLOGIC PARAMETERS, TISSUE CONTAMINANTS, AND TISSUE THIAMINE IN MORBID AND HEALTHY CENTRAL FLORIDA ADULT AMERICAN ALLIGATORS (ALLIGATOR MISSISSIPPIENSIS)

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PATHOLOGY, PHYSIOLOGIC PARAMETERS, TISSUE CONTAMINANTS, AND TISSUE THIAMINE IN MORBID AND HEALTHY CENTRAL FLORIDA ADULT AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*)

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ABSTRACT: An investigation of adult alligator (*Alligator mississippiensis*) mortalities in Lake Griffin, central Florida, was conducted from 1998–2004. Alligator mortality was highest in the months of April and May and annual death count peaked in 2000. Bacterial pathogens, heavy metals, and pesticides were not linked with the mortalities. Blood chemistry did not point to any clinical diagnosis, although differences between impaired and normal animals were noted. Captured alligators with signs of neurologic impairment displayed unresponsive and uncoordinated behavior. Three of 21 impaired Lake Griffin alligators were found to have neural lesions characteristic of thiamine deficiency in the telencephalon, particularly the dorsal ventricular ridge. In some cases, lesions were found in the thalamus, and parts of the midbrain. Liver and muscle tissue concentrations of thiamine (vitamin B₁) were lowest in impaired Lake Griffin alligators when compared to unimpaired alligators or to alligators from Lake Woodruff. The consumption of thiaminase-positive gizzard shad (*Dorosoma cepedianum*) is thought to have been the cause of the low tissue thiamine and resulting mortalities.

Key words: Alligator, blood chemistry, crocodilian, gizzard shad, heavy metals, histopathology, mortality, organochlorine pesticides, thiaminase.

INTRODUCTION

An unusually large number of wild adult alligators (*Alligator mississippiensis*) were found dead in Lake Griffin in central Florida from unknown causes in the late 1990s. Prior to death, affected alligators were unresponsive and exhibited uncoordinated behavior. These alligators appeared to be normal, robust, healthy animals except for their behavior. Examination of 10 impaired alligators collected in 1997 and 1999 revealed that they were affected by a severe neurologic disease of unknown cause. Histologic evidence of neuropathy was minimal, although a diagnosis of peripheral neuropathy was based on decreased conduction velocity (Schoeb et al., 2002). Organochlorine pesticides and heavy metal levels in these alligators were

within expected normal parameters. No definitive cause of death was apparent from this study, but dead alligators continued to found into the early 2000s.

For decades, Lake Griffin has been subjected to the effects of agricultural and urban runoff, as well as to lake level stabilization for boating access. The lake is part of the St. John's River/Ocklawaha River drainage and is one of a chain of connected lakes and wetlands that includes Lake Apopka. The upper Ocklawaha drainage has been the subject of both extensive monitoring and studies of the impacts of anthropogenic disruption and contamination, including the effects on alligators (Guillette et al., 1999; Pickford et al., 2000). However, Schoeb et al. (2002) found no link with the toxic contaminants in morbid alligators from

Lake Griffin. Rather, they suggested nutritional deficiency as a possible unexplored cause of alligator morbidity and mortality. Chief among these causes is thiamine deficiency, which is recognized to cause both neuropathy and encephalopathy in several species (Jubb and Huxtable, 1993), including crocodilians (Jubb 1992; Huchzermeyer 2003).

The discovery of thiamine deficiency in salmonid species in the Laurentian Great Lakes of North America and the Baltic Sea has many parallels to the Lake Griffin alligator situation (McDonald et al., 1998; Hill and Nellbring, 1999; Blazer and Brown, 2005). Originally, it was thought that salmonid fry mortality, referred to as early mortality syndrome (EMS), was caused by exposure to toxic chemicals. Studies showed that fry mortality from yolk sac edema was linked to halogenated hydrocarbon exposure (Spitsbergen et al., 1991; Walker and Peterson, 1991). After careful review of the report by Mac et al. (1985), the signs and symptoms of dioxin toxicity were not consistent with that of EMS (Horning et al., 1998; Marcquenski and Brown, 1997); thiamine deficiency was definitively shown to be the cause of salmonid fry mortality (Honeyfield et al., 2005). In the case of Lake Griffin, there are no data on the thiamine status of adult alligators that were dying, but observations from affected alligators (Schoeb et al., 2002) were consistent with reports related to thiamine deficiency in salmonids; these included specific neurologic lesions in Atlantic salmon (Lundström et al., 1999), impaired motor coordination (abnormal swimming behavior; Marcquenski and Brown, 1997), and adult mortality (Brown et al., 2005b).

The cause of the thiamine deficiency in salmonids was linked to consumption of thiaminase-rich alewife (*Alosa pseudoharengus*) in the Great Lakes basin and herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) in the Baltic salmonids (Brown et al., 2005a; Ikonen, 2006). Gizzard shad (*Dorosoma cepedianum*), like alewife and herring, contain thiaminase, an enzyme

that degrades thiamine (Tillitt et al., 2005). Gizzard shad are an abundant fish in many Florida lakes and are eaten by alligators (Delany and Abercrombie, 1986; Delany et al., 1999; Rice, 2004).

These data were collected as an ongoing field investigation with the following objectives: 1) to document the yearly incidence of adult alligator mortality; 2) to collect data on the pathologic condition of the animals; 3) to record physiologically important measures associated with animal health such as blood chemistry, neural chemistry, and concentrations of common contaminants and metals; and 4) to assess tissue thiamine concentrations in central Florida alligators experiencing abnormal behavior and mortality.

