Arch Environ Contam Toxicol (2016) 71:347–358
DOI 10.1007/s00244-016-0301-9

Chronic Ingestion of Coal Fly-Ash Contaminated Prey and Its Effects on Health and Immune Parameters in Juvenile American Alligators (*Alligator mississippiensis*)

John W. Finger Jr.[1,2,3,5] · Matthew T. Hamilton[2,4] · Brian S. Metts[2,6] · Travis C. Glenn[1,3] · Tracey D. Tuberville[2,3]

Received: 6 May 2016 / Accepted: 6 July 2016 / Published online: 30 July 2016

Abstract Coal-burning power plants supply approximately 37 % of the electricity in the United States. However, incomplete combustion produces ash wastes enriched with toxic trace elements that have historically been disposed of in aquatic basins. Organisms inhabiting such habitats may accumulate these trace elements; however, studies investigating the effects on biota have been primarily restricted to shorter-lived, lower-trophic organisms. The American alligator (*Alligator mississippiensis*), a long-lived, top-trophic carnivore, has been observed inhabiting these basins, yet the health or immune effects of chronic exposure and possible accumulation remains unknown. In this study, we investigated how chronic dietary ingestion of prey contaminated with coal combustion wastes (CCWs) for 25 months, and subsequent accumulation of trace elements present in CCWs, affected juvenile alligator immune function and health. Alligators were assigned to one of four dietary-treatment groups including controls and those fed prey contaminated with CCWs for one, two, or three times a week. However, no effect of Dietary Treatment ($p > 0.05$) was observed on any immune parameter or hematological or plasma analyte we tested. Our results suggest that neither exposure to nor accumulation of low doses of CCWs had a negative effect on certain aspects of the immune and hematological system. However, future studies are required to elucidate this further.

✉ John W. Finger Jr.
jwf0016@auburn.edu

1 Department of Environmental Health Science, University of Georgia, Athens, GA 30602, USA

2 Savannah River Ecology Laboratory, University of Georgia, Aiken, SC 29802, USA

3 Interdisciplinary Toxicology Program, University of Georgia, Athens, GA 30602, USA

4 Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602, USA

5 Present Address: Department of Biological Sciences, Auburn University, Auburn, AL 36849, USA

6 Present Address: Grovetown Middle School, Grovetown, GA 30813, USA

Environmental stressors, such as anthropogenically derived contaminants, are known to affect a multitude of physiological and behavioral processes (Finger and Gogal 2013; Hopkins et al. 1998; Rowe et al. 2002). One area of concern is exposure to contaminants associated with the combustion of fossil fuels for energy production and the subsequent waste produced. In particular, coal-burning power plants supply approximately 37 % of the electricity consumed in the United States of America (US EIA 2016), However, incomplete combustion produces solid waste materials, termed "coal combustion wastes" (CCWs), that must be disposed of (NRC 2006). Collectively, CCWs may be comprised of approximately 20 trace elements and, depending on the source, may include copper (Cu), lead (Pb), selenium (Se), mercury (Hg), cadmium (Cd), and arsenic (As; Hopkins et al. 1999; NRC 2006; Rowe et al. 2002). Waste management of CCWs may occur by way of a variety of routes including disposal in landfills or mines or use in structural products (i.e., cement or concrete; NRC 2006). Residues may also be released into artificial aquatic settling basins (or "ash basins") where CCWs and water are amalgamated and subsequently deposited in water-containing impoundments (NRC 2006; Rowe et al. 2002). The ash-water slurry flows into a primary settling basin, from which surface water flows into a secondary basin and is then typically released without further treatment into nearby rivers or other water bodies. Although this method currently constitutes only 21 % of disposal facilities (Lemly and Skorupa 2012), almost 66 % of all CCWs were disposed by this method before 1980 (Rowe et al. 2002).

Many types of animals and plants have been documented to reside, breed, or forage in ash basins (Hopkins et al. 1999; Roe et al. 2004; Rowe et al. 2001, 2002) leading to increased risk of contaminant exposure. In fact, many of the elements found in ash basins have been shown to accumulate in both plants and animals (Rowe et al. 2002), thus increasing the risk in higher-trophic organisms. However, the effects of CCW exposure have been mainly investigated in shorter-lived organisms (Hopkins et al. 1998, 1999, 2002; Rowe et al. 1996, 2001), and the effects of chronic exposure in longer-lived organisms are less well known. Moreover, the majority of studies investigating exposure often use organisms of lower-trophic status (Hopkins et al. 1998; Rowe et al. 2001), thus failing to account for how accumulation may affect higher-trophic organisms and possibly provide insight into human health effects. Therefore, investigations into the effects of CCWs on longer-lived, higher-trophic organisms are necessary to elucidate how these compounds may affect organismal and environmental health.

Crocodilians, semiaquatic archosaurs, have been previously suggested as useful environmental sentinels for contaminant exposure due to their life history (i.e., long-lived) and top-trophic placement (Campbell 2003; Finger and Gogal 2013; Milnes and Guillette 2008), thus facilitating bioaccumulation and biomagnification of compounds. Most of the studies investigating the effects of contaminants on crocodilians have focused on the American alligator (*Alligator mississippiensis*; Campbell 2003), a species endemic to the southeastern United States. Most studies have focused on mercury or endocrine-disrupting chemicals, but few have investigated trace elements present in CCWs (Burger et al. 2000; Campbell et al. 2010). Importantly, however, alligators have been observed in ash basins on the Savannah River Site (SRS) near Aiken, South Carolina, potentially exposing them to CCW contaminants through he ingestion of water and contaminated prey items (Finger, *personal observation*; Roe et al. 2004). Given the historically common practice of aquatic disposal of CCW and the widespread geographic distribution of the American alligator throughout the southeastern United States (Rowe et al. 2002), alligators may be likely inhabitants of ash basins in regions where they co-occur. Furthermore, previous studies have showed accumulation in other species of vertebrates, some of which alligators may prey on (Hopkins et al. 1998, 1999, 2002; Rowe et al. 1996, 2002). However, accumulation may be affected by a number of factors including individual age, sex, or feeding ecology (see Campbell 2003; Campbell et al. 2010). The only study that has investigated the effects of CCW on wild alligators found tat increased Se in ovo was associated with decreased clutch viability in hatchlings and eggs from a female alligator nesting downstream from a coal-burning power plant (Roe et al. 2004), presumably due to accumulation of Se in the female and subsequent maternal transfer to the offspring. As of yet, no study has directly investigated the role of CCWs on crocodilian physiology, nor have the long-term effects of CCW exposure been elucidated. Therefore, further research is necessary to understand how CCWs may impact a long-lived, top-trophic predator.

