

Exposure to residual concentrations of elements from a remediated coal fly ash spill does not adversely influence stress and immune responses of nestling tree swallows

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Anthropogenic activities often produce pollutants that can affect the physiology, growth and reproductive success of wildlife. Many metals and trace elements play important roles in physiological processes, and exposure to even moderately elevated concentrations of essential and non-essential elements could have subtle effects on physiology, particularly during development. We examined the effects of exposure to a number of elements from a coal fly ash spill that occurred in December 2008 and has since been remediated on the stress and immune responses of nestling tree swallows. We found that nestlings at the site of the spill had significantly greater blood concentrations of Cu, Hg, Se and Zn in 2011, but greater concentrations only of Se in 2012, in comparison to reference colonies. The concentrations of elements were below levels of significant toxicological concern in both years. In 2011, we found no relationship between exposure to elements associated with the spill and basal or stress-induced corticosterone concentrations in nestlings. In 2012, we found that Se exposure was not associated with cell-mediated immunity based on the response to phytohaemagglutinin injection. However, the bactericidal capacity of nestling plasma had a positive but weak association with blood Se concentrations, and this association was stronger at the spill site. Our results indicate that exposure to these low concentrations of elements had few effects on nestling endocrine and immune physiology. The long-term health consequences of low-level exposure to elements and of exposure to greater element concentrations in avian species require additional study.

Key words: bactericidal capacity, cell-mediated immunity, element, stress response, tree swallow

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Introduction

Humans are rapidly altering the environment and while these changes may threaten the persistence of species and populations (Vitousek *et al.*, 1997), they may also have subtle,

non-lethal effects on individuals. Exposure to anthropogenic pollutants can affect physiology (Acevedo-Whitehouse and Duffus, 2009; Martin *et al.*, 2010), compromise reproductive performance (Heinz, 1996; Baos *et al.*, 2012) and affect development of vertebrates (Markman *et al.*, 2011). Elements,

including heavy metals, metalloids and trace elements, are one form of pollution that wildlife are exposed to through a number of anthropogenic processes, such as intensive agriculture (Ohlendorf *et al.*, 1986; Orłowski *et al.*, 2010), mining (Weech *et al.*, 2012), coal combustion (Rowe *et al.*, 2002) and metal smelting (Janssens *et al.*, 2001). Some of these elements are of known toxicological importance, such as arsenic (As), lead (Pb) and mercury (Hg), as well as elements that are nutritionally important but become toxic at elevated concentrations, such as copper (Cu), iron (Fe), selenium (Se) and zinc (Zn). At optimal dietary concentrations, the latter elements affect a variety of physiological processes, acting as enzyme cofactors and antioxidants and enhancing the immune response (Reilly, 2006; Wintergerst *et al.*, 2007). However, at higher concentrations they can cause oxidative stress (Janz *et al.*, 2010; Koivula and Eeva, 2010), increase susceptibility to infection (Sherman, 1992; Wintergerst *et al.*, 2007) and inhibit reproductive performance (Baos *et al.*, 2012). Given the important physiological functions of elements, individuals may be more sensitive to element contamination during early development, when exposure could permanently alter some physiological processes (Nyholm, 1998; Baos *et al.*, 2006a). Thus, exposure to even moderately elevated concentrations of some elements during development may affect physiological processes, such as stress and immune responses, which are directly relevant to survival and reproduction.

The stress response is one aspect of physiology that may be affected by exposure to elements. This response is regulated by the hypothalamic–pituitary–adrenal axis (HPA axis), which controls the release of glucocorticoids in vertebrates (Wingfield and Romero, 2001). Basal glucocorticoid concentrations are responsible for regulating energy balance, blood glucose and fatty acid levels (reviewed by Landys *et al.*, 2006), while stress-induced glucocorticoids are released in response to unexpected challenges and cause changes in behaviour and physiology that enhance the probability of survival (reviewed by Wingfield *et al.*, 1998; Sapolsky *et al.*, 2000). Exposure to elements can potentially affect the regulation of glucocorticoids via an array of mechanisms acting on different levels of the HPA axis. For example, release of glucocorticoids can be affected by altering the release of, or response to, corticotrophin-releasing hormone or adrenocorticotrophic hormone from the hypothalamus and pituitary, respectively (Potmis *et al.*, 1993; Handy, 2003; Gagnon *et al.*, 2006). Elements such as Cu, Fe, Mn and Zn are also important components of many enzymes (Marmioli and Maestri, 2008), and increases in their concentrations above normal dietary levels could potentially affect enzymes associated with glucocorticoid metabolism (Hopkins *et al.*, 1997).