METHODS

Study areas

Study areas included Lake Griffin in Lake County, Florida and Lake Woodruff National Wildlife Refuge in Volusia County, Florida. Lake Griffin (28° 50'N, 81° 51'W) occupies 5,742 ha, of which 3,964 ha are open water and the remainder are inundated wetlands and marsh. The lake is part of the St. John's River/Ocklawaha River drainage and is one of a chain of connected lakes and wetlands that includes Lake Apopka. The lake has an avg. depth of 2.67 m and is characterized by complete wind-driven mixing and eutrophic conditions. The population estimate of adult (>1.8 m total length) alligators in Lake Griffin during this study was approximately 2,200 (A. Woodward and D. Carbonneau, pers. comm.). Lake Woodruff National Wildlife Refuge (hereafter referred to as Lake Woodruff), is part of, and continuous with, the St. John's River drainage. Lake Woodruff (29° 06' 00"N, 81° 25' 00"W) consists of several lakes and streams (1,269 ha) bordered by extensive marsh and hardwood swamp (6,550 ha). Lake Woodruff has a mean depth of 1.84 m, and the open water shoreline perimeter of our study area

was 52 km (32.6 miles). The estimated population of adult alligators in Lake Woodruff and immediately adjacent connected waters during this study was approximately 1,200 (A. Woodward and D. Carbonneau, pers. comm.). Lake Woodruff has been subject to only moderate levels of anthropogenic impact. Neither of the study areas are closed systems and both exchange water, nutrients, and alligators with surrounding wetlands and associated river flows.

Alligator mortality surveys

Between 1998 and 2004, the entire shoreline of Lake Griffin and adjacent waters were surveyed by airboat every 2 wk (Woodward et al. 1996). Dead alligators were located both visually and sometimes by smell, and were occasionally reported by lakeside residents. Dead alligators were recorded and marked with spray paint to avoid counting animals more than once. Regular visits to Lake Woodruff were also conducted, both day and night, through the same period of 1998–2004, and the Lake Woodruff alligator population, with few alligator mortalities (<5 per yr) reported, served as the reference in this study.

Capture and handling of live alligators was conducted under Special Use Permit WXO1261b issued by the Florida Fish and Wildlife Conservation Commission and with Animal Care and Handling Approval D005 issued by the University of Florida, Gainesville, Florida, USA. Wild alligators were collected on Lake Griffin between February and October, from 1999–2002. Alligators were located during the day and assessed for physiologic motor impairment by their vigor, or lack of it, in escaping close-approach by an airboat. Normal, healthy alligators usually cannot be closely approached during the day. Alligators showing impaired motor function were uncoordinated, unresponsive, and could be closely approached; these were captured by hand lasso or capture dart, brought to the boat, secured, sexed, and measured. In addition, specimens of

unimpaired alligators were located at night with a spotlight and captured in the same manner. We also collected healthy alligators at night from Lake Woodruff, a relatively pristine lake 22 miles (35 km) distant, to establish reference levels of apparently normal alligators. The sex of captured alligators was established by manual palpation of the cloaca and specimens were measured by both total length (TL) and snout-vent length (SVL). Most specimens were weighed to the nearest kg on a spring scale either at capture or following euthanasia.

Immediately following capture, a blood sample (approximately 20 ml) was collected from the vertebral sinus. Blood samples were decanted to lithium heparin (two tubes) and plain (one tube) vacutainer tubes and held on ice for 2–6 hr before preparation.

Captured alligators were lavaged for stomach contents (Rice, 2004; Rice et al., 2005), held restrained overnight, and euthanized the next morning by cervical section and exsanguination.

Pathology

Alligators were transported to a necropsy facility and a complete post-mortem examination and tissue collection were performed. Major organs were examined for gross lesions and samples obtained for additional analysis. Tissue specimens of fat, muscle, liver, kidney, brain, nerve, and gonad were collected into 10% neutral buffered formalin solution for histopathology, and representative tissues were placed into whirlpaks® and frozen for toxicology and other analyses. Frozen tissues were maintained at –70 C or on dry ice until thawed for analysis.

Histopathology

Fresh tissue samples of approximately 0.5 cm³ volume were collected at necropsy and fixed and stored in 10% neutral buffered formalin. The remainder of the brain was fixed in formalin. Tissues were prepared for light microscopy using rou-

tine methods for processing, embedding, sectioning, and staining (with hematoxylin and eosin) by the histology laboratory of the Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida, USA. Slides were then sent to the consulting pathologists at the University of Alabama at Birmingham, Birmingham, Alabama, USA or the Disney's Animal Programs, Orlando, Florida, USA for examination and interpretation.

Microbial pathogens

Blood was collected from the heart through the pericardium at necropsy using sterile procedures. Approximately 2 ml of fresh blood was introduced to a standard pediatric blood culture medium bottle (Fisher catalog #L4371580) using a clean, sterile needle. Culture bottles were stored at 0 C and bacterial cultures were then submitted to the Microbiology Department at the State Veterinary Diagnostic Lab in Kissimmee, Florida, USA for routine pathogen screening.

Neurologic cholinesterase activity

Alligator samples ($n=18$) collected prior to 2001 were analyzed at the Institute of Environmental and Human Health, Texas Tech University, Lubbock, Texas, USA for cholinesterase activity and reactivation to evaluate exposure to organophosphates (OP). Brain and spinal cord tissue samples of 0.2 g to 0.4 g were macerated and homogenized in 1:9 weight:volume Tris 0.05 M pH 7.4 buffer. Cholinesterase in two forms (acetylcholinesterase, AchE; butyrylcholinesterase, BchE) was released with Triton-X 100 (1%). Cholinesterase activity was measured using the method of Ellman et al. (1961), as modified by Gard and Hooper (1993) for use on a SPEC-TRAmx 96-well spectrophotometer plate reader (Molecular Devices, Sunnyvale, California, USA). Reactivation analysis was performed with pralidoxime chloride (2-PAM) to displace cholinesterase inhibiting organophosphates. Cholinesterase enzymes treated with 2-PAM will increase

the measurable enzyme activity if the sample has previously been inhibited with OP exposure (Petroianu et al., 2004). The presence of carbamate-inhibited cholinesterase activity was determined by comparing enzyme activity before and after incubation for 1 hr at 25 C, both with and without 2-PAM. Spontaneous reactivation above 20% of the pre-incubation sample was taken as an indication of carbamate inhibited cholinesterase activity.

