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Variations in hepatic biomarkers in American alligators (*Alligator mississippiensis*) from three sites in Florida, USA

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Abstract

Sub-individual biomarkers are sub-lethal biological responses commonly used in the assessment of wildlife exposure to environmental contaminants. In this study, we examined the activity of glutathione-s-transferase (GST) and lactate dehydrogenase (LDH), and metallothionein (MT) concentrations among captive-raised alligator hatchlings, wild-caught juveniles, and wild-caught adults. Juveniles and adults were collected from three locations in Florida (USA) with varying degrees of contamination (i.e. Lake Apopka (organochlorine polluted site), Merritt Island National Wildlife Refuge (NWR) (metal polluted site), and Lake Woodruff NWR (reference site)). We examined whether changes in the response of these three biomarkers were age and sex dependent or reflected site-specific variations of environmental contaminants. Juvenile alligators from Merritt Island NWR had higher MT concentrations and lower GST activity compared to those from the other two sites. This outcome was consistent with higher metal pollution at this location. Sexually dimorphic patterns of MT and GST ($F > M$) were observed in juvenile alligators from all sites, although this pattern was not observed in adults. GST activity was lower in captive-raised alligators from Lake Apopka and Merritt Island NWR as compared to animals from Lake Woodruff NWR, suggesting a possible developmental modulator at these sites. No clear patterns were observed in LDH activity. We concluded that GST and MT demonstrate age and sex specific patterns in the alligators inhabiting these study sites and that the observed variation among sites could be due to differences in contaminant exposure.

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Keywords

Glutathione-s-transferase; Lactate dehydrogenase; Metallothionein; American alligator; Biomarker; Sex differences; Sexual dimorphism

Introduction

Alligators provide useful animal models for the assessment of wildlife exposure to environmental contaminants due to their potential for bioaccumulation as they are top predators, have long life spans, and exhibit highly conserved regulatory and signaling pathways (Crain and Guillette, 1998; Lance, 2003; Milnes and Guillette, 2008). Prior studies have found altered sex steroid concentrations, changes in sex-related expression of certain hepatic enzymes, tissue lesions in ovarian and testicular tissues, and reduced phallus sizes in American alligator (*Alligator mississippiensis*) populations inhabiting contaminated lakes in Florida and it has been postulated that these physiological alterations result from exposure to organochlorine and other chemicals, although alternative mechanisms have been proposed as contributing factors (i.e. thiamine deficiency) (Crain, 1997; Guillette et al., 1994; Gunderson et al., 2004a; Gunderson et al., 2001; Gunderson et al., 2004b; Rauschenberger et al., 2009; Woodward et al., 2011).

Juvenile and adult alligators are exposed to contaminants through both diet (Smith et al., 2007). Such exposure can result in temporarily altered gene expression, protein expression, or enzymatic activities that can alter sexually dimorphic patterns of expression (Gunderson et al., 2006; Gunderson et al., 2001; Mortensen et al., 2011; Wilson and LeBlanc, 1998; Wilson et al., 1999). However, contaminant exposure also occurs during reptilian development through the transfer of accumulated maternal toxicants to the egg, along with the nutrients and materials necessary for normal development (Guirlet et al., 2008; Guirlet et al., 2010; Roe et al., 2004). Exposure to toxicants during critical stages of development has been shown to alter gene expression and steroid concentrations at later life stages (Milnes et al., 2004; Moore et al., 2010). Because of the multimodal routes of exposure in wildlife, it is important to consider both the extent to which physiology is permanently altered by developmental contaminant exposure (organizational-epigenetic regulation) and to which it is reversibly altered in response to xenobiotics at later life stages (activational regulation) when examining biomarkers that can serve as indicators of contaminant exposure (Guillette et al., 1995).