Given that elements have the potential to influence the production, release and clearance of glucocorticoids, it is not surprising that studies examining the effects of elements on basal and stress-induced glucocorticoid concentrations have produced mixed results. For example, in some avian studies, Hg exposure may reduce (Franceschini *et al.*, 2009; Herring *et al.*, 2012) or elevate basal glucocorticoid concentrations

(Wada *et al.*, 2009). Basal glucocorticoid concentrations have not been significantly associated with mixtures of elements, including cadmium (Cd), Hg and Se [common eiders (*Somateria mollissima*), Wayland *et al.*, 2003], As, Cd, Cu, Pb and Zn [white storks (*Ciconia ciconia*), Baos *et al.*, 2006a], or As, Cu, nickel (Ni), Pb and Zn [pied flycatchers (*Ficedula hypoleuca*), Eeva *et al.*, 2005], but Cd, Se and Hg may influence basal glucocorticoids in interactions with other elements or body condition [lesser scaup (*Aythya affinis*), Pollock and Machin, 2009]. Effects on induced glucocorticoid concentrations produced using standardized handling restraint are also variable. Exposure to elements including Pb and Cd can enhance the stress-induced release of glucocorticoids (Wayland *et al.*, 2002; Baos *et al.*, 2006a), while exposure to Hg and Se can inhibit it (Wayland *et al.*, 2002; Wada *et al.*, 2009). In these same or very similar studies, other elements, including As, Cd, Hg, Se and Zn, appeared to have no effect on stress-induced glucocorticoids (Wayland *et al.*, 2002, 2003; Baos *et al.*, 2006a). Given that animals may be exposed to a variety of elements that may interact in complex ways (Marmioli and Maestri, 2008; Zwolak and Zaporowska, 2012), it is important to understand how elements commonly found in the environment influence the HPA axis as well as other physiological processes.

Exposure to elements can also affect immune responses and disease resistance. Many immune responses are affected by nutritional condition (Alonso-Alvarez and Tella, 2001; Lifjeld *et al.*, 2002; Ponton *et al.*, 2013), and exposure to high concentrations of elements may reduce an individual's nutritional condition and hence their immune response (Ritchie *et al.*, 1994; Massányi *et al.*, 1999). Element exposure may directly affect the immune system by impairing immune cell function, altering protein synthesis, or through cytotoxic effects on immune organs (Koller, 1973; Blakley *et al.*, 1980; Dan *et al.*, 2000). Exposure to elements such as Pb increases disease prevalence in house sparrows (*Passer domesticus*, Bichet *et al.*, 2013), providing indirect evidence that element exposure impairs immunity. Exposure to elevated concentrations of Pb or a mixture of elements including As, Cd, Cu, Pb, Hg, Se and Zn was associated with reduced humoral immunity [zebra finch (*Taeniopygia guttata*) and great tit (*Parus major*), Snoeijs *et al.*, 2004, 2005], while cell-mediated immunity can be impaired by exposure to Hg in tree swallows (*Tachycineta bicolor*, Hawley *et al.*, 2009). However, other studies have detected no effect of these same elements on humoral (Wayland *et al.*, 2003; Biser *et al.*, 2004; Hawley *et al.*, 2009) or cell-mediated immunity (Snoeijs *et al.*, 2005; Baos *et al.*, 2006b), and a few studies have even detected stimulatory effects of Se exposure on these immune responses (Wayland *et al.*, 2002; Surai, 2006; Brady *et al.*, 2013). Robust innate and cell-mediated immune responses require adequate dietary concentrations of Cu, Fe, Se and Zn (reviewed by Maggini *et al.*, 2007; Wintergerst *et al.*, 2007), and it is possible that moderate increases in these elements, below levels associated with toxic effects, are responsible for the enhanced immune responses detected in some studies.

We examined the effects of environmental exposure to a mixture of elements from a recently remediated coal fly ash spill on the stress and immune responses of nestling tree swallows. Coal fly ash is one of the largest solid waste streams produced globally and represents a significant source of an array of elements to aquatic systems (Rowe *et al.*, 2002; NRC, 2006). Fly ash spills, such as the one that took place at our study site, represent extreme circumstances, but aquatic disposal of fly ash continually introduces elements into streams and rivers around the world. Fly ash contains elevated concentrations of several elements including As, Hg, Se, V and others that pose health risks to humans and wildlife (Rowe *et al.*, 2002; NRC, 2006). At the time of our study, remediation efforts at our site were largely completed and the concentrations of elements in this system were below levels associated with negative effects on reproduction or survival in many avian species (Ohlendorf, 2003; Dauwe *et al.*, 2005; Brasso and Cristol, 2008). However, we hypothesized that exposure during development to low concentrations of elements associated with coal fly ash would affect avian physiology. In light of the discrepancies amongst study systems highlighted above, we focused on the relationship between element exposure and the HPA axis and immune responses in an effort to contribute to this growing body of literature.

Methods

Study species

Tree swallows are one of the primary model species used to address the movement of contaminants from aquatic to terrestrial ecosystems (Custer, 2011) and are used extensively in field studies of physiology, life history and behaviour (Robertson *et al.*, 2011). Tree swallows are aerial insectivores and, when breeding in riparian areas, feed primarily on emerging aquatic insects (Custer *et al.*, 2010; Custer, 2011; Beck *et al.*, 2013). A few studies have examined the effects of one element, Hg, on the stress and immune responses of tree swallows. Some of these studies have detected reduced basal corticosterone concentrations with greater Hg exposure (Franceschini *et al.*, 2009) and others increased basal but reduced stress-induced corticosterone concentrations with greater Hg exposure (Wada *et al.*, 2009). The cell-mediated immune response was negatively affected by exposure to Hg, but Hg exposure did not affect the humoral immune response (Hawley *et al.*, 2009). Although these studies demonstrated that Hg exposure related to aspects of tree swallow physiology, no studies have examined the effects of many of the elements found in fly ash on the immune or stress responses of tree swallows.