Blood chemistry

Chilled blood samples from the field were sent to the laboratory within 2–6 hr of collection, centrifuged at $2,500 \times G$ for 5 min, and supernatant plasma was removed and stored frozen in 2-ml cryovials. Blood plasma from field-collected or euthanized alligators was stored frozen at –70 C and then submitted to the College of Veterinary Medicine Teaching Hospital, University of Florida, for standard exotic animal/reptile blood chemistry screen LV2OR.

Organochlorine analysis

Samples of 20–80 g frozen alligator tail muscle were shipped on dry ice to En-Chem Laboratories, Green Bay, Wisconsin, USA for organochlorine pesticide extraction (EPA SW-846; Environmental Protection Agency [EPA] 1996a) and gas chromatography analysis (EPA SW-846; EPA 1996b). Samples of 20–80 g of visceral body fat were shipped on dry ice to Mississippi State University Chemical Laboratory, Starkville, Mississippi, USA for organochlorine (including toxaphene) analysis (Cromartie et al., 1975). Fat samples were analyzed from five normal Lake Woodruff alligators, five impaired Lake Griffin alligators, and one normal Lake Griffin alligator.

Metals analysis

Samples of 20–80 g frozen liver were shipped on dry ice to the University of Pennsylvania, Philadelphia, Pennsylvania, USA for analysis of 16 metals using

inductively coupled plasma argon emission spectroscopy (ICPAES). Liver (2.5 g) was weighed into a Tuffainer® and 5 ml of 5% nitric acid was added and sample was digested for 3 hr at 90 C. Samples were cooled to room temperature. Deionized water was added to the sample to bring the final volume to 25 ml in a volumetric flask. Each sample was transferred to a 50 ml disposable tube, 2 ml of iso-octane were added, the tube was shaken, and the layers allowed to separate. The iso-octane was removed and the sample incubated for 20 min in a 60 C water bath. Samples were then analyzed by ICPAES analysis using an INTEGRA XM2 (GBC Scientific Equipment Inc., Arlington Heights, Illinois, USA). Calibration curves were generated from two standard solutions of the elements of interest, and the instrument response was determined against standard reference material (NIST 1557b; National Institute of Science and Technology [NIST], Gaithersburg, Maryland, USA).

Thiamine analysis

Analyses were completed as described in Brown et al. (1998) with minor modifications of the elution gradient. All reagents were made with Burdick Jackson HPLC grade water (VWR Scientific Products, S. Plainfield, New Jersey, USA). The binary gradient was generated by mixing 25 mM ammonium phosphate (Sigma Chemicals, St. Louis, Missouri, USA) buffer and dimethyl formanid (DMF). The following binary gradient was set: (Time/DMF%) 0 min to 1 min/0%; 5 min/0.5%; 7 min/10%; 9 min/30%; 9 min/35%; 10 min/35%; and 10.1 min/0%. Total run time was 22 min. For tissue thiamine analysis, 0.1–0.2 g liver and 0.3–0.6 g muscle were used.

Statistical analysis

Analysis of variance, means, and standard error (SE) of the means were determined (Statistical Analysis System [SAS], 2003). Differences in mean values with more than two means were tested with Duncan multiple range test ($P < 0.05$).

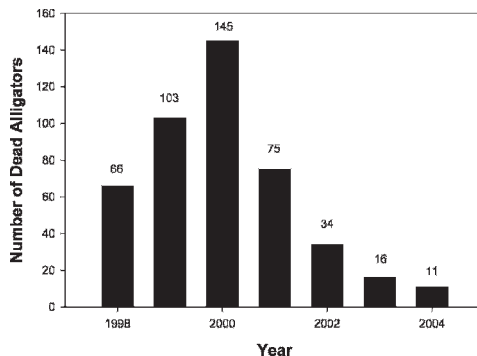


FIGURE 1. Yearly numbers of dead alligators observed on Lake Griffin, Florida.

RESULTS

Alligator mortality

For Lake Griffin, annual mortality of adult alligators peaked in 2000 and then declined to normal levels by 2004 (Fig. 1). No morbid alligators demonstrating poor coordination or any other symptoms were observed in 2004 or 2005. During the years of high mortality (1997–2001), dead alligators were found in all months of the year with a distinct peak occurring in April and May (Fig. 2).