As part of another study (Tuberville et al. 2016), we investigated the effects of chronic (25 months) dietary ingestion of CCW-contaminated prey and subsequent accumulation of CCW trace elements on the immune system and health of the American alligator. Accumulation of trace elements found in CCWs was previously shown to increase the metabolic rate in crayfish and snakes (Hopkins et al. 1999; Rowe et al. 2001). However, increased metabolic rate is most likely an adaptive response to counteract the deleterious effects of contaminants, which consequently may induce tradeoffs with other systems such as immune function or growth (Hopkins et al. 1999; Rowe et al. 2001). Furthermore, exposure to CCWs and its constitutive trace elements was previously shown to deleteriously affect various physiological processes including endocrine signaling, embryonic development, and/or reproduction (Hopkins et al. 1997, 2004; Roe et al. 2004). However, less is known about how the immune system may be affected by CCW exposure. Importantly, however, many of the trace elements present in CCWs, such as Se and As, have been shown to have deleterious effects on the immune system (e.g., Beck et al. 2014; Dangleben et al. 2013; Hoffman 2007), thus necessitating further research of how longer-lived, top-trophic organisms are affected. Therefore, we hypothesized that chronic exposure to CCWs would negatively affect immune parameters in American alligators. The ability of an organism to fend off pathogens is essential for survival. However, tradeoffs associated with mounting a physiologically costly immune response can negatively affect other functions such as reproduction or growth (see Demas et al. 2011; Finger and Gogal 2013; Finger et al. 2013, 2015a). Therefore, the potential direct and indirect effects of exposure to CCWs on fitness may result in profound population-level effects. In this study, our objectives were to understand how exposure to CCWs may impact the immune system and health of the American alligator.

Methods and Materials

Husbandry and Contaminant Exposure

Husbandry, experimental design, and dietary-exposure treatments have previously been described (Tuberville et al. 2016). Briefly, 1-year-old American alligators obtained from Rockefeller Wildlife Refuge (RWR) in Grand Chenier, Louisiana, USA, were transported to the University of Georgia's Savannah River Ecology Laboratory on the SRS. Thirty-six alligators were randomly distributed among and housed in groups of six individuals in six $270 \times 56 \times 57$ cm^3 tanks in a concrete block frame aquatic animal facility with fiberglass roofing for natural light but with limited climate control (ambient temperatures ranged from 18 to 30 °C throughout the experiment Finger et al. 2015b).

Before initiation of the contaminant-exposure treatments, alligators were fed ad libitum with Mazuri crocodilian chow (PMI Nutrition International LLC, Brentwood, Missouri, USA). For 2 years (beginning 13 June 2011) animals in each tank were exposed to one of four Dietary Treatments, whereby food was deposited into each tank (i.e, alligators were not directly administered food orally). Although all animals were fed three times per week, treatments varied in relative frequency of contaminated relative to uncontaminated food and ranged from zero to three times per week among the four treatments (see Tuberville et al. 2016 for a more detailed explanation). Although the diet of alligators inhabiting the SRS ash basins has not been characterized, small fish and invertebrates, including mosquitofish and crayfish, are frequently consumed by juvenile alligators (Delany 1990; Delany and Abercrombie 1986). Both mosquitofish and crayfish have been documented from coal fly ash settling basins on the SRS (Cherry et al. 1976; Rowe et al. 2001) and were thus used to expose alligators to CCWs. Contaminated and uncontaminated food pellets contained the same ratio of ground crayfish, ground mosquitofish, and Mazuri crocodilian chow, but they differed in whether prey were collected from coal fly ash settling basins on the SRS (CCW-contaminated food) or uncontaminated reference wetlands on the SRS (control or uncontaminated food). Se [mean ± SE (SE) concentration; contaminated pellets = 12.26 ± 0.20 ppm; control pellets = 1.5 ± 0.001 ppm], As (contaminated pellets = 5.05 ± 0.06 ppm; control pellets = 1.85 ± 0.04 ppm), and Cd [contaminated pellets = 1.95 ± 0.03 ppm; control pellets lower than the limit of detection (LOD)] were all significantly greater in CCW-contaminated food than in control food (see Tuberville et al. 2016). Animals in the control treatment ($n = 1$ tank, control tank) were fed only uncontaminated food. Animals in the low-dose treatment [$n = 1$ tank (LOW)] received contaminated food only once per week resulting in an average dose of 5.09 ppm Se, 2.92 ppm As, and 0.81 ppm Cd. Animals in the medium-dose group [$n = 2$ tanks (MED-A and MED-B)] received contaminated food twice per week resulting in an average dose of 8.67 ppm Se, 3.98 ppm As, and 1.38 ppm Cd. Animals in the high-dose group [$n = 2$ tanks (HIGH-A and HIGH-B)] were fed contaminated prey three times per week. For further explanation of how diets were prepared and administered, see Tuberville et al. (2016).