Study site

In December 2008, a coal fly ash impoundment at the Tennessee Valley Authority fossil plant in Kingston, TN, USA (35.8722°N, 84.5250°W) ruptured, releasing 4.1 million m³ of coal fly ash slurry into the Emory River, which then flowed into the Clinch and Tennessee Rivers (TVA, 2009). In the 2.5 years following the spill, most of the coal fly ash was

removed from the river system but ~400 000 m³ remained at the time of our study (TVA, 2011a).

We studied tree swallows along an element contamination gradient and at several reference colonies in Roane and Loudon Counties, TN, USA, from May to July 2011 and 2012 (Fig. 1). We placed nest boxes at the spill site (SS)

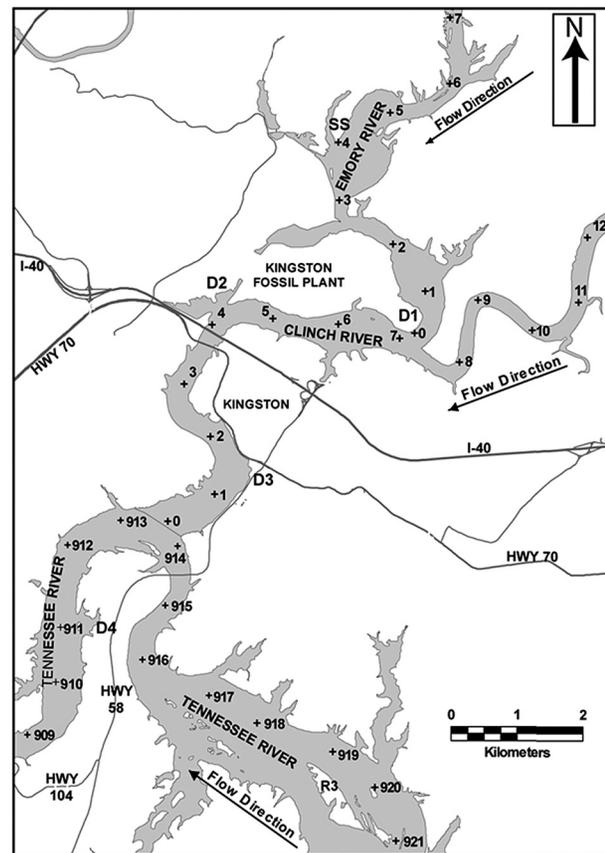


Figure 1: Tree swallow colonies located near Kingston, TN, USA. The study area consisted of two highly impacted colonies located on the Emory River. One was located at the site of the spill (spill site, SS, $n = 94$) and the second at the confluence of the Clinch and Emory River (downstream 1, D1, $n = 31$) 4 km downstream from the spill. Two moderately impacted colonies were located on the Clinch River at downstream 2 (D2, $n = 31$) and downstream 3 (D3, $n = 43$) and were 3.0 and 7.0 km, respectively, downstream from the confluence with the Clinch River. A low-impacted colony was located downstream on the Tennessee River (D4, $n = 51$) 2.5 km from the confluence with the Clinch and Tennessee Rivers. We used three reference colonies; two were located near Lenoir City, TN, USA 30.5 km east of Kingston. Reference 1 (R1, $n = 46$) was located at Ft Loudoun Dam on the Tennessee River and reference 2 (R2, $n = 53$) at Tellico Dam on the Little Tennessee River. Reference 3 was located on Long Island (R3, $n = 53$) on the Tennessee River 5.5 km upstream from the confluence with the Clinch River. We also placed boxes at Melton Hill Dam (MD, $n = 68$) on the Clinch River, which served a role analogous to a positive control. The sites MD, R1 and R2 are not pictured here. n refers to the number of nest boxes located at each colony. River kilometres are given in each river.

and at four colonies located downstream from the spill site (hereafter 'downstream', D1–D4). Colony D1 was located ~3.5 km by river from the spill site, while D4 was located ~14 km by river from the spill site. We had three reference colonies; two located ~30.5 km east of Kingston at Ft Loudoun Dam (Reference 1) and at Tellico Dam (Reference 2), while Reference 3 (R3) was located on the Tennessee River ~6 km by river upstream from the confluence with the Clinch River, at Long Island. We also placed boxes at Melton Hill Dam (MD) on the Clinch River, which served a role analogous to a positive control because preliminary data gathered prior to this study indicated that tree swallows are exposed to ash-related contaminants such as Se at this colony (ARCADIS, 2011). The source(s) of this contamination is unclear, but could include the Bull Run Fossil Plant (Stantec, 2009; TVA, 2011b), a former coal ash storage pond associated with the Y-12 Security Complex (Cook *et al.*, 1999), or other non-point source pollution (USDA, 2009). We grouped these colonies into four types, the spill site, all downstream colonies (D1–D4), all reference colonies (R1–R3) and Melton Hill (MD), based on how they were impacted by the fly ash spill. By sampling nestlings from these colonies, we were able to examine the physiological responses of nestlings across a range of low to moderately elevated element concentrations.