Fifty-eight adult alligators were collected on Lakes Griffin and Woodruff between 1999 and 2002. From Lake Griffin, 21 apparently normal and 21 impaired alligators were collected, while 16 normal animals were collected from Lake Woodruff. The mean SVL of all specimens was 116.5 cm (SE=3.2 cm), representing animals averaging 2.3 m total length. The smallest specimen was 76 cm SVL. Mean SVL was similar among Lake Griffin impaired alligators (121.5 SE=6.6 cm), normal Lake Griffin alligators (118.9 SE=4.2 cm) and normal Lake Woodruff alligators (112.8 SE=6.6). Differences in mean weight were not statistically significant; the mean weight of specimens in our sample was 57.9 kg (SE=5.5 kg). Specimens from Lake Woodruff (mean weight 49.7; SE=8.1 kg) were slightly smaller than those from Lake Griffin. Impaired specimens (74.6 kg; SE=13.1 kg)

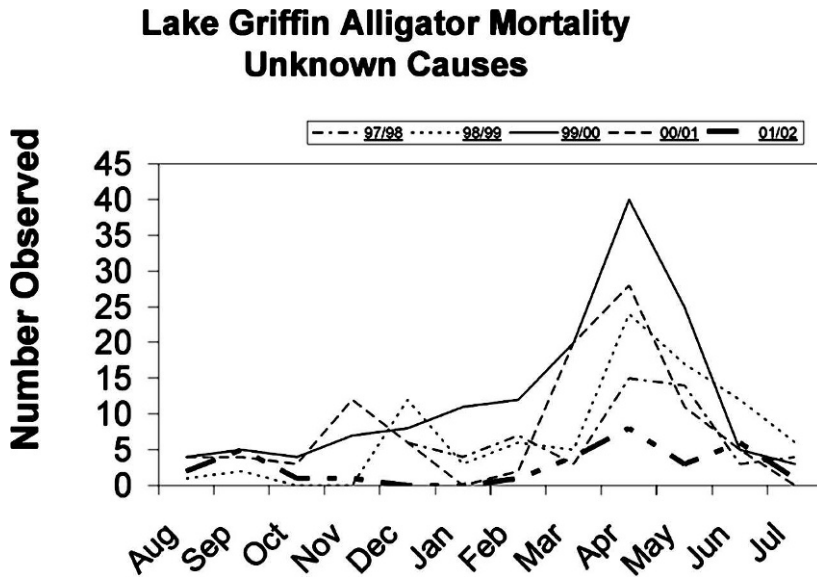


FIGURE 2. Monthly incidence of dead alligator observed in Lake Griffin, Florida, from 1998 through 2002, counted in biweekly surveys.

tended to be larger than healthy animals (51.3, SE=4.5 kg). Fulton's K condition index, calculated from weight and length of alligators between apparently normal and impaired Lake Griffin alligators, was not significantly different and has been reported elsewhere (Rice, 2004). Sex ratio of these alligators was not significantly different from 1:1 and was not different between groups.

Gross pathology

Post-mortem examination revealed few external or internal lesions to which death could be attributed. Five alligators collected alive from Lake Woodruff showed no abnormalities. Of the 21 live Lake Griffin specimens, 12 were judged impaired in the field. None of these showed significant gross pathology lesions, with the exception of minor lesions and stomach ulcers (two individuals). Five of the Lake Griffin specimens that were judged unimpaired showed no abnormalities. Three of the Lake Griffin alligators showed obvious signs of traumatic injury; two with extensive boat propeller damage to the body and the other with alligator bite wounds to the head and penetrating the

thoracic cavity. Two alligators found freshly dead had evidence of water and, in one case, mud in the lungs, indicating drowning as the proximal cause of death. We also observed abnormal behavior in the field that could lead to drowning; other affected alligators refused to enter the water. These observations suggested that progressive neurologic impairment may lead to poor coordination, inability to swim, and drowning.

Histology

Three hundred eighty-six slides were examined from five normal Lake Woodruff, five normal Lake Griffin and 10 impaired Lake Griffin alligators. These included major organs and structural tissue from each animal, including 113 slides of brain, 49 spinal cord and 39 neural tissue. In most cases, there were no significant findings, that is, findings that would suggest a clinically important condition. Exceptions were three alligators of 10 impaired alligators with necrotizing encephalopathy similar to that previously seen in Lake Griffin alligators with clinical neurologic impairment (Schoeb et al., 2002). Histopathologic lesions were not

TABLE 1. Mean (\pm SE) brain, spinal cord, and serum enzyme activity for total (TchE), acetyl (AChE), and butyl (BchE) cholinesterase activity (μ m acetylthiocholine hydrolyzed/minute/g) in alligators from Lakes Griffin and Woodruff, Florida.

Tissue	<i>n</i>	TchE	SE	AChE	SE	BchE	SE
Brain							
Lake Griffin	4	1.04	\pm 0.192	0.97	\pm 0.220	0.07	\pm 0.011
Lake Woodruff	5	1.78	\pm 0.634	1.68	\pm 0.576	0.10	\pm 0.074
<i>P</i> value		0.35		0.33		0.75	
Spinal cord							
Lake Griffin	6	0.47	\pm 0.081	0.42	\pm 0.071	0.06	\pm 0.012
Lake Woodruff	3	0.64	\pm 0.023	0.60	\pm 0.042	0.04	\pm 0.023
<i>P</i> value		0.20		0.13		0.50	
Serum							
Lake Griffin	8	0.61	\pm 0.078	0.03	\pm 0.004	0.58	\pm 0.074
Lake Woodruff	10	0.68	\pm 0.047	0.03	\pm 0.003	0.65	\pm 0.047
<i>P</i> value		0.43		0.43		0.45	

limited to the midbrain; areas affected included parts of the telencephalon, particularly the dorsal ventricular ridge, but also extending ventrally into the subpallium in some cases; the thalamus; parts of the midbrain, usually the torus semicircularis but also the tegmentum in one case; the granular layer and white matter of the cerebellum; and the medulla (one case). The most consistently affected areas were the dorsal ventricular ridge of the telencephalon and the torus semicircularis of the midbrain (Figs. 3 and 4).

Two alligators had lymphocytic meningoencephalitis similar to that previously seen in one clinically ill Lake Griffin alligator. This is an inflammatory, rather than degenerative, process and therefore is likely to have a different cause from the necrotizing encephalopathy. Very mild encephalitis, localized to one region of the telencephalon, was observed in one alligator. It is unlikely that this lesion was fatal. Other lesions listed were considered to be minor incidental findings typical of parasite infections and other conditions common to wild animals.