Sample Collection and Analysis

Only the 21 (control, $n = 3$; LOW $n = 4$; MED, $n = 7$; HIGH, $n = 7$) alligators remaining at the end of the 25-month feeding experiment were included in the current study of immune and health parameters. Twelve of the 36 alligators (2/tank) were killed and dissected after 12 months of CCW exposure treatment as part of another study (see Tuberville et al. 2016). In addition, 3 alligators died (one from control, one from MED-A, and one from HIGH-A) before the end of the study and are also not included in analysis. At the conclusion of the 25-month exposure period, alligators were captured from their respective tanks, and a blood sample was obtained from the occipital sinus within 260 s (mean ± SE 84.62 ± 10.02) of capture for each individual using a 25-gauge, 2.54-cm heparinized needle (Hamilton et al. 2016a). Whole blood (0.25–0.75 mL) was immediately aliquoted into a 1.5-mL tube for subsequent trace-element analysis (see further details in Tuberville et al. 2016). The remaining whole blood sample was placed in lithium heparin tubes (Becton Dickson, San Antonio, Texas, USA), inverted three times, and placed on ice for subsequent processing within 30 min of sample collection (see later text).

First, approximately 0.75 mL whole blood from each lithium-heparin tube (as described previously) was placed into a heparinized microhematocrit capillary tube (15401-628; VWR Scientific Inc. Drummond Scientific Co., Broomall, Pennsylvania, USA) to measure hematocrit [i.e., packed cell volume (PCV)]. Microhematocrit tubes were centrifuged for 5 min at $3636\times g$ in a LW Scientific ZIPocrit microcentrifuge (Lawrenceville, Georgia, USA),and PCV (in %) was determined using the provided ZIPocrit reading chart. The remaining blood sample in the lithium-heparinized tubes was centrifuged (Cole-Parmer, Vernon Hills, Illinois, USA) for 1 min at $1640\times g$. The plasma portion was aliquoted into 1.5-mL tubes and frozen at −60 °C for subsequent immune assays and plasma biochemistry (described in later text).

Phytohaemagglutinin Assays

After blood sampling, phytohaemagglutinin (PHA; PHA-P no. L8754; Sigma-Aldrich, St Louis, Missouri, USA) was used to investigate the effects of CCW treatment on the cellular (inflammatory) immune response according to the protocol of Finger et al. (2013) for saltwater crocodiles (*Crocodylus porosus*). Because it was previously shown in saltwater crocodiles that 2 mg/mL PHA elicited a significant swelling response after injection (Finger et al. 2013, 2015a), the same dose was used in this study. Briefly, 20 µL of 2 mg/mL PHA and 20 µL of sterile phosphate-buffered saline (PBS) were injected into the right and left hind toe webs, respectively, between the first and second digits with a 0.3-mL syringe with a 29-gauge needle. PBS was used as a control solution because injection alone is likely to trigger an inflammatory response (e.g., Brown et al. 2011; Finger et al. 2013). Web thickness of both webs was measured to the nearest 0.01 mm before injection (at 0 h, which was immediately after blood collection) and 24 h after injection using a dial thickness gauge (Peacock G-1A; Ozaki Manufacturing Ltd, Japan) with three rapid, sequential measurements taken at each time point (Finger et al. 2013). To minimize errors, one person (J. W. F.) performed injections and measurements for standardization. The three measurements were averaged to determine the average thickness of each individual at the respective time points (Brown et al. 2011; Finger et al. 2013). Swelling response due to injection with PHA or PBS was quantified for each injection site by subtracting the time-0 measurement from the 24-h postinjection measurement.

Bacterial Killing Assay

Bacterial killing assays (BKAs) were performed with *Esherichia coli* according to the protocol of Finger et al. (2015a). Briefly, thawed plasma was aliquoted into CO_2-independent media and subsequently mixed with *E. coli*. An aliquot of this mixture was then immediately plated onto tryptic-soy agar (TSA) plates corresponding to time point "0." The remaining mixture was incubated at room temperature for 60 min (after initial addition of bacteria), and then another aliquot was plated out corresponding to time point "60." Plates were inverted and incubated at 37 °C for 24 h, and colonies were enumerated. Percent bacterial killed was calculated using the following formula (Finger et al. 2015a): $\left(1 - \frac{\text{Colonies 60 min}}{\text{Colonies 0 min}}\right) \times 100$.

Plasma Biochemistry

Thawed frozen plasma was analyzed using an Avian/Reptile Profile Plus rotor in a VetScan VS2 analyzer (Abaxis North America, Union City, California, USA) to determine plasma biochemistry parameters as described by Hamilton et al. (2016b). To prevent any biochemical changes in plasma due to refreezing/thawing samples for analysis, plasma samples used for BKAs and plasma biochemistry were analyzed on the same day. Analytes measured included aspartate aminotransferase (AST), bile acids (BA), uric acid (UA), total protein (TP), albumins (ALB), globulins (GLB), glucose (GLU), phosphorous (Ph), creatine kinase (CK), calcium (Ca), potassium (K^+), and sodium (Na^+). For one individual in HIGH-A, a large-enough blood sample could not be obtained for analysis on the VS2 or with BKAs and thus was not used in analysis.

Killing, Morphometrics, Dissection, and Splenic Mass Determination

After capture, blood sampling, and PHA injection, all individual alligators were measured for snout vent length (SVL) and mass as described by Isberg et al. (2005) and Webb and Messel (1978) and sexed as described by Allsteadt and Lang (1995). Body condition indices (BCIs) were generated using Fulton's condition factor with SVL and mass (see Hamilton et al. 2016b).

After sampling, measuring, and sexing, alligators were killed with tricaine methanesulfonate (MS222) as described by Conroy et al. (2009). Alligators were then dissected, and organs (kidney, liver, spleen) were removed. Samples from the liver and kidney were stored at −60 °C until trace-element analysis. The spleen was weighed to the nearest 0.01 g as a routine immunotoxicological measure of immune function. Splenic mass could not be obtained for two individuals (one in LOW, one in HIGH-A), and one splenic value was excluded from analysis due to only a portion of the organ being weighed (thus, overall $n = 19$).