We placed clean nest boxes in each area when tree swallows were arriving at the breeding grounds and prospecting for nest sites. All of the colonies were established at least 1 year prior to this study except for D1, which we established in 2011. Nest boxes were 25 cm × 20 cm × 41 cm with a 2.5 cm entrance hole and were mounted 1.5 m above the ground on metal conduit. All entrances were oriented toward the water, and boxes were located within 70 m of the shore to facilitate foraging on emerging aquatic insects. We spaced nest boxes 15 m apart when in a single row, or 20 m apart with a staggered alignment of two or more rows. We checked nest boxes every 4 days, beginning in late March, for signs of nesting activity and to obtain basic reproductive data. When nestlings were 13 days old, we banded them and obtained blood samples for the corticosterone assay, immune challenge or element analysis (see below). For each nestling, we measured the length of the left and right tarsus (each tarsus was measured twice) and body mass.

Response to handling stress

In 2011, we examined the effect of element exposure on the stress response of 13-day-old nestlings by subjecting them to a standardized handling stress protocol (after Wingfield and Romero, 2001). We obtained a blood sample (~60 µl) from up to half of the nestlings in a brood within 3 min of disturbing the box. These nestlings were then held alone in a cloth bag for 30 min, after which a second blood sample was obtained. Samples were stored in a cooler containing ice blocks before being centrifuged for 5 min at 9783 g, and the plasma fraction was removed and stored at –20°C in the field-house and stored long-term at –80°C.

In the autumn of 2011, we randomly selected plasma from one nestling from each nest that we subjected to the handling stress protocol and quantified basal and induced corticosterone concentrations in 125 nestlings across all colonies. By using a single sample per nest, we avoided issues with pseudoreplication. We used Enzo Life Sciences enzyme immunoassay kits (catalogue no. 901-097) using a procedure previously validated for tree swallows by Wada *et al.* (2009). We haphazardly distributed samples from different colonies equally across 10 96-well plates. We diluted 12 µl of plasma with an equal volume of 3% steroid displacement buffer and then diluted samples 1:20 with assay buffer. On each plate, a standard curve that ranged from 15.6 to 2000 pg/ml was run in triplicate. A 500 pg/ml corticosterone standard was also run in triplicate on each plate, and each plasma sample was run in duplicate. The assay had a detection limit of 1.1 ng/ml, and any samples ($n = 49$) that fell below this were assigned half of the detection limit for their corticosterone concentration. Samples that fell below the detection limit were equally distributed among Melton Hill, the spill site, downstream and reference colonies ($\chi^2 = 3.04$, d.f. = 3, $P = 0.385$) and assays ($\chi^2 = 8.55$, d.f. = 7, $P = 0.287$). Three nestlings had induced corticosterone concentrations that were below the detection limit, and we ran statistical tests including and excluding these samples. We calculated intra-assay variation as the average coefficient of variation between duplicate samples on each plate and inter-assay variation as the coefficient of variation among the standards on every plate. Intra-assay variation was 11.2% and inter-assay variation 13.3%.

Immune response

We examined the effects of elements on aspects of the immune response in nestling tree swallows in June and July 2012. We randomly selected a single nestling from each nest to avoid issues with pseudoreplication. We quantified the response of 37 13- to 14-day-old tree swallow nestlings to phytohaemagglutinin (PHA; Sigma Aldrich, St Louis, MO, USA) by injecting the patagium (wing web) of nestlings with 0.15 mg of PHA dissolved in 30 µl of phosphate-buffered saline (PBS; after Smits *et al.*, 1999). The injection of PHA leads to a localized swelling due to the influx and proliferation of T cells and leukocytes at the injection site (Martin *et al.*, 2006) and a build-up of free radicals that are produced during phagocytosis by components of the innate immune system (Peretz, 1989). Feathers were first cleared from the wing web, and the area was sterilized using 70% ethanol. One individual held the nestling with its right wing extended in a standardized position, while a second individual (J.J.H.) made all measurements and performed the injections. Prior to and 24 h following injection, the thickness of the wing web was quantified to the nearest 0.01 mm using a micrometer. To avoid bias, the micrometer dial was not visible to the measurer while making the measurement. We made five pre- and five post-injection measurements at the injection point, discarded the lowest and highest values in each set and used the remaining three values to produce average pre- and

post-injection thicknesses. We divided the difference between the post- and pre-injection measurements by the pre-injection measurement and multiplied this value by 100 to calculate the percentage increase in swelling caused by the injection and used this as our measure of the cell-mediated immune response.