In this study, we measured metallothionein (MT) concentrations, glutathione-s-transferase (GST) activity, and lactate dehydrogenase (LDH) activity in hatchlings that were incubated as eggs at a female producing temperature (30 °C) and raised under identical conditions in captivity for approximately one year, as well as in wild-caught juvenile and adult alligators. Animals and eggs were collected from three sites in Florida with varying histories of organochlorine and heavy metal contamination (Burger et al., 2000; Garrison et al., 2010; Guillette et al., 1999; Heinz et al., 1991). MT, GST, and LDH are regulated by sex steroids and can be sensitive indicators of contaminant exposure in numerous taxa. MT is a small cysteine rich protein responsible for the sequestration of heavy metals and the scavenging of

free radicals at the cellular level. Increased levels of MT can be indicative of heavy metal exposures, which include Cd, Hg, Ag and Cu (Hogstrand and Haux, 1990; Klaassen and Liu, 1998; Livingstone, 1993; Roch, 1985; Simoniello et al., 2010a; Simoniello et al., 2013; Simoniello et al., 2011; Simoniello et al., 2010b). In rodent models, MT can be induced by exposure to estrogen and estrogen-like compounds, (Hofer et al., 2010; Rivera-Gonzalez et al., 1998) making MT a potentially sensitive biomarker for several classes of contaminants to which alligators in this study are exposed. GST is a hepatic phase II enzyme, which facilitates toxicant removal through the conjugation of xenobiotic compounds to glutathione (Vos and Vanbladeren, 1990). Some studies have reported both increased and decreased GST activities in contaminant-exposed organisms, that were dependent upon the chemical nature of xenobiotics and exposure level (Elia et al., 2003; Frasco and Guilhermino, 2002; Gallagher et al., 2001). For example, dimethoate, cadmium, and nickel have all been demonstrated to decrease GST activity (Frasco and Guilhermino, 2002; Iscan et al., 2002) where exposure to β -naphthoflavone (BNF) leads to an increase in enzyme activity (Frasco and Guilhermino, 2002). LDH, which converts pyruvate to lactate, is the final enzyme catalyst in the anaerobic-glycolytic pathway. Changes in LDH activity may be indicative of oxidative stress, direct interaction of the toxicant with the enzyme, cellular leakage due to tissue damage, or increased energetic demands for detoxification processes (Albertsson et al., 2007; Frasco and Guilhermino, 2002; Vieira et al., 2008; Yadav et al., 2007). Interestingly, estrogen and estrogenic compounds (DES and octyl-phenol) elevate LDH in rodent models (Cay and Ulas, 2010; Hong et al., 2006). Taken together, these studies suggest that long-term exposure of alligators to environmental contaminants may result in altered activational and/or organizational regulatory pathways, which can lead to serious impairment of toxicant detoxification as well as nutrient metabolism (e.g., glycolysis).

Previous work demonstrated that juvenile and adult alligators collected from Merritt Island National Wildlife Refuge (NWR) exhibited mildly elevated tissue levels of metals, PCBs, and PBDEs when compared to alligators collected from Lake Apopka (organochlorine and metal polluted site) and Lake Woodruff NWR (reference site)(Horai et al., 2014; Isobe, Unpubl. data). The goals of this study were to 1) determine whether MT, GST and LDH varied in the animals analyzed from the above mentioned sites, potentially serving as early indicators of contaminant induced alterations in physiology 2) examine whether these endpoints exhibit sexually dimorphic patterns of expression and 3) to assess whether the site-specific variation of these toxicological endpoints is conserved during ontogenia. To do this, eggs were collected from the three sampling sites and incubated under laboratory conditions. The selected biomarkers were then measured in approximately one year old animals that had been housed in captivity under identical conditions.

Materials and Methods

Study Sites—Lake Woodruff NWR (lat. 29°06'N, long 81°25'W) served as our reference population because previous studies have documented that this site is characterized by lower levels of organochlorine compounds than Lake Apopka (lat. 28°40'N, long. 81°38'W) and lower metal concentrations in alligator tissues than Merritt Island NWR (lat. 28°59'N, long. 80°61'W)(Boggs et al., 2013; Burger et al., 2000; Garrison et al., 2010; Guillette et al.,

1999; Guillette et al., 1994; Heinz et al., 1991; Horai et al., 2014). Lake Apopka is heavily contaminated with organochlorine pesticides from an industrial spill in the 1980s (e.g. DDT and its metabolites, dicophol) in addition to a wide variety of contaminants from sewage treatment facilities and agricultural runoff (Garrison et al., 2010; Guillette et al., 1999; Guillette et al., 1994). The third site, Merritt Island NWR, is located on a barrier island on the east coast of Florida and surrounds the Kennedy Space Center. There are numerous freshwater impoundments here, and the alligators inhabiting this area are regularly found in both saltwater and freshwater environments. Chemical analysis of juvenile and adult alligators caught at this site showed mildly elevated tissue concentrations of metals (Li, Fe, Ni, Sr, In, Sb, Hg, Pb, Bi), as well as PCBs and PBDEs, when compared to alligators from the other two sampling sites (Horai et al., 2014; Isobe, Unpubl. data).