Microbial pathogens

As in previous years (Schoeb et al., 2002), bacterial cultures were unremarkable, showing only occasional colonies of common

bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* spp., *Proteus mirabilis*); these are expected to occur in, and on, alligators. There were no indications of any bacterial infection causing illness or death.

Neurologic cholinesterase activity

Nine brain, nine spinal cord, and 18 serum samples were assessed for cholinesterase activity and reactivation (Table 1). Enzyme activities are expressed as μ m acetylthiocholine hydrolyzed/min/g. No differences were observed in total cholinesterase (TchE), AChE, or BchE, activity in brain tissue, spinal cord, or serum between alligators from Lake Woodruff and Lake Griffin. One of nine alligators from Lake Woodruff demonstrated exposure to a low level of OP pesticides. The serum sample from another alligator showed 8.7% inhibition of cholinesterase activity consistent with exposure to OP pesticides. However, brain and spinal chord samples from this animal showed no reactivation. One alligator from Lake Griffin showed 22.7% spontaneous reactivation indicating carbamate exposure. This animal was classified as an impaired alligator; however, it did not have brain lesions as reported by Schoeb et al. (2002). There was no indication of OP exposure in the remaining samples,

TABLE 2. Mean (\pm SE) concentration of organochlorine (OC) pesticides^{a,b} (ppm) in alligator fat from Lakes Griffin and Woodruff, Florida.

Pesticide	Lake Griffin	SE	Lake Woodruff	SE	P value
<i>n</i>	6		5		
p,p'-DDE	0.92	± 0.577	1.88	± 0.632	0.29
PCB, total	0.53	± 0.154	1.32	± 0.169	0.01
Dieldrin	0.02	± 0.023	0.07	± 0.025	0.18
Mirex	0.00	± 0.003	0.01	± 0.004	0.03
Toxaphene	2.07	± 0.976	0.00	± 1.069	0.18
trans-nonachlor	0.20	± 0.050	0.04	± 0.054	0.60
cis-nonachlor	0.10	± 0.008	0.03	± 0.009	0.01
alpha-chlordane	0.06	± 0.016	0.01	± 0.017	0.06
p,p'-DDD olefin	0.06	± 0.016	0.01	± 0.017	0.06
oxy-chlordane	0.03	± 0.007	0.04	± 0.008	0.40
Hept+Epox	0.02	± 0.005	0.02	± 0.006	0.97
p,p'-DDD	0.01	± 0.003	0.00	± 0.004	0.03

^a p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE); total polychlorinated biphenyls (PCB); p,p'-dichlorodiphenyldichloroethane olefin (p,p'-DDD olefin); Heptachlor+Epoxide (Hept.Epox); and p,p'-dichlorodiphenyldichloroethane (p,p'-DDD).

^b The following compounds were not detected in alligator fat (lowest detectable limit 0.010 ppm): p,p'-dichlorodiphenyltrichloroethane (p,p'-DDT); o,p'-dichlorodiphenyltrichloroethane (o,p'-DDT); o,p'-dichlorodiphenyldichloroethylene (o,p'-DDE); hexachlorobenzene (HCB); gamma-chlordane; o,p'-dichlorodiphenyldichloroethane (o,p'-DDD); alpha-hexachlorocyclohexane (alpha BHC); gamma-hexachlorocyclohexane (gamma BHC); beta-hexachlorocyclohexane (beta BHC); delta-hexachlorocyclohexane (delta BHC); endrin; endosulphan I; endosulphan II; endosulphan sulphate; and methoxychloradane.

including samples recorded as neural impaired or with brain lesions.

Organochlorine pesticides

Organochlorine (OC) pesticides in alligator muscle were generally below the detection limits (<0.005 ppm). The only exception was 4,4'-DDE that was detectable in six of seven impaired Lake Griffin alligators with a mean value of 0.046 ppm (SE=0.022 ppm) and in three of four Lake Woodruff alligators with a mean of 0.024 ppm (SE=0.017). In muscle, DDE was undetectable (<0.005 ppm) in one apparently normal Lake Griffin alligator and one normal Lake Woodruff alligator.

Although significant differences between groups in alligator-fat OC pesticides were detected (polychlorinated biphenyls [PCB], Mirex, cis-nonachloridane and p,p'-dichlorodiphenyldichloroethane [p,p'-DDD; Table 2]), concentrations were low. Of the 27 organochlorines screened, 14 were below the detectable limit in all samples. Thirteen OCs occurred in detectable quantities in at least some of the

samples (data not shown). Only one OC was detected in every fat sample; p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE).

Metals and minerals

Measured levels of metals in alligator liver, including toxic metals (e.g., arsenic, cadmium, chromium, mercury, lead, selenium, and tin) were similar to levels previously reported in Schoeb et al. (2002). Additional data on metals have been presented elsewhere (Kuharik et al. (2003). One animal had slightly elevated lead. No differences in heavy metals were evident between impaired and normal alligators or between alligators from Lake Griffin and Lake Woodruff. Mercury, a pervasive contaminant in Florida aquatic systems, was at or below background levels. Among the nutritionally important minerals, no differences were found in the liver content of five minerals (mean of all animals in ppm: calcium, 60; iron, 1,205; magnesium, 166; molybdenum, 0.03; zinc, 24.50). Concentrations of liver copper (8.0 ppm and

TABLE 3. Lake Griffin, Florida alligator mean (\pm SE) plasma chemistry of neurologically impaired and apparently healthy alligators, 2001.