We examined innate immunity in nestlings by evaluating the bactericidal capacity of plasma (Liebl and Martin, 2009). Blood samples were obtained from 96 nestlings, one from each nest, 13 days post-hatch. The area around the puncture site was cleansed with 70% ethanol prior to blood collection from the brachial vein. We collected 120 μl of blood in heparinized capillary tubes; 60 μl was reserved for element analysis and the other 60 μl used to assess bactericidal capacity. Samples were stored in coolers with ice packs in the field (≤ 4 h) and were refrigerated prior to being centrifuged (≤ 2 h) at 9783 g for 5 min for the bactericidal assay. The plasma fraction was placed in a sterile 0.5 ml tube and refrigerated until all samples gathered that day were centrifuged. All samples were run on the day of collection in order to minimize degradation. The majority of samples were run in triplicate and occasionally duplicate ($n = 2$) by diluting 3.5 μl of plasma with 31.5 μl of sterile PBS (1:10 dilution). We added 12.5 μl of 10^5 bacteria/ml *Escherichia coli* solution (ATCC 8739, Epower microorganisms; Microbiologics®, St Cloud, MN, USA) to each tube and vortexed each sample. Samples were incubated at 37°C for 30 min, then 250 μl of tryptic soy broth (TSB; Sigma Aldrich) was added to each tube, and samples were incubated for an additional 12 h at 37°C. Positive controls were prepared in triplicate by adding 12.5 μl of 10^5 bacteria/ml *E. coli* solution to 250 μl of TSB, and we prepared duplicate blanks by combining 50 μl of PBS with 250 μl of TSB. We prepared an additional control in duplicate that contained 3.5 μl of plasma, 250 μl of TSB and 50 μl of PBS to check that bacteria were not introduced during bleeding or sample processing.

Following the 12 h incubation, samples were vortexed, and a Nanodrop Spectrophotometer (ND-2000; Thermo Scientific, Pittsburgh, PA, USA) was used to measure the absorbance of each sample at an optical density of 300 nm (Liebl and Martin, 2009). The absorbance of each sample and the positive controls were each averaged and used to calculate the proportion of bacteria killed as one minus (average sample absorbance/average positive control absorbance). The Nanodrop arm was cleansed between each sample with 70% ethanol, and the entire work area was cleansed with ethanol before and after each work day.

Analyses of elements

Blood samples from nestlings were shipped overnight on dry ice to the Trace Element Analysis Core at Dartmouth College (Hanover, NH, USA). Concentrations of As, Ba, Cd, Cr, Cu, Fe, Mn, Hg, Se, Sr, Tl, V and Zn present in blood were quantified for each sample using inductively coupled mass spectrometry following EPA method 6020A (EPA, 2008). Samples were digested using an open vessel acid digestion with 0.5 ml

of 9:1 HNO_3 :HCl (Optima, Fisher Scientific, St Louis, MO, USA) using microwave heating at 105°C for 45 min. After cooling, 0.1 ml H_2O_2 was added to the samples and they were taken through a second heating step (adapted from EPA, 1996). The samples were then diluted to 10 ml with deionized water. Digested samples were analysed for element concentrations by collision cell inductively coupled mass spectrometry (7700x; Agilent, Santa Clara, CA, USA). Concentrations of As, Ba, Cd, Cr, Cu, Fe, Mn, Sr, Tl, V and Zn (He collision mode), Se (reaction mode) and Hg (normal mode) were quantified in each sample. Digestion quality control measures included digestion blanks, fortified blanks and reference materials at a frequency of one each per 20 samples. There was insufficient blood to allow for digestion of duplicates or spikes. Analytical sample duplicates and spikes were performed at a frequency of one each per 20 samples. Additional quality control consisted of reporting limit checks, interference checks and initial and continuing calibration checks and blanks.

Arsenic, Cd, Cr, Tl and V concentrations were below detection limits (BDL) in over half of the nestling blood samples from all colonies in both years and were not considered further (Table 1). In 2011, Mn concentrations were BDL and in 2012, Hg concentrations were BDL in over half of the samples from each colony and were excluded from analyses in those years. The average relative percentage difference for eight elements over five analysis duplicates was $12 \pm 2\%$. The average percentage recovery for 13 elements over five analysis spiked samples was $97 \pm 21\%$. The average percentage recovery for As, Cd, Cu, Fe, Mn, Hg, Se, Sr and Zn was $100 \pm 13\%$ for five separate digestions of the standard reference material NIST 2976. Digestion blanks were less than reporting limits and fortified spike recoveries were generally 90–110% throughout the digestion batches. Other elements were not certified in the NIST standard.