Animal Collection—Animals were collected under State of Florida Fish and Wildlife Conservation Commission Permit # AMP08X-01 and United States Department of the Interior Fish and Wildlife Service permit #s 08-002 (NWR), 2006 SUP 55. Collection procedures were reviewed and approved by the University of Florida IACUC committee (protocol approval numbers 200801660, 2008018050200801238). Female juvenile alligators (snout-vent length = 53.8 cm $\pm$ 10 cm, mean $\pm$ SD) were collected at night by airboat on April 9–11, 2008. Male juvenile alligators (snout-vent length = 47.0 cm $\pm$ 8.3 cm, mean $\pm$ SD) were collected in the same manner on April 15–21, 2009. Adult alligators (snout-vent length: females = 128.0 $\pm$ 46.8 cm, males = 152.4 $\pm$ 19.8 cm, mean $\pm$ SD) were collected from the same locations from March 31 to April 9, 2010. Alligator eggs were collected from the three sites in Florida in June of 2008 and were incubated at the University of Florida at an all-female producing temperature (30 °C). They were raised under identical captive conditions to a mean snout-vent length of 12.8 $\pm$ 0.5 cm (mean $\pm$ SD) and were between 325 and 360 days old at the time of sacrifice. All animals were killed through injection of a lethal dose of sodium pentobarbital into the vertebral vein. Hepatic tissues for enzymatic activity analysis were harvested, flash frozen, and stored at –80 °C until analysis.

Biomarker Analysis

Metallothionein (MT)—MT concentrations were determined with minor modifications to a previously published protocol (Linde and Garcia-Vazquez, 2006). Briefly, male juvenile liver tissues (1 g) were homogenized by hand in 3 ml of homogenization buffer (0.5 M sucrose, 20 mM Tris-HCl, 0.01% β -mercaptoethanol, pH 8.6) in a Kontes Glass Co. homogenizer. All other liver tissues (0.30 – 0.33 g) were homogenized with 1 ml of homogenization buffer with 10–15 steel beads using a Bullet Blender (speed 7 for 4 minutes) (Next Advance, Inc., BBUC3137). The homogenate was centrifuged at $39,086 \times g$ (4 °C) for 20 minutes (L8-80M Beckman Ultracentrifuge). Cold (–20 °C) absolute ethanol (1.05 ml) and chloroform (80 μ l) were added to supernatants (1 ml), and the extracts were mixed and centrifuged at $3,716 \times g$ (4 °C) for 20 minutes (Sorvall Legend RT centrifuge). The supernatant was collected and ice cold absolute ethanol was added (3:1 v/v). Samples were stored at –20 °C for at least one hour, after which they were centrifuged at $3,716 \times g$ (4 °C) for 20 minutes. The resulting pellets were washed with 100 μ l of an ethanol:chloroform:homogenization buffer mix (87:1:12 v/v). Finally, the samples were centrifuged at $3,716 \times g$ (4 °C) for 20 minutes and the resulting pellets were dried under a

nitrogen stream. The pellets were suspended in 300 μ l of re-suspension buffer (5 mM Tris HCl, 1 mM EDTA, pH 7) and added to 4.2 ml of DTNB buffer (0.43 mM 5-5'-dithiobis-2-nitrobenzoic acid, 0.2 M phosphate buffer, pH 8). The samples were evenly divided between 2 wells on a 24-well plate and incubated for 30 minutes at room temperature. Absorbance was read with a Spectramax 190 spectrophotometer at a wavelength of 412 nm. A glutathione (GSH) standard curve was prepared as a reference cysteine rich standard (1 cysteine/molecule) to estimate MT concentrations, as previously described (Linde and Garcia-Vazquez, 2006).