Trace-Element Analysis

As, Se, and Cd concentrations in kidneys and livers were determined by analyzing on an inductively coupled plasma mass spectrometer after freeze-drying, grinding, and microwave digestion in 70 % nitric acid. Detailed methods and treatment results were presented previously in Tuberville et al. (2016). However, trace-element concentrations are used in this study to relate health and immune parameters to contaminant levels in individual alligators. All three trace elements were previously shown to be increased in biota exposed to CCWs, including the study system where prey were collected for this study, and are known to cause deleterious sublethal effects (Tuberville et al. 2016 and references therein).

Statistics

All analyses were performed in GenStat Version 16.1(VSN International, Hemel Hempstead, UK) and JMP Pro 12.1 (SAS, Cary, North Carolina, USA).

Before this study, the PHA assay had never been validated in other crocodilian species in addition to *C. porosus* (Finger et al. 2013). Therefore, it was important to first determine if alligators mounted a similar response to injection (i.e., PHA-induced swelling was greater than PBS). Web Thickness, the thickness of the injection site as determined by the thickness gauge, was used the main outcome variable. Time (0 vs. 24 h) and PHATreatment (PHA vs. PBS) were included as factors to examine their affect on Web Thickness. Animalid was used as a random effect to account for repeated measures (i.e., times 0, 24 h) of Web Thickness on the same individual (Finger et al. 2013).

To understand the effect of CCWs on immune and hematological parameters, Dietary Treatment ($n = 4$)—representing the number of times (0, 1, 2, or 3) individuals were fed CCWs per week—was used as the main factor in analysis using a univariate linear mixed model (restricted maximum likelihood). Tank ($n = 6$) was included as a random effect to account for any inter-tank variation (i.e., as a blocking term). The following outcome variables were each investigated in separate analyses: SwellingDiff [difference in PHA- and PBS-induced swelling 24 h after injection (PHA24-PBS24)], percent *E. coli* killed (EcoliBKA), splenic mass, and plasma biochemical and hematological parameters. Some variables were log-transformed to meet assumptions of normality (i.e., LnAST). Handling time between capture and obtaining a blood sample was included in models when it was a significant covariate. Although no difference in size (SVL and mass), growth rate, or BCI (see later text) was observed among Dietary Treatment groups for animals exposed for 25 months (Tuberville et al. 2016), BCI was used as a covariate to account for the effect of alligator condition on immunological and health parameters. Sex was not used in analysis due to the disproportionate number of females (total, $n = 18$) relative to males (total, $n = 3$) in treatment groups (control, $n = 3$ females, $n = 0$ males; LOW, $n = 3$ females, $n = 1$ male; MID, $n = 6$ females, $n = 1$ male; HIGH, $n = 6$ females, $n = 1$ male). In addition to mixed models, we regressed liver and kidney concentrations of As, Cd, and Se against immune parameters (i.e., SwellingDiff, EcoliBKA, and splenic mass).

Results are presented as means $\pm$ 1 SE. If data were initially log-transformed to improve normality, then reported values were back-transformed. Values for a particular dependent variable were removed if residuals were >4 SDs from the mean of the residual. Least significant differences (LSDs) at the 5 % level (i.e., 5 % LSD) were used to compare means in post hoc analysis (when significant). A value of $p < 0.05$ was considered significant.

Results

Morphometrics

No effect of Dietary Treatment was found on either SVL or mass after exposure to CCW-laden or uncontaminated prey for 25 months (Fig. 1; Tuberville et al. 2016). Dietary Treatment also had no effect on BCI ($p = 0.954$). However, to account for how alligator condition affected each parameter (independent of Dietary Treatment), BCI was used as a covariate in subsequent analyses.

PHA Validation

To evaluate the successful implementation (i.e., swelling greater in PHA injected) of PHA injection in American alligators, the effect of Time (0 vs. 24 h) and PHATreatment (PHA vs. PBS) on Web Thickness was analyzed independent of Dietary Treatment. There was a significant Time $\times$ PHATreatment interaction ($p < 0.001$) on Web Thickness (Fig. 2) with Web Thickness at PHA24 (1.13 $\pm$ 0.02 mm) being significantly greater than at both PBS24 (0.76 $\pm$ 0.02 mm) and PHA0 (0.73 $\pm$ 0.02 mm; 5 % LSD). Web Thickness at PBS24 was also significantly greater than that at PBS0 (0.70 $\pm$ 0.02 mm) showing that injection alone triggers a swelling response (5 % LSD).

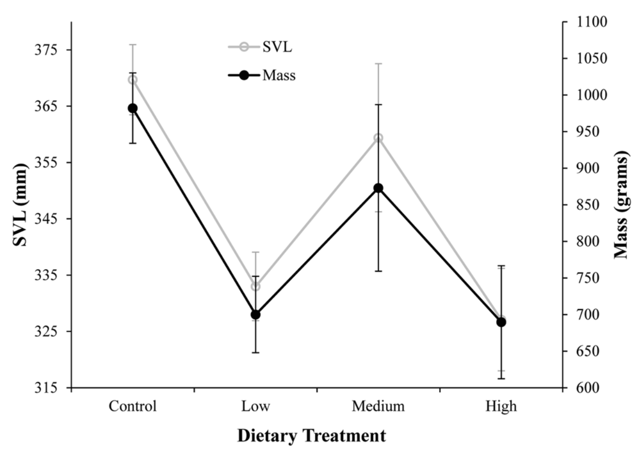

Fig. 1 The effect of Dietary Treatment with CCWs for 25 months on American alligator SVL and mass. Circles are raw means ($\pm$1 SE) of respective treatments (control, $n = 3$; LOW, $n = 4$; MED, $n = 7$; HIGH $n = 7$). SVL (in mm) is indicated by open *grey circles* and a *grey line*. Mass is indicated by *black shaded circles* and a *black line*. The *x*-axis denotes Dietary Treatments based on the number of times each week the group of alligators was fed CCW-laden prey: *control* 0 times, *LOW* 1 time, *MED* 2 times, *HIGH* 3 times