Statistical analysis

We used Kolmogorov–Smirnov tests and normality plots to determine whether variables met the assumptions of parametric tests. Element concentrations and basal and induced corticosterone concentrations were not normally distributed and were log transformed prior to analysis, which successfully normalized the data. We first compared element concentrations among colony types (reference, spill site, downstream and Melton Hill) using a MANOVA followed by univariate ANOVAs and Tukey's tests to determine which elements were significantly elevated in the system due to the fly ash spill. Only elements that were found at the spill site at significantly higher concentrations than all reference colonies were included in the analysis that examined the effects of element exposure on the stress and immune responses of nestlings. Given that Hg concentrations were BDL in 2012 and Mn concentrations were BDL in 2011, we made these comparisons separately for each year. We calculated body condition for nestlings as the residuals of a regression of mass on tarsus length ($r^2 = 0.243$, d.f. = 124, $P < 0.001$). While the use of

Table 1: Mean values and standard errors for blood element concentrations (in micrograms per gram wet mass) in nestling tree swallows among colonies in 2011 and 2012

Element	Reference	Melton Hill	Spill site	Downstream	F	P value	Average detection limit
Ba 2011	0.74 ± 0.07	0.94 ± 0.09	0.94 ± 0.08	0.79 ± 0.05	2.15	0.10	0.049
2012	0.90 ± 0.08	0.61 ± 0.09	0.65 ± 0.08	0.69 ± 0.07	0.98	0.41	0.015
Cu 2011	0.28 ± 0.01	0.31 ± 0.02	0.35 ± 0.01	0.29 ± 0.01	6.20	0.001	0.080
2012	0.42 ± 0.07	0.32 ± 0.07	0.29 ± 0.07	0.39 ± 0.05	2.10	0.11	0.044
Fe 2011	367.7 ± 11.9	380.2 ± 14.6	393.5 ± 13.3	349.0 ± 9.1	2.99	0.03	8.22
2012	481.3 ± 34.7	405.2 ± 38.3	353.4 ± 33.9	379.7 ± 28.3	2.69	0.05	1.47
Mn 2011	BDL	BDL	BDL	BDL	NA	NA	0.068
2012	0.065 ± 0.007	0.056 ± 0.008	0.040 ± 0.007	0.046 ± 0.006	3.00	0.04	0.015
Hg 2011	0.013 ± 0.003	0.008 ± 0.004	0.014 ± 0.003	0.010 ± 0.002	5.69	0.001	0.029
2012	BDL	BDL	BDL	BDL	NA	NA	0.029
Se 2011	0.85 ± 0.11	2.65 ± 0.14	1.79 ± 0.13	0.99 ± 0.09	29.45	<0.001	0.312
2012	0.98 ± 0.11	0.88 ± 0.12	1.74 ± 0.11	1.05 ± 0.09	14.11	<0.001	0.012
Sr 2011	0.094 ± 0.020	0.071 ± 0.024	0.096 ± 0.022	0.116 ± 0.015	2.06	0.11	0.020
2012	0.069 ± 0.007	0.049 ± 0.007	0.068 ± 0.007	0.051 ± 0.005	2.75	0.05	0.037
Zn 2011	6.21 ± 0.20	5.68 ± 0.24	6.64 ± 0.22	5.54 ± 0.15	6.26	0.001	2.17
2012	8.34 ± 0.61	6.11 ± 0.68	5.73 ± 0.60	6.63 ± 0.50	5.73	0.001	1.47

Concentrations of several elements were below the detection limit (2011 detection limit/2012 detection limit) in both years and were not considered further, as follows: As (0.009/0.006), Cd (0.009/0.007), Cr (0.098/0.088), Tl (0.009/0.001) and V (0.016/0.015). Number of nests sampled at each colony, 2011: reference = 30, Melton Hill Dam = 20, spill site = 24 and downstream = 51; and 2012: reference = 22, Melton Hill Dam = 18, spill site = 23, and downstream = 33 (2011 d.f. = 3, 121; 2012 d.f. = 3, 92). Abbreviations: BDL, below detection limit; and NA, not assessed.

residuals as a measure of body condition is controversial (Green, 2001), studies have shown that residuals do correlate well with lipid reserves (Ardia, 2005; Schulte-Hostedde *et al.*, 2005) and other studies have shown that residual body mass is related to the immune and the stress responses in avian species (Pollock and Machin, 2009; Palacios *et al.*, 2012).

We used linear regressions and backward elimination of non-significant terms to examine the effects of clutch initiation date, element concentrations, body condition and two-way interactions between condition and element concentrations on the immune and stress responses of nestlings. We eliminated non-significant interaction terms first, followed by main effects that were not significantly related to physiology. We allowed terms to remain in the model as long as $P \leq 0.10$, but considered their contribution to be statistically significant only when $P \leq 0.05$. For the stress response, we performed separate regressions with basal and induced corticosterone as the dependent variables because these two parameters represent distinct physiological responses that engage different receptor types (Kloet, 1991). To examine the immune responses, we used the percentage of bacteria killed and wing web swelling as dependent variables. Given that much of the variance in element concentrations, particularly for Se, was attributable to variation at the spill site, we ran an

additional iteration of any statistically significant model that focused only on samples collected at this colony. Given that element concentrations and colony type were confounded in the analysis, we did not include colony type in the regression models. Rather, we also compared corticosterone concentrations and immune responses among colony types using an ANCOVA, with clutch initiation date included as a covariate. All statistical tests were two-tailed, with $\alpha = 0.05$. All statistical analyses were performed using PASW 18 (SPSS, 2009).