Glutathione-s-transferase (GST)—GST activity (EC 2.5.1.18) was analyzed using previously published protocols, with slight modifications to the procedure (Gunderson et al., 2004b). Male juvenile liver tissue (1 g) was homogenized by hand in a Kontes Glass Co. hand homogenizer with 3 ml of cold Tris buffer (pH 7.4). Liver tissue (0.33 g) from all other animals was homogenized in 1 ml of cold Tris buffer (pH 7.4) with 15–20 zirconium oxide beads using a Bullet Blender (Next Advance, Inc., BBUC3137)(Speed 7 for 4–8 minutes). No difference was found in enzymatic activities due to the manner of tissue homogenization. Samples were centrifuged at $100,350 \times g$ (4 °C) for 60 minutes (L8-80M Beckman Ultracentrifuge). The cytosolic fraction was collected and stored at –80 °C. Total protein concentrations were determined using a Bradford assay (Bradford, 1976). Samples were run in triplicate in a 96 well plate with 30 μ g of protein. Absorbance was read using a Spectromax 190 at 340nm and activity calculated as previously described (Gunderson et al., 2004b).

Lactate Dehydrogenase (LDH)—LDH activity (EC 1.1.1.27) was measured with slight modifications to previously described procedures (Diamantino et al., 2001). Male juvenile liver tissues (0.5–1 g) were homogenized by hand in 3ml of cold Tris buffer (0.2 M Tris – HCl, pH 7.3) in a Kontes Glass Co. homogenizer. For all other samples, liver tissue (0.33 g) was homogenized in 1 ml of cold Tris buffer with 15–20 zirconium oxide beads in a Bullet Blender (speed 7 for 4 minutes). The homogenate was centrifuged at $2,916 \times g$ (4 °C) (L8-80M Beckman Ultracentrifuge) for 5 minutes. Protein concentrations were determined with a Bradford assay (Bradford, 1976). Samples were diluted with Tris buffer to a concentration of 2.5 μ g/ μ l of total protein. Each well contained 270 μ L of Tris buffer, 10 μ L diluted sample (2.5 μ g/ μ l), 10 μ l of 6.6mM NADH and 10 μ l of 30mM sodium pyruvate was added to start the reaction. The plate was mixed and read with a Spectramax 190, at a wavelength of 340nm at 0 and 3 minutes. A NADH standard curve was used to calculate the change in NADH over time, and activity was reported in μ M NADH oxidized/ μ g total protein/min.

Statistical Analysis

All statistical analyses were conducted using SigmaPlot for Windows 12.5.0 (Systat Software, Inc.). One way ANOVA with Holm-Sidak method for multiple comparisons or Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method for multiple comparisons were used for comparisons among the three sites depending on whether the datasets were parametric or non-parametric as determined by a Shapiro-Wilk Normality test and an equal variance test. Likewise, an unpaired two-tailed t-test or Mann-Whitney Rank Sum Test was

used to determine whether sexually dimorphic patterns of expression were apparent within each site and to examine differences in cases where only two sites were sampled.

Results

Metallothionein Concentrations

Site-, sex- and age- specific variations of liver MT concentrations are reported in Figure 1. A pronounced sexual dimorphism ($F > M$) was observed in wild-caught juvenile alligators from all of the sites ($p < 0.002$). Male juveniles from Merritt Island NWR exhibited slightly higher MT concentrations than male alligators from Lake Woodruff NWR ($P < 0.05$), a finding consistent with mildly elevated metal concentrations previously reported in these animals (Horai et al., 2014). Conversely, no difference was observed among wild-caught juvenile females alligators ($p = 0.74$). Sexually dimorphic patterns were not observed in adult alligators from Lakes Woodruff ($p = 0.07$) and Apopka ($p = 0.11$). There was no evidence of organizational regulation of MT as no difference in concentrations were observed among captive-raised alligator hatchlings from the three sites ($p = 0.95$).