Plasma component	Units	Impaired Lake Griffin		Normal Lake Griffin		P value
	<i>n</i>	9		21		
Alk.Phos ^a	u/l	23.67	\pm 2.91	15.95	\pm 1.80	0.03
AST ^a	u/l	284.33	\pm 41.15	190.57	\pm 20.55	0.02
ALT/GPT ^a	u/l	33.33	\pm 7.02	23.71	\pm 3.46	0.14
Total bilirubin	mg/dL	0.14	\pm 0.02	0.16	\pm 0.04	0.85
Total protein	g/dL	7.44	\pm 0.46	5.61	\pm 0.23	0.01
Albumin	g/dL	1.82	\pm 0.16	1.19	\pm 0.07	0.01
Albumin/globulin	ratio	0.32	\pm 0.02	0.28	\pm 0.01	0.08
Globulin	g/dl	5.62	\pm 0.31	4.42	\pm 0.18	0.01
Calcium	mg/dl	16.30	\pm 1.17	13.54	\pm 0.64	0.03
Phosphorus	mg/dl	5.94	\pm 0.56	6.17	\pm 0.40	0.76
Creatinine	mg/dl	0.54	\pm 0.08	0.49	\pm 0.05	0.52
BUN ^a	mg/dl	1.28	\pm 0.59	2.54	\pm 0.38	0.09
Glucose	mg/dl	167.22	\pm 16.37	108.33	\pm 10.71	0.01
Cholesterol	mg/dl	81.00	\pm 6.66	77.95	\pm 4.32	0.70
Uric acid	mg/dl	2.62	\pm 0.37	1.06	\pm 0.24	0.01
Sodium	meq/l	145.11	\pm 3.49	154.62	\pm 2.28	0.03
Potassium	meq/l	5.01	\pm 0.42	5.42	\pm 0.23	0.42
Chloride	meq/l	96.78	\pm 2.99	109.10	\pm 1.96	0.01
Carbon dioxide	meq/l	13.33	\pm 1.36	11.57	\pm 0.89	0.29
Anion gap ^a	meq/l	40.33	\pm 4.42	39.48	\pm 2.90	0.87

^a Alk.Phos = alkaline phosphatase; AST = aspartate aminotransferase; ALT/GPT = alanine aminotransferase; BUN = blood urea nitrogen; anion gap = sum of anion and cation values.

16.0 ppm) and manganese (1.75 ppm and 0.99 ppm) differed between Lakes Griffin and Woodruff alligators, respectively.

Blood chemistry

Differences in plasma chemistry between the normal and impaired Lake Griffin alligators were observed for alkaline phosphatase (ALP), aspartate aminotransferase (AST), total protein, albumin, globulin, blood urea nitrogen (BUN), uric acid, sodium, and chloride (Table 3). The impaired group had mild to moderate elevations in the metabolic enzymes ALP and AST. Concentrations of total protein, globulin, glucose and uric acid were elevated in impaired Lake Griffin alligators compared with normal Lake Griffin alligators. Whereas blood urea nitrogen (BUN) sodium and chloride were lower.

Tissue thiamine

Alligator liver thiamine pyrophosphate and total thiamine concentration were

lower in the year 2000 as compared to 2001 (Table 4). Muscle thiamine pyrophosphate, the active form of thiamine, was higher in 2001 than 2000. When data were analyzed by alligator condition (normal or impaired) and lake, liver thiamine monophosphate and total thiamine were significantly lower in impaired Lake Griffin alligators compared to the normal alligators from Lake Woodruff (Table 5). All forms of thiamine were found to be significantly lower in the muscle of impaired Lake Griffin alligators. Low thiamine values were found to be consistent with observed impaired motor function (Fig. 5).

DISCUSSION

Our results confirm the findings of Schoeb et al. (2002) and implicate nutritional deficiency as the cause of alligator morbidity and mortality. Based on a process of elimination, data support thia-

TABLE 4. The effect of year on Lake Griffin alligator mean (\pm SE) liver and muscle concentration (pmol/g) of thiamine pyrophosphate (TPP), thiamine monophosphate (TP), unphosphorlated thiamine (T), and total thiamine (Total T).

Tissue	Year	n	TPP	SE	TP	SE	T	SE	Total T	SE
Liver	2000	22	1231	\pm 83	534	\pm 55	327	\pm 76	2092	\pm 159
	2001	23	1856	\pm 155	610	\pm 60	234	\pm 31	2700	\pm 214
	P value		0.01		0.05		0.67		0.01	
Muscle	2000	22	130	\pm 25	49	\pm 9	22	\pm 5	202	\pm 34
	2001	23	219	\pm 31	26	\pm 3	2	\pm 1	247	\pm 35
	P value		0.01		0.05		0.01		0.04	

mine deficiency as the cause of mortality in Lake Griffin alligators. Mortality was observed in adults of both sexes, with an increased incidence in the spring. Mortality of alligators observed between 1998 and 2000 began to wane in 2001 and dropped to a near-normal level indistinguishable from natural mortality during 2003; no mortality was observed in 2005. Several environmental factors changed beginning in the winter of 2001. After several years of low winter conditions in Lake Griffin (0.5–1.0 m) water returned to normal levels with the rains in winter 2001 and summer 2002. Coincident with this change, water quality (particularly chlorophyll levels as an index of phytoplankton blooms) improved. After water levels returned to normal, in 2003, phosphorus levels in Lake Griffin were the lowest reported for several years; monthly mean concentration fell below 50 μ g/L

(W. Goodwin, St. John's River Water Management District, pers. comm.). Although these changes coincided with a reduction in the number of dead alligators, we have no plausible explanation to directly link these lake conditions with the reduced mortality observed after 2002; a possible indirect link may have involved changes in the available prey species in the food web.