Results

Concentrations of elements among colonies

We first compared concentrations of elements among the four colony types, i.e. reference, the spill site, downstream and Melton Hill. In 2011, we found that concentrations of Ba and Sr did not differ significantly among colony types (Table 1; all $P \geq 0.10$). However, we found that Cu, Fe, Hg, Se and Zn concentrations differed significantly among colonies, and *post hoc* tests indicated that the spill site had greater concentrations than the reference colonies for all of these elements ($P \leq 0.001$) except for Fe ($P = 0.47$). Given that only Cu, Hg, Se and Zn were significantly elevated at the spill site in comparison to reference colonies, we focused on the effect

of these elements on the basal and induced plasma corticosterone concentrations of nestlings. Copper, Se and Zn concentrations were not significantly correlated with each other (all $r \leq 0.11$, all $P \geq 0.23$), but Hg concentrations correlated positively with Cu ($r = 0.27$, $P = 0.002$) and were almost significantly correlated with Se concentrations ($r = 0.16$, $P = 0.07$). In order to reduce the number of tests performed while avoiding issues with collinearity, we performed two separate backward elimination regressions with different combinations of the elements, one that included Cu, Se and Zn and a second that used only Hg.

In 2012, we found that concentrations of Ba and Cu did not differ significantly among colony types (Table 1; all $P \geq 0.06$). Concentrations of Fe, Mn, Sr and Zn differed significantly among colony types, but not in ways that indicated an association with the fly ash spill. *Post hoc* tests indicated that concentrations of Fe, Mn, Sr and Zn were significantly greater at reference colonies than those at the spill site (all $P \leq 0.05$). Only concentrations of Se remained significantly elevated at the spill site in comparison to reference colonies ($P < 0.001$). Thus, all of the immune challenge analyses focused on nestling blood Se concentrations.

Stress response

In 2011, we examined the effect of element exposure on the stress response of nestling tree swallows. Basal corticosterone concentrations averaged 2.7 ± 0.24 ng/ml (range 0.57–17.4 ng/ml) and induced corticosterone concentrations averaged 11.8 ± 0.87 ng/ml (range 0.57–62.1 ng/ml) in all of the colonies combined. Basal corticosterone concentrations differed significantly among colony types (Table 2; $F_{3,120} = 3.6$, $P = 0.02$), and Tukey's *post hoc* tests indicated that basal corticosterone concentrations were significantly greater at downstream colonies than at reference colonies. Induced corticosterone concentrations also differed significantly among colony types (Table 2; $F_{3,117} = 3.7$, $P = 0.01$), and this result did not change when the three individuals with induced corticosterone concentrations below the assay detection limit were included in the analysis ($F_{3,120} = 4.7$, $P = 0.004$). Induced

corticosterone concentrations were significantly lower at downstream colonies than at reference colonies or at the spill site (both $P \leq 0.04$). Element concentrations and their interaction with condition were unrelated to basal corticosterone concentrations (Table 3; full model with Cu, Se and Zn, $r^2 = -0.07$, d.f. = 116, $P = 0.37$; full model with Hg, $r^2 = -0.06$, d.f. = 120, $P = 0.13$). The only term that remained in the final version of these models was nestling condition, which had a very weak but statistically significant negative relationship with basal corticosterone concentrations (final models, $r^2 = -0.03$, d.f. = 123, $P = 0.05$). Likewise, induced corticosterone concentrations were unrelated to element exposure and the interactions between condition and element exposure (Table 3; full model with Cu, Se and Zn, $r^2 = 0.09$, d.f. = 113, $P = 0.19$; full model with Hg, $r^2 = -0.07$, d.f. = 117, $P = 0.06$), and this did not change if the three nestlings with induced corticosterone concentrations below the assay detection limit were included in the analysis for the model including Cu, Se and Zn ($r^2 = 0.10$, d.f. = 116, $P = 0.14$). While the full model including Hg was statistically significant when these three individuals were included ($r^2 = -0.09$, d.f. = 120, $P = 0.03$), this was caused by an association between induced corticosterone concentrations and measurement date rather than Hg exposure (Table 3). For both groups of elements, clutch initiation date remained in the final models and had a weak, negative relationship with induced corticosterone concentrations (Table 3; final models, $r^2 = -0.05$, d.f. = 123, $P = 0.01$), and including or excluding the individuals with induced corticosterone below the assay detection limit did not influence this relationship.