Glutathione-s-transferase Activity

Figure 2 shows site, sex and age specific variations of liver GST activity. Captive-raised yearling female alligators from Lake Apopka and Merritt Island NWR exhibited lower GST activity ($p < 0.05$) than animals from the reference location (Lake Woodruff NWR). Lower GST activity was also found in both wild female juvenile alligators ($p < 0.05$) and wild male juvenile alligators ($p < 0.05$) from Merritt Island NWR. Interestingly, juvenile alligators from Lake Apopka did not differ ($p > 0.05$) from Lake Woodruff NWR alligators, despite the depressed activity observed in yearling captive raised animals collected as eggs from Lake Apopka ($p < 0.05$). This could suggest the presence of an activational environmental GST inducer in Lake Apopka. Adult male alligators from Merritt Island NWR exhibited higher GST activity ($p = 0.007$) compared to Lake Woodruff males, with no difference being observed between males from Lakes Woodruff and Apopka ($p = 0.19$). Similarly, no difference in GST activity was observed between female adult alligators from Lakes Woodruff and Apopka ($p = 0.309$). Sexually dimorphic patterns of GST activity ($F > M$) were observed in wild-caught juvenile alligators collected from all sites ($p < 0.006$). This pattern was not observed in adult alligators collected from Lakes Woodruff ($p = 0.14$) or Apopka ($p = 0.07$).

Lactate Dehydrogenase Activity

We observed no sex-, age- or site- specific variations in LDH activity among captive raised yearlings ($p = 0.28$) or male field-caught juveniles ($p = 0.15$) (Figure 3). Wild female juvenile alligators were not analyzed, due to a shortage of tissues. No differences among sites were observed in adult alligators, although sexually dimorphic expression ($F > M$) was observed in animals collected from Lake Apopka ($p = 0.03$). This pattern was not present in Lake Woodruff adults ($p = 0.11$).

Discussion

In this study, we examined the impact of biological (sex and age) and environmental (contaminants) variables on three common biomarkers of contaminant exposure. Furthermore, we explored whether there was evidence of organizational regulation, based on the expression of the markers in animals from eggs that were collected from sites with a marked difference in pollution history.

MT is a cysteine rich protein present in eukaryotic organisms that plays many cellular roles, which include scavenging of free radicals, maintenance of essential metal homeostasis, and sequestration of toxic metals within the cell (Atif et al., 2005a; Atif et al., 2008; Atif et al., 2006; Atif et al., 2005b; Baerwald, 2013; Baerwald et al., 2008; Bell and Vallee, 2009; Egli et al., 2006a; Egli et al., 2006b; Klaassen et al., 2009; Kumari et al., 1998; O'Connor et al., 2014; Palmiter, 1998, 2004). Increases in MT can be indicative of metal (Cd, Hg, Ag, Cu, etc.) or chemical exposure, as these cysteine-rich proteins protect the cell against damage caused by high levels of essential trace and toxic metals (Klaassen and Liu, 1998; Livingstone, 1993; Simoniello et al., 2010a; Simoniello et al., 2013; Simoniello et al., 2011). Interestingly, estrogen induces MT in rat kidney and uterus, as well as enhances the uptake of cadmium and the corresponding induction of MT in rats (Blazka and Shaikh, 1991; Hofer et al., 2010; Rivera-Gonzalez et al., 1998). These findings make MT an interesting sub-lethal endpoint, as metal contamination has been observed in alligators sampled from Merritt Island NWR and as organochlorine contaminants (found in Lake Apopka), acting as weak estrogens, bind to the alligator estrogen receptor (Burger et al., 2000; Garrison et al., 2010; Guillette et al., 1999; Guillette et al., 2006; Horai et al., 2014; Vonier et al., 1996).

Our results are consistent with the hypothesis that MT expression, in response to metal exposure, is activationally modulated in wild juvenile alligators inhabiting Merritt Island NWR. MT levels were significantly higher in wild juvenile male alligators collected from Merritt Island NWR compared to those from Lake Woodruff NWR, although no significant differences existed among females. Furthermore, sexually dimorphic patterns were observed in wild-caught juveniles from each of the sites; higher MT expression existed in females than males. The mildly elevated MT in male juveniles from Merritt Island NWR could be indicative of heavy metal exposure, as induction of MT by exposure to heavy metals has been observed in reptiles (Simoniello et al., 2010a; Simoniello et al., 2011; Simoniello et al., 2010b) and slightly elevated concentrations of metals (Li, Fe, Ni, Sr, In, Sb, Hg, Pb, Bi) have previously been reported in these animals (Horai et al., 2014). The sexually dimorphic patterns are interesting, as estrogen has been reported to up-regulate MT in other taxa and would be consistent with differences in circulating estrogen concentrations between males and females (Hofer et al., 2010; Rivera-Gonzalez et al., 1998). Additionally, estrogen has been shown to enhance the uptake of heavy metals in rats (Blazka and Shaikh, 1991) that could lead to sex specific patterns of metal toxicity if females exhibit enhanced uptake of metals from the environment. Interestingly, no differences existed among sites in MT concentrations, nor were sexually dimorphic patterns of expression observed in adult alligators collected from Lakes Woodruff and Apopka. These observations suggest that differences in life stage specific expression, circulating hormone concentrations among age groups (Guillette et al., 1997; Hamlin et al., 2010; Hamlin et al., 2014; Lance, 2003; Rooney