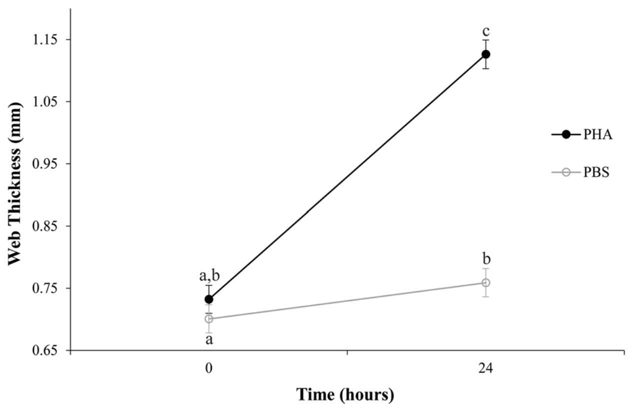

Fig. 2 The effect of PHA and PBS on Swelling [Web Thickness (in mm)] of juvenile American alligator toe webs before (0 h) and 24 h after injection. *Circles* are predicted means (±1 SE) of respective treatments (control, $n = 3$; LOW, $n = 4$; MED, $n = 7$; HIGH $n = 7$). Swelling associated with PHA injection is indicated with a *black line* and *black-shaded circles*, PBS with a *grey line* and *open grey circles*. Means with different *letters* are significantly different (5 % LSD)

There were no differences in Web Thickness before injection in either toe (PHA0 vs. PBS0; 5 % LSD).

Effects of Dietary Treatment on Immune Parameters

Dietary Treatment, however, had no effect ($p = 0.40$) on SwellingDiff 24 h after injection (Fig. 3). Mean SwellingDiff (±SE) after injection in control and those fed once, twice, or thrice weekly were 0.41 ± 0.04, 0.33 ± 0.12, 0.31 ± 0.04, and 0.48 ± 0.07 mm, respectively. BCI had no effect on SwellingDiff ($p = 0.621$).

Similar to PHA, Dietary Treatment had no effect on EcoliBKA ($p = 0.725$). The highest bactericidal capacity was observed in individuals fed CCWs three times per week (0.89 ± 0.03 %), whereas the lowest was observed in control individuals (0.75 ± 0.06 %; Fig. 4). BCI also had no effect on EcoliBKA ($p = 0.354$).

Dietary Treatment also had no effect on splenic mass ($p = 0.49$). Individuals fed CCWs three times per week displayed the greatest mass (0.97 ± 0.14 g). The lowest splenic mass was observed in individuals fed CCWs once per week (0.55 ± 0.06 g). BCI, however, had a significant positive effect on splenic mass ($p = 0.043$; 0.49 ± 0.22).

Plasma Biochemistry

Mean biochemistry and PCV values are listed in Table 1. There was no effect of Dietary Treatment on any biochemical parameter (AST $p = 0.81$; CK $p = 0.29$; Ca^{2+} $p = 0.101$; GLU $p = 0.68$; Ph $p = 0.55$; K^+ $p = 0.79$; Na^+ $p = 0.96$; and TP $p = 0.43$) or PCV ($p = 0.91$). AST ($p = 0.043$) and Ph ($p = 0.008$) were significantly affected by BCI, but no other biochemical parameters or PCV were affected. BCI was negatively related to AST levels (−1.35 ± 0.19), but it was positively related to Ph levels (1.75 ± 0.58).

Levels of BAs, UAs, and ALBs were lower than their respective LOD on the VS2 and thus could not be analyzed. Chemistry suppression due to interference within samples prevented obtainment of GLB levels (calculated from total ALBs and TP). Therefore, these (BAs, ALBs, UAs, and GLB) data are not shown.

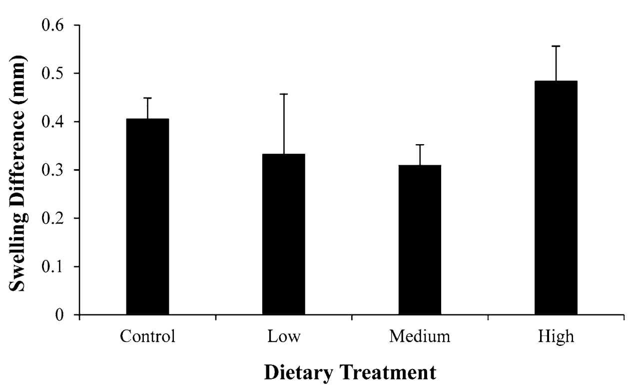

Fig. 3 The effect of Dietary Treatment with CCWs for 25 months on the difference in swelling 24 h after injection in American alligators. *Shaded bars* represent raw means (±1 SE) of swelling difference [PHA minus PBS (in mm)] 24 h after initial injection. The *x*-axis denotes Dietary Treatments based on the number of times each week the group was fed CCW-laden prey: *Control* 0 times, *LOW* 1 time, *MED* 2 times, and *HIGH* 3 times (control, $n = 3$; LOW, $n = 4$; MED, $n = 7$; HIGH, $n = 7$)

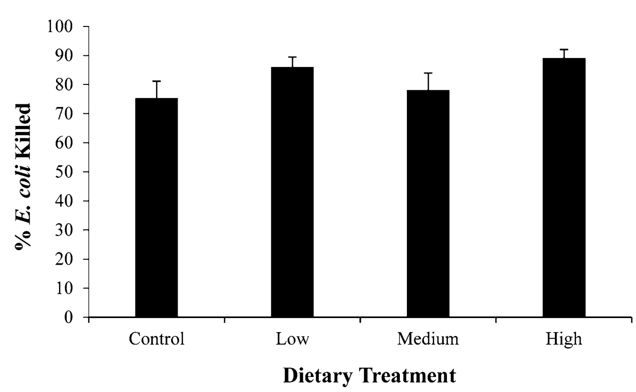

Fig. 4 The effect of Dietary Treatment with CCWs for 25 months on percent EcoliBKA in American alligators. *Shaded bars* represent means (±1 SE) of EcoliBKA. The *x*-axis denotes Dietary Treatments based on the number of times each week the group of alligators was fed CCW-laden prey: Control = 0 times, LOW = 1 time, MED = 2 times, and HIGH = 3 times (control, $n = 3$; LOW, $n = 4$; MED, $n = 7$; HIGH, $n = 7$)