In early spring of 2002, commercial fishermen removed more than 500,000 kg of gizzard shad and garfish (*Lepisosteus* sp.) from Lake Griffin. Adult alligator mortality has remained low since then. Gizzard shad are a rich source of thiaminase (Tillitt et al., 2005) and are reported to be an important dietary component for alligators (Rice, 2004; Rice et al., 2007). Lake Griffin gizzard shad (avg. fork length 19.23 cm, SE, 1.4 cm) were found to be high in thiaminase (15.6 nmol thiamine destroyed/g/min, SE, 1.2 nmol/g/min). A

TABLE 5. The effect of alligator physical status on mean (\pm SE) concentration (pmol/g) of liver and muscle thiamine pyrophosphate (TPP), thiamine monophosphate (TP), unphosphorlated thiamine (T), and total thiamine (Total T) from Lakes Griffin and Woodruff, Florida, sampled in 2000 and 2001.

Tissue	n	TPP	SE	TP	SE	T	SE	Total T	SE
Liver									
Normal Griffin	9	1747	\pm 184	607 ^{ab}	\pm 107	236 ^a	\pm 74	2589 ^a	\pm 312
Impaired Griffin	21	1354	\pm 154	443 ^b	\pm 52	179 ^a	\pm 29	1975 ^b	\pm 209
Normal Woodruff	15	1708	\pm 164	734 ^a	\pm 49	447 ^b	\pm 95	2889 ^a	\pm 169
Muscle									
Normal Griffin	9	194 ^{ab}	\pm 70	40 ^a	\pm 10	5 ^a	\pm 4	240 ^{ab}	\pm 74
Impaired Griffin	21	120 ^a	\pm 18	18 ^b	\pm 5	8 ^a	\pm 2	145 ^a	\pm 21
Normal Woodruff	15	243 ^b	\pm 35	63 ^c	\pm 9	21 ^b	\pm 7	327 ^b	\pm 38

^{a,b,c} Liver or muscle mean thiamine in a column with different letters are significantly different using Duncan multiple range test ($P < 0.05$).

detailed analysis of fish diet in Lake Griffin and Lake Woodruff in 2000–2002 (Rice, 2004; Rice et al., 2007) indicated that alligators ate almost no gizzard shad in 2002–2003, although they continued to eat other fish species. Thiamine deficiency in salmonids has been linked to consumption of thiaminase-rich alewife in the Great Lakes basin and herring and sprat in the Baltic salmonids (Brown et al., 2005a; Ikonen, 2006). Therefore, after the larger shad containing thiaminase were removed from Lake Griffin and lake conditions presumably favored a broader supply of food, alligator mortality declined.

By the process of elimination, other possible explanations can be discounted. There was no evidence of a bacterial etiology nor was there evidence of environmental contaminants such as the toxicity associated with heavy metals or OCs. No difference in cholinesterase activity was found between the groups of alligators; the use of 2-PAM, which re-activates enzyme activity suppressed by pesticide exposure, also did not suggest significant previous exposure to pesticides (Petroianu et al., 2004). A number of substances can damage both the central nervous system and peripheral nervous system by attacking myelin (Koestner and Norton, 2001), but in these alligators, no demyelination was seen in histologic evaluations. Detectable concentrations of OCs were below concentrations normally associated with mortality in other wildlife (Eisler and Jacknow, 1985; Blus, 1996; Marburger, et al. 2002). In all cases where OC pesticides were detected, the highest OC concentrations measured were below or comparable to concentrations recorded for alligators in other Florida lakes with no major mortality events (Guillette et al., 1999; Sepúlveda et al., 2004). There was no indication of exposure to OCs or their breakdown products, being responsible for observed neural impairment or mortality of alligators. Only one OC (p, p'-DDE) was detected in every alligator fat sample and

probably represent persistent breakdown of agricultural pesticides that are no longer in use but commonly detected in animal tissues throughout Florida. Concentrations of metals in alligator liver, including toxic metals (e.g. arsenic, cadmium, chromium, mercury, lead, selenium and tin) agree with previous findings (Schoeb et al., 2002; Kuharik et al., 2003) and were generally unremarkable.

Impaired or sick Lake Griffin alligators had elevated alkaline phosphatase, aspartate aminotransferase, total protein, albumin, globulin and glucose (Table 3), all indicative of abnormal health. One known cause of elevated alkaline phosphatase is high steroids (i.e. during periods of stress). In the absence of liver pathology, stress due to capture or undiagnosed illness may have been more pronounced in sick than healthy alligators. Whether elevated total protein in impaired alligators was due to dehydration or resulted from systemic inflammatory from a pathogen remains unknown, however, no microbial pathogens were found. Healthy alligator plasma total protein values were similar to published data (Lance et al. 1983, 2001; Guillette et al. 1997). The cause for increased plasma globulin concentration in impaired Lake Griffin alligators is not known but acute tissue injury or long-term production of antibodies (immunoglobulins) have been associated.

Elevated blood glucose, is an indicator of stress and, in this case, may have been related to thiamine deficiency. Without adequate tissue thiamine, energy production from glucose via the Krebs cycle would be dramatically slowed, and intuitively lead to higher plasma glucose. Healthy Lake Griffin alligator plasma glucose values were normal compared to published data (Lance et al., 2001; Schoeb et al., 2002). Collectively, clinical evidence of abnormal alligator health in the impaired Lake Griffin alligators was found in the blood chemistry but was of little diagnostic assistance.