et al., 2004), contaminant exposure, or dietary shifts impact MT concentrations in alligators. Ontogenetic dietary shifts — from freshwater and estuarine diets among females and juveniles to a predominantly marine diet among large adult males — have been described in the Merritt Island NWR alligator population (Boggs et al., 2016). Given that the freshwater systems at Merritt Island NWR are predominantly isolated systems, such as ponds and dikes, metal contamination in these systems may be greater than in the estuarine and marine systems that are more open to dilution. Therefore, differences in MT, seen among juveniles from different sites, may be removed among the adult males due to dietary shift. However, the small sample sizes for adult female alligators and the scope of the study limit the conclusions that can be drawn.

In this study, we found no evidence that MT is impacted through organizational regulatory pathways. MT levels were not significantly different among captive-raised alligators from the three sites, suggesting that MT expression is not impacted by developmental factors that might include *in ovo* xenobiotic exposure (organizational regulation). Studies in the wall lizard (*Podarcis sicula*) report increases in MT in both embryos whose eggs were incubated in soils containing cadmium and in adult animals exposed to cadmium, either through intraperitoneal injection or diet (Simoniello et al., 2010a; Simoniello et al., 2013; Simoniello et al., 2011). However, adult *Podarcis carbonelli* chronically exposed to a Cd-rich diet showed no statistical differences in both liver and gut MT concentrations between controls and treated groups (Mann et al., 2007). Although we know that organochlorine pesticides and their metabolites are sequestered in the yolk of alligator eggs (Heinz et al., 1991), little is known about metal concentrations in eggs. In adult animals, one-time intraperitoneal injections lead to temporarily increased MT mRNA expression, which returned to control levels by 14 days following exposure, demonstrating an activational, reversible response in adult animals. Even if MT concentrations were elevated in embryos (which we did not examine), the response could return to baseline once the exposure ceased (Simoniello et al., 2010a). Maternal transfer of nutrients, metals, and other toxicants to the egg is another pathway through which xenobiotic exposure can occur, and reptilian eggs have been shown to contain metals and chlorinated contaminants (Guirlet et al., 2008; Guirlet et al., 2010; Heinz et al., 1991; Nagle et al., 2001; Roe et al., 2004).

GST is a phase II detoxification enzyme that is sensitive to contaminant exposure, as well as natural variability factors (van der Oost et al., 2003). Thus interpretation of results is a difficult task in situations in which wild animals are exposed to complex mixtures of chemicals over different periods of time and at different life stages. Some laboratory studies have documented decreased GST activity as an indicator of organophosphorus pesticide or metal exposure, whereas an increase of this detoxifying enzyme activity is associated to PAH exposure (Frasco and Guilhermino, 2002; Iscan et al., 2002). Previous work by our research group reported no differences in GST activity among three sites in south Florida with varying degrees of contamination and agricultural activity in the surrounding areas (Gunderson et al., 2004b). In this study, we report evidence suggestive of organizational and activational regulation of GST activity, sexually dimorphic patterns of activity, and differences in activity patterns between juvenile and adult alligators.