Table 1 Biochemical and hematological parameters

Parameter	Unit	Mean	SE	Range	Previously reported mean[a]	Previously reported range[a]
Aspartate aminotransferase (AST)	(U/L)	267.05	12.60	205–401	221.30	140–314
Creatine kinase (CK)	(U/L)	915.25	328.12	147–6743	511[b]	177–3126
Glucose (GLU)	(mg/dL)	62.60	1.31	55–76	102.60	82–130
Phosphorous (Ph)	(mg/dL)	5.47	0.21	3.80–7.60	5.30	4.20–7.50
Calcium (Ca)	(mg/dL)	9.96	0.11	9.20–11	10.80	9.90–12
Potassium (K^+)	(mmol/L)	4.38	0.11	3.80–5.40	4.90	3.80–6.10
Total protein (TP)	(g/dL)	3.96	0.07	3.40–4.60	3.65[b]	2.90–4.40
Sodium (Na^+)	(mmol/L)	138.10	0.51	135–144	140.30	133–148
Hematocrit (PCV)	(%PCV)	22.05	2.42	2–39	16	13–21

Summary of plasma biochemical and hematological parameter from 21 alligators exposed to one of four Dietary Treatments for 25 months. Reported are overall mean (with EM) and range values for each parameter irrespective of Dietary Treatment. The units of each measured parameter are immediately to the right of each respective parameter. Values unable to be determined are not included. Our previously reported means and ranges of each parameter from healthy wild-caught American alligators are also provided (Hamilton et al. 2016a, b)

[a] Values from Hamilton et al. (2016a, b)

[b] Median values of nonnormally distributed data (see Hamilton et al. 2016a, b)

Simple Regression: Trace Elements Versus Immune Parameters

In our previous study, we observed that kidney and liver Se (liver 4.83–17.47 µg/g dry mass, kidney 6.56–28.61 µg/g dry mass, 25 month exposure animals only), As (liver 0.53–1.48 µg/g dry mass, kidney 0.71–2.35 µg/g dry mass, 25 month exposure animals only), and Cd concentrations (liver 0.05–0.72 µg/g dry mass, kidney 0.18–3.33 µg/g dry mass, 25 month exposure animals only) varied significantly among Dietary Treatment in a dose-dependent manner (Tuberville et al. 2016). Therefore, we performed simple regression, irrespective of Dietary Treatment, with the final concentrations of Se, As, and Cd in both the liver and kidney against the immune parameters SwellingDiff, EcoliBKA, and splenic mass.

SwellingDiff was not affected by Liver Se ($p = 0.098$), Liver As ($p = 0.148$), Liver Cd ($p = 0.215$), Kidney Se ($p = 0.388$), Kidney As ($p = 0.419$), or Kidney Cd ($p = 0.356$) concentrations. EcoliBKA was also not affected by Liver Se ($p = 0.594$), Liver As ($p = 0.822$), Liver Cd ($p = 0.07$), Kidney Se ($p = 0.290$), Kidney As ($p = 0.621$), or Kidney Cd ($p = 0.072$) concentrations. Splenic mass was similarly not affected by Liver Se ($p = 0.406$), Liver As ($p = 0.355$), Liver Cd ($p = 0.723$), Kidney Se ($p = 0.540$), Kidney As ($p = 0.254$), or Kidney Cd ($p = 0.825$) concentrations.

Discussion

In this study, alligators were chronically fed prey contaminated with CCWs for 25 months, thus accumulating significant trace-element concentrations that varied among Dietary Treatments (Tuberville et al. 2016). Because previous studies suggested that trace elements present in CCWs suppress immune function (e.g., Dangleben et al. 2013; Fairbrother and Fowles 1990), we hypothesized that chronic dietary exposure to CCW-contaminated prey would negatively affect the immune system of alligators. In contrast, however, our results suggest that neither chronic exposure to nor accumulation of CCWs detrimentally affects the immune system or health of juvenile American alligators, at least for the tissue concentrations reported here.

PHA

Injection with PHA (nonpathogenic, polyclonal antigenic lectin) has been used in a multitude of studies as a routine measure of an individual's ability to mount an immune response and thus trigger inflammation, which is manifested macroscopically by an increase in localized swelling after injection (usually 24 h after injection; Brown et al. 2011; Finger et al. 2013, 2015a). Our aim in this study was first to successfully implement PHA injection in American alligators and second to use PHA injection to evaluate the effect of CCWs on the innate, inflammatory potential of an individual. In the course of infection, pathogens are recognized and ingested by innate cells (i.e., phagocytes; Abbas et al. 2010). These innate cells, such as dendritic cells, subsequently present degraded pathogenic antigens to lymphocytes, the mediators of the adaptive immune system, which consequently facilitate a number of responses aimed at microbial elimination (Abbas et al. 2010). Decreased responsiveness would have obvious deleterious consequences to an individual and pose increase disease susceptibility. Therefore, an understanding of how accumulated trace elements present in CCWs affect the immune response is of the upmost importance.

Expectedly, at 24 h after injection, all individuals in our study (regardless of Dietary Treatment) exhibited a significantly increased swelling response after PHA injection relative to PBS injection. This is a finding we have previously observed in saltwater crocodiles (Finger et al. 2013, 2015a). Furthermore, this indicates successful implementation of the technique for the first time in American alligators.