In the absence of published thiamine

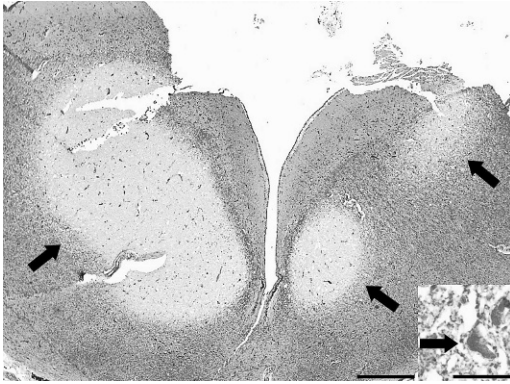


FIGURE 3. Histologic section of midbrain from an impaired Lake Griffin alligator collected in 2000. Arrows point to multiple pale foci of necrosis. Bar=670 μ m. Inset: same animal; arrow points to necrotic neurons. Bar=33 μ m.

data for alligator or related species, we compared data among alligator groups and from reports describing thiamine deficient fish (McDonald et al., 1998; Blazer and Brown, 2005). Direct evidence to support thiamine deficiency as the cause of the observed mortality and morbidity include higher incidence of adult alligator mortality in 2000 than in 2001 concurrent with reduced liver thiamine pyrophosphate, thiamine mono-phosphate and total thiamine (2000). Also, muscle thiamine pyrophosphate, the active form of thiamine and total thiamine, was lower in 2000 compared to 2001. Low thiamine values were found to be consistent with observed impaired motor function. We classified the apparent degree of motor impairment in the field on a scale of 1 to 4 with 1 being completely normal and 4 being comatose and unmoving. Alligators showing a more severe lack of motor coordination had significantly lower thiamin levels than normal alligators (Fig. 5). Impaired animals marked in the field invariably died within a few days. From their location and size, it was deduced that some impaired alligators died quickly. Also several of our captured impaired alligators died while we were holding them.

Impaired Lake Griffin alligators had the lowest thiamine concentrations, and brain

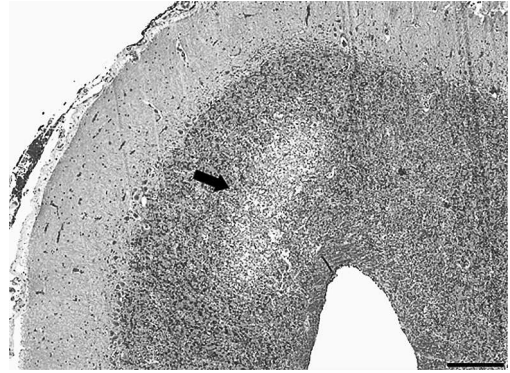


FIGURE 4. Histologic section of the cerebellum from an impaired Lake Griffin alligator collected in 2000. Arrow points to focal necrosis of granular layer. Bar=330 μ m.

lesions were found only in impaired animals. Histologic lesions (Figs. 3 and 4) are consistent with thiamine deficiency in other species (Jubb and Hustable, 1993). In a study investigating egg mortality from Lake Griffin, embryo death also was reported to be associated with low thiamine (Sepúlveda et al., 2004), and in another study, some hatchlings examined from Lake Griffin eggs had central nervous system signs and necrotizing encephalopathy similar to that observed in adult alligators (Richey, 2001).

Alligator mortality on Lake Griffin shows a striking similarity to salmonids dying from thiamine deficiency on the Great Lakes, where no evidence of disease, OC pesticides or harmful management practices were observed. (Honeyfield et al., 1998). Furthermore, Honeyfield et al. (2005) experimentally reproduced early mortality syndrome in lake trout fry and observed adult mortality (as previously reported by Brown et al. [2005b]). Signs and symptoms of EMS were observed when fish were fed diets containing thiaminase (Honeyfield et al., 2005). Salt water crocodiles with signs and symptoms similar to Florida alligators have been reported to be thiamine responsive, although the cause of the deficiency was not thiaminase related (Jubb, 1992). We believe that the tissue thiamine content,

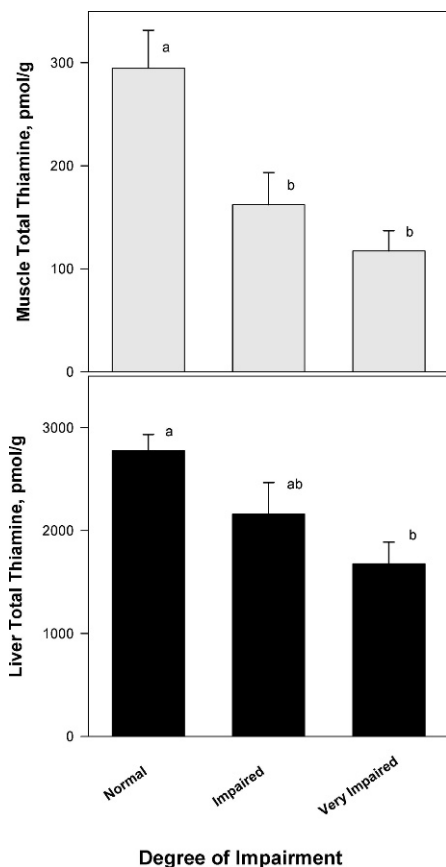


FIGURE 5. Mean muscle and liver total thiamine concentration for the varying degrees of impairment observed: Normal alligators showing unimpaired behavior, impaired alligators demonstrating reduced daylight wariness to close human approach, and seriously impaired alligators demonstrating obvious motor impairment (see text Methods). Mean values with different superscripts (^{a,b}) were significantly different ($P < 0.05$) using Duncan multiple range test.

clinical signs, and the pathology observed in alligators from Lake Griffin support the hypothesis of thiamine deficiency. Further experimental work is needed to examine if thiamine deficiency can be induced under controlled conditions. Work is also needed to evaluate the relationship between gizzard shad thiaminase levels from other Florida aquatic habitats and to determine if thiamine deficiency occurs in other vertebrates that feed heavily on thiaminase-rich food.

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