GST activity was significantly lower in Lake Apopka and Merritt Island NWR female alligators raised in captivity as compared to those from Lake Woodruff NWR, suggesting that GST activity could be altered in response to developmental factors, such as exposure to xenobiotics (although other physiological factors, such as genetic differences among the populations and other possible enzymatic modulators, cannot be ruled out at this time). This observed GST depression is consistent with findings in flounder larvae and juveniles exposed to cadmium while developing (Cao et al., 2010). However, studies spanning both vertebrates and invertebrates have failed to find significant alteration of GST activity in developing organisms exposed to contaminants (Crane et al., 2002; Hirthe et al., 2001; Huang et al., 2010). The differences in GST responses might be explained by different properties of the environmental contaminants to which the organisms were exposed, differences in the time scale of the studies, or differences in the organisms' responses to contaminants. Furthermore, prior studies did not examine GST activity after exposure to particular contaminants had ceased, making it difficult to conclude if observed alterations permanently or temporarily affected activity.

GST activity was significantly lower in male and female wild-caught juvenile alligators collected from the Merritt Island NWR as compared with those from the other two sites. This suggests that contaminants present at Merritt Island NWR cause depression of GST activity at this life stage and is consistent with studies reporting lower GST activity in fish exposed to a mixture of metals (Cu, Cd, Fe and Ni) and frogs exposed to Cr and Cd (Kostaropoulos et al., 2005; Pandey et al., 2008). Elevated metal concentrations (Li, Fe, Ni, Sr, In, Sb, Hg, Pb, Bi) (Horai et al., 2014) as well as slightly higher MT concentrations in male juveniles from Merritt Island NWR suggests heavy metal exposure and could be one explanation for the lower GST activity in juvenile alligators observed in this study. Additionally, we observed a sexual dimorphism in GST activity, with higher activity in juvenile wild-caught female alligators than males in all three sites. This differs from prior studies in which no sexual dimorphism was observed in this endpoint in juvenile alligators (Gunderson et al., 2004b). Previously reported GST activity values in male juvenile alligators from Lake Okeechobee ranged between 358–399 $\mu\text{M}/\text{ug}/\text{min}$ (Gunderson et al., 2004b) whereas juvenile males from all sites in this study exhibited activities below 200 $\mu\text{M}/\text{ug}/\text{min}$. GST activities in juvenile female alligators from Lake Okeechobee ranged from 361 $\pm$ 48–442 $\pm$ 57 $\mu\text{M}/\text{ug}/\text{min}$ (Gunderson et al., 2004b) with juvenile females in this study exhibiting activities of 438 $\pm$ 65.3 $\mu\text{M}/\text{ug}/\text{min}$ (Lake Woodruff NWR), 254 $\pm$ 37.7 $\mu\text{M}/\text{ug}/\text{min}$ (Merritt Island NWR) and 450.6 $\pm$ 51.3 $\mu\text{M}/\text{ug}/\text{min}$ (Lake Apopka). Thus suggests that all of the values fall roughly within the same ranges with the exception of females from Merritt Island NWR which appear to be slightly lower (statistically significant in the this study). More populations need to be screened in order to establish normal GST activity ranges in wild alligators.

Comparisons between results from the captive raised alligators with those from wild juveniles suggest that contaminant exposure through a pathway, such as diet, would lead to the induction of GST activity in female alligators from Lake Apopka. Though GST activity reaches physiological conditions comparable to our reference population, it is possible that the ability of Lake Apopka animals to remove toxicants through this mechanism is limited

by the lower basal activity observed in the developmental study. However, it is beyond the scope of this study to make such a conclusion.

Adult alligator GST activity exhibited different patterns among sites, with males collected from Merritt Island NWR having slightly higher GST activity than animals from the other two sites and with animals from Lakes Woodruff and Apopka lacking the sexually dimorphic patterns of activity that were observed in the wild juveniles that we analyzed. The loss of sexual dimorphism in GST activity between the juveniles and adults is interesting to note given that we previously reported a loss of sexually dimorphic patterns of testosterone biotransformation enzyme activities in juvenile alligators inhabiting contaminated sites in Florida (Gunderson et al., 2001). Based on the limited adult female sample sizes from Lakes Woodruff and Apopka and no females being collected from Merritt Island NWR, it is difficult to draw conclusions, although dietary shifts, exposure to different classes of contaminants, bioaccumulation of contaminants, or life-stage dependent changes in GST activity could all provide potential mechanisms to explain the shift in patterns. It is worth noting that a similar loss of sexual dimorphism with age was also observed in MT concentrations, as discussed above, and future studies should continue to examine these apparent age-dependent shifts in biomarker expression.