Although we originally hypothesized that CCW ingestion and accumulation would suppress immune function, we observed no effect of Dietary Treatment or trace-element (As, Se, Cd) concentrations on the difference in swelling (PHA–PBS) 24 h (i.e., SwellingDiff) after injection. These results suggest that chronic exposure to low levels CCWs may not affect the ability of juvenile alligators to mount an immune response to novel antigens. Similarly, Fairbrother and Fowles (1990) observed no effect of Se administration (neither selenomethionine nor sodium selenite) on PHA-induced swelling in mallards (*Anas platyrhynchos*) relative to controls even though birds accumulated significant amounts of Se. Primary PHA injections, as were performed in this study, measure the innate, inflammatory potential of an individual, whereas subsequent injections may provide insight into specific, acquired immunity to repeated exposure to the same antigen (Brown et al. 2011; Finger et al. 2013). In contrast to the PHA results, mallards exhibited a significantly decreased delayed type hypersensitivity (DTH) reaction to tuberculin after selenomethionine administration (Fairbrother and Fowles 1990). Similar to subsequent (i.e., more than primary) PHA injections, DTH reactions investigate acquired immunity to previously encountered pathogens. It may be that elements present in CCWs affect the two arms of the immune system (innate vs. acquired) disparately or that a particular immune parameter is more or less sensitive to accumulation of a particular element. For example, the PHA response of wild northern common eiders (*Somateria mollissima borealis*) was significantly and positively related to hepatic Se concentrations, whereas there was no relationship between Cd concentrations and PHA response (Wayland et al. 2002).

BKAs

The BKA measures constitutive immune mediators present in the plasma including antimicrobial proteins and peptides, antibodies, and complement proteins (Demas et al. 2011; Finger et al. 2015a), and killing ability is related to the decrease in the number of colonies after incubation with an individual's plasma. Because stressors that induce perturbations in constitutive innate immune mediators may be important in affecting pathogen infection and disease susceptibility, we used BKAs as another measure to evaluate the effects of CCW ingestion and accumulation on the immune system.

Similar to PHA, bactericidal capacity (i.e., EcoliBKA) was also unaffected by Dietary Treatment or trace-element concentrations in liver and kidney, thus suggesting that chronic ingestion of CCWs may not affect constitutive innate defenses present in the plasma of juvenile American alligators. Complement proteins (CPs), which are an essential component in innate defense against microbes present in plasma (Finger and Isberg 2012), are primarily produced by hepatocytes; however, other types of cells—such as fibroblasts and epithelial cells (Morgan and Gasque 1997; Qin and Gao 2006)—may also produce CPs. Alligators fed CCW-contaminated prey accumulated substantial amounts of trace elements in liver. Therefore, a logical postulation is that accumulation (in the liver) may perturb CP production and consequently affect plasma bactericidal capabilities. However, this was not observed in our study.

Splenic Mass

The spleen is a peripheral immune organ that aids in blood filtration, pathogen-immune interactions, and removal of senescent erythrocytes (Abbas et al. 2010; Finger and Isberg 2012). In fact, because crocodilians apparently do not possess lymph nodes or germinal centers (Finger and Isberg 2012), the importance of the spleen in pathogenic defense may be paramount (Rooney et al. 2003). As a routine immunotoxicological test, the spleen was weighed to examine how CCW exposure may affect splenic mass and consequently its function. For example, changes in organ mass, such as those that may manifest as organ hypertrophy or atrophy, may reflect toxicity (see Sellers et al. 2007). In particular, atrophy of the spleen is associated with decreased immune function in a number of reptiles (Rooney et al. 2003 and references therein). Therefore, a decrease in splenic mass due to CCW exposure may manifest as decreased immune function and increased disease susceptibility.

No effect of Dietary Treatment was observed on splenic mass. Similarly, liver and kidney As, Cd, and Se concentrations also had no effect on splenic mass. However, although these results suggest that chronic ingestion and accumulation of CCWs does not affect juvenile alligator splenic mass, histological examination after administration of CCWs would provide further evidence of the impact (or lack thereof) of CCWs on splenic function (Haley et al. 2005; Sellers et al. 2007). Analogous to the PHA and BKA findings, the null effect of Dietary Treatment on splenic mass of juvenile alligators provides further evidence that ingestion and accumulation of CCWs may not affect immune function.

Similar to our results, Fairbrother and Fowles (1990) found that Se treatment had no effect on splenic mass in mallards. Ingestion of pellets containing high (300 μg/g) or low (50 μg/g) levels of Cd for 4.5 months also had no effect on splenic mass in Pekin ducks (*A. pekin*; Hughes et al. 2000). Neither hepatic Se nor renal Cd concentrations had an effect on spleen mass in wild eider ducks (Wayland et al. 2002). Administration of 10 ppm sodium arsenite to wild cotton rats (*Sigmodon hispidus*) for 6 weeks also had no effect on spleen mass (Savabieasfahani et al. 1998).

BCI had a significant positive effect on splenic mass indicating that juvenile alligators of higher body condition have a larger spleen. As already mentioned, the spleen likely plays an important immune and hematological role in alligators (Rooney et al. 2003). Therefore, healthier alligators (as measured by BCI) would be expected to have a larger spleen. Future research is necessary to understand how body condition may impact splenic mass, and subsequently alligator health.

Plasma and Blood Parameters

Blood biochemical and hematological analytes were measured to better understand how CCW exposure may affect alligator health (Table 1). In this study, we found no effect of Dietary Treatment on any plasma analyte or on PCV indicating that ingestion and accumulation of CCWs has no effect on plasma biochemical or hematological parameters in juvenile American alligators.

We recently published preliminary reference intervals for wild alligators captured on the Savannah River Site that were analyzed using the same avian/reptile rotor used in this study (Hamilton et al. 2016b). In general, the values presented in this study are similar to those reported by Hamilton et al. (2016b; see Table 1). That our values for experimentally exposed animals fell within the range of values reported for wild-caught, healthy juvenile alligators provides additional support that exposure to low levels of CCWs through dietary ingestion may not affect juvenile alligator health. However, mean CK levels of alligators in this study were almost 2 times greater than those of wild alligators, and GLU levels were half as high as those observed in wild alligators (Hamilton et al. 2016b). Because no effect of Dietary Treatment was observed on both CK and GLU levels, this suggests that other factors may have influenced these values. Indeed, many variables can affect biochemical values including animal size, sample-collection protocol, habitat, type of prey ingested, season, or reproductive status (Guillette et al. 1997; Hamilton et al. 2016b; Lance et al. 1983; Lance and Elsey 1999; Lovely et al. 2012; Thrall et al. 2012; Zayas et al. 2011). Higher levels of CK could be indicative of muscle damage through increased exertion associated with capture, whereas the lower levels of GLU observed may be related to nutritional status (i.e., time since last meal) of individuals (Thrall et al. 2012). Similar to Hamilton et al. (2016b), we observed a negative effect of BCI on AST. The reason why the negative effect was more pronounced in our previous study may be related to size of the animals, environmental conditions (i.e., captive vs. wild-caught), or some other unknown factor (Guillette et al. 1997; Hamilton et al. 2016b; Lance et al. 1983; Lance and Elsey 1999; Lovely et al. 2012; Thrall et al. 2012; Zayas et al. 2011). In contrast, to Hamilton et al. (2016b), we observed a positive effect of BCI on Ph. Ph levels are often an indicator of renal function, but Ph is also important in energy, protein, and nucleic acid synthesis (Thrall et al. 2012). Therefore, greater levels of Ph likely promote improved body condition in alligators.

Interpretational Caveats

Whilst our results suggest that chronic ingestion of prey contaminated with low doses of CCWs may not detrimentally affect the health or the immune system of juvenile American alligators, our observations may have been affected by other unforeseen or unaccounted for factors. First, because alligators are long-lived and slow maturing reptiles possibly living up to approximately 50 years (see Woodward et al. 1995), the exposure period of 25 months may not have been long enough for detrimental immune effects to manifest. Juvenile alligators may be less likely to exhibit or experience immune perturbations than adult alligators, which feed at a higher trophic level and have potentially longer exposure periods (Finger and Gogal 2013; Milnes and Guillette 2008). Second, although alligators accumulated significant amounts of CCW trace elements throughout the course of the study (see Tuberville et al. 2016), the dosage accumulated may not have been high enough to manifest in immunotoxicity (i.e., the dose dictates the toxicity). In fact, some of the trace elements present in CCWs may display immunological hormesis (e.g., see Fairbrother et al. 1994; Wayland et al. 2002), whereby lower concentrations enhance a particular immune parameter (see PHA section in preceding text). Moreover, reptiles in particular may be less susceptible to metal-induced toxicity and subsequent aberrations (e.g., Hopkins et al. 2002; Tuberville et al. 2016; Warner et al. 2016). However, this has yet to be fully investigated in crocodilians, requiring future study. Third, because alligators were housed communally within respective tanks and crocodilians establish hierarchies at a young age, this may have prevented equal proportioning of food between each individual (see Tuberville et al. 2016). This in turn could have affected trace-element accumulation and consequently immune/health parameters. Due to this, future studies should administer alligators individually to ensure that all subjects receive an equivalent dose. In addition, the disproportionate number of females relative to males may have influenced the results because females and males may display sex-specific differences in response to CCW exposure (e.g., Hopkins et al. 2002). Alligators, like all crocodilians, exhibit temperature-dependent sex determination, and temperature for alligators while in ovo in this study ranged from 29 to 31.8 °C, temperatures at which females usually predominate (Lang and Andrews 1994). Therefore, this likely accounts for the disproportionate number of females after random sampling of individuals at RWR. Last, the immune techniques implemented in this study may not have been sensitive enough to accurately reflect the impacts of CCW accumulation on an individual's immune function (see Finger et al. 2013, 2015a). Because many hematological, biochemical, and immunological parameters or assays may be affected by a host of extrinsic variables (Franklin et al. 2003; Finger and Isberg 2012; Glassman et al. 1979; Sykes and Klaphake 2008), we attempted to mitigate this by standardizing ambient conditions, sampling individuals of the same size, and obtaining blood samples rapidly after capture to prevent corticosterone-induced perturbations in hematological or immunological parameters (Romero and Reed 2005).

Conclusions

In conjunction with the study by Tuberville et al. (2016), this is one of the first studies to investigate how trace elements present in CCWs may affect the American alligator. Moreover, this is the first study to measure the effect of chronic CCW exposure on the immune system of a long-lived, top-trophic carnivore. Last, to our knowledge, this is also the first study to use commonly implemented (in other species) ecoimmunology techniques to evaluate immunotoxicity in a crocodilian. Therefore, species-specific comparisons with other studies are not possible (see preceding text) and require future study.

Our results suggest that chronic ingestion of CCW-contaminated prey, as well as subsequent accumulation of trace elements present in CCWs, does not detrimentally affect the immune system or the health of alligators. Future studies should be undertaken with the implementation of novel ecoimmunological techniques not yet implemented in crocodilians (see Demas et al. 2011; Finger et al. 2013, 2015a) along with more sensitive assays (e.g., Finger et al. 2015b) and repeated samplings to better understand the effects of CCW accumulation.

Acknowledgments Support was provided in part by Award Number DE-FC09-07SR22506 from Department of Energy to the University of Georgia Research Foundation. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia (Approval No. A2010 11-561-Y3-A3). J. W. F. was funded by the Interdisciplinary Toxicology Program and the Department of Environmental Health Science at the University of Georgia. MTH and all sample analysis was funded by a grant from the Area Closures Project to T. D. T. We thank David E. Scott and Stacey L. Lance for their assistance with project design and dissections. Brett DeGregorio was instrumental in setting up the experimental tanks; Caitlin Kupar assisted with the collection of prey, and Matthew Atkinson helped conduct PHA assays on alligators. We would also like to thank John Seaman at SREL for guidance on tissue digestions and data interpretation. Thanks must also be extended to Suresh Benedict at Berrimah Veterinary Laboratories in Berrimah, NT, Australia, for advisement on BKAs and to Peter C. Thomson at the University of Sydney for statistical insight.

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