We found no evidence for differences based on developmental exposure to mixtures *in ovo*. This is consistent with a previous study that reported that pesticide mixture exposure during development in caimans had no effect on serum LDH (Poletta et al., 2011). Furthermore, we found no evidence for activational regulation of LDH activity in wild-caught juvenile or adult alligators in association with reported contaminant concentrations and land-use practices in and around the three sites sampled in this study. We observed no difference between sexes in Lake Woodruff adult animals, which is consistent with a study on caiman that reported finding no sexual dimorphism in LDH activity at 2 or 12 months of age (Poletta et al., 2011). Interestingly, Lake Apopka adults exhibited a pronounced sexual dimorphism in LDH activity ($F > M$). Lake Apopka, as previously noted, has a history of organochlorine pesticide contamination, of which many compounds have been shown to exhibit estrogenic properties (Garrison et al., 2010; Guillette et al., 1999; Guillette et al., 2006; Vonier et al., 1996). LDH has been shown to increase in response to estrogens and estrogenic compounds (DES and octyl-phenol) in rodent models (Cay and Ulas, 2010; Hong et al., 2006), making it reasonable to predict that sexually dimorphic patterns of expression would be observed in reference animals and that estrogenic contaminants could conceivably alter these patterns, although given the scope of this study and limited sample sizes, we do not have enough information to draw conclusions. Future studies should continue to examine variation in this endpoint between sexes and among age classes, as well as help determine whether it serves as a useful biomarker indicative of contaminant exposure in crocodilians.

Conclusions

In this study, we measured detectable levels of MT, GST and LDH in alligators from three different age groups and described variation in these endpoints between sexes, among size classes, and among three sites with different land use practices taking place in the surrounding areas. Our results stress the importance of identifying a reference value, of

considering sexes separately, and of factoring in age when measuring biomarkers that are sensitive to contaminant exposure. Clear sexually dimorphic patterns ($F > M$) were observed in GST activity and MT concentrations in juvenile alligators, which is consistent with observations in other taxa that demonstrates that estrogens and other hormones may play a regulatory role in the expression of these endpoints. The slightly elevated MT concentrations and lower GST activities observed in juvenile alligators collected from Merritt Island NWR is consistent with the higher levels of metal contamination previously reported in the juvenile and adult animals examined in this study (Horai et al., 2014; Iscan et al., 2002; Livingstone, 1993). The results suggest that physiological alterations in MT and GST due to environmental factors could be occurring in juvenile and adult alligators in this study. They may further suggest that developmental exposure to toxicants can affect GST activity at later life stages, although determining the physiological significance of these subtle differences is beyond the scope of this study. Further work is needed to examine the normal ranges and regulation of these endpoints by hormones, environmental variables, contaminants, and age-related factors.

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Highlights

- Site specific patterns in MT and GST were observed.
- Sexually dimorphic patterns of MT and GST were present in juvenile alligators.
- MT and GST patterns were consistent with metal exposure.
- Possible developmental modulation of GST could explain patterns.
- No clear patterns in LDH were present among sites or between sexes.

Figure 1. Hepatic MT concentrations (mean $\pm$ SE) in a) captive-raised alligator hatchlings, b) wild-caught juvenile alligators, and c) adult alligators collected from Lake Woodruff NWR, Merritt Island NWR, and Lake Apopka. Line above bars denotes significant differences ($p < 0.05$) between males and females within a site. Different letters above bars (a, b) denote significant differences among males.

Figure 2.

Hepatic GST activities (mean + SE) in a) captive-raised alligator hatchlings, b) wild-caught juvenile alligators, and c) adult alligators collected from Lake Woodruff NWR, Merritt Island NWR, and Lake Apopka. Line above bars denotes significant differences ($p < 0.05$) between males and females within a site. Different letters above bars (^{a,b}) denote significant differences among males.

Figure 3.

Hepatic LDH activity (mean + SE) in a) captive-raised alligator hatchlings, b) wild-caught juvenile alligators, and c) wild-caught adult alligators collected from Lake Woodruff NWR, Merritt Island NWR, and Lake Apopka. Line above bars denotes significant differences ($p < 0.05$) between males and females within a site.