Immune response

In 2012, we evaluated the effects of Se exposure on the cell-mediated and innate immune responses of nestling tree swallows. Among all of the colonies, the average response (percentage increase in swelling) to PHA injection was $73 \pm 6\%$ (range 17–160%), and we found no significant differences among colony types in the PHA-induced swelling (Table 2). The cell-mediated immune response of nestlings

Table 2: Mean values and standard errors for stress and immune responses in nestling tree swallows among colonies

Response	Reference	Melton Hill	Spill site	Downstream	<i>F</i>	<i>P</i> value
Basal corticosterone (ng/ml)	1.80 ± 0.47	2.25 ± 0.58	1.99 ± 0.52	3.69 ± 0.36	3.6	0.02
Induced corticosterone (ng/ml)	16.12 ± 1.7	9.64 ± 2.1	13.98 ± 1.9	9.10 ± 1.3	4.7	0.004
Corticosterone samples (<i>n</i>)	30	20	24	51		
PHA (%)	58.3 ± 15.5	78.9 ± 12.2	75.8 ± 9.6	72.5 ± 10.4	0.41	0.75
PHA samples (<i>n</i>)	5	8	13	11		
BKA (%)	17.2 ± 2.5	18.4 ± 2.7	17.3 ± 2.4	20.6 ± 2.0	0.55	0.65
BKA samples (<i>n</i>)	22	18	23	33		

The stress response was quantified in 2011, while the immune responses were quantified in 2012. Least-squares means are given for basal and induced corticosterone concentrations because Julian clutch initiation date had a significant influence on both the basal and induced corticosterone concentrations. Basal and induced corticosterone, d.f. = 3, 120; phytohaemagglutinin (PHA), d.f. = 3, 33; and bactericidal killing assay (BKA), d.f. = 3, 92. *n* refers to the number of nests sampled at each colony.

Table 3: Full and reduced model results from multiple regressions examining the effects of element exposure on the stress responses of nestling tree swallows

Term	β	P value
Basal corticosterone full model		
Intercept	0.864	0.12
Cu	-0.263	0.58
Se	-0.034	0.83
Zn	-0.345	0.40
Condition	-0.026	0.11
Clutch initiation date	-0.003	0.14
Cu \times condition	0.085	0.53
Se \times condition	-0.003	0.95
Zn \times condition	0.077	0.62
Basal corticosterone full model Hg		
Intercept	0.533	0.28
Hg	-0.095	0.56
Condition	-0.029	0.07
Clutch initiation date	-0.003	0.14
Hg \times condition	-0.024	0.74
Basal corticosterone final model both		
Intercept	0.191	<0.001
Condition	-0.032	0.04
Induced corticosterone full model		
Intercept	1.788	<0.001
Cu	-0.184	0.63
Se	0.121	0.33
Zn	-0.327	0.33
Condition	-0.003	0.85
Clutch initiation date	-0.004	0.03
Cu \times condition	0.171	0.12
Se \times condition	-0.021	0.58
Zn \times condition	-0.075	0.56
Induced corticosterone full model Hg		
Intercept	1.630	<0.001
Hg	-0.041	0.75
Condition	-0.001	0.94
Clutch initiation date	-0.005	0.01
Hg \times condition	0.098	0.10
Induced corticosterone final model both		
Intercept	1.700	<0.001
Clutch initiation date	-0.005	0.01

From the full model, we used backward elimination, beginning with interaction terms, to remove terms that did not contribute significantly to the model fit until only statistically significant terms remained. Basal and induced corticosterone models converged on the same final models (indicated by 'both' in the table) that included only nestling condition or clutch initiation date, respectively. For the analysis, induced corticosterone models exclude the three individuals with induced corticosterone concentrations below the assay detection limit; however, including these individuals produced nearly identical results.

was not related to clutch initiation date, body condition, Se concentrations or any of the interaction terms (Table 4; full model $r^2 = -0.06$, d.f. = 32, $P = 0.75$; final model $r^2 = -0.02$, d.f. = 35, $P = 0.45$). The mean bactericidal capacity of nestling plasma was $18.7 \pm 1.17\%$ (range 10–49%), and we found no differences among colonies in bactericidal capacity (Table 2; $P = 0.65$). The bactericidal capacity of nestling plasma was not influenced by clutch initiation date, residual body mass or the interaction between Se exposure and condition, but was positively related to Se exposure (Table 4; full

Table 4: Full and reduced model results from multiple regressions examining the effects of selenium exposure on the immune responses of nestling tree swallows

Term	β	P value
PHA full model		
Intercept	-1.267	0.84
Se	-0.067	0.84
Condition	-0.017	0.56
Clutch initiation date	0.011	0.75
Se \times condition	0.149	0.28
PHA final model		
Intercept	0.750	<0.001
Se	-0.220	0.45
BKA full model		
Intercept	0.038	0.84
Se	0.050	0.01
Condition	0.006	0.18
Clutch initiation date	0.001	0.62
Se \times condition	-0.005	0.53
BKA final model		
Intercept	0.128	<0.001
Se	0.050	0.01
SS BKA full model		
Intercept	0.739	0.25
Se	0.430	0.02
Condition	-0.020	0.19
Clutch initiation date	-0.004	0.28
Se \times condition	-0.092	0.35
SS BKA final model		
Intercept	0.062	0.12
Se	0.529	0.001

From the full model, we used backward elimination, beginning with interaction terms, to remove terms that did not contribute significantly to model fit. Abbreviations: BKA, bactericidal killing assay; PHA, phytohaemagglutinin; SS, spill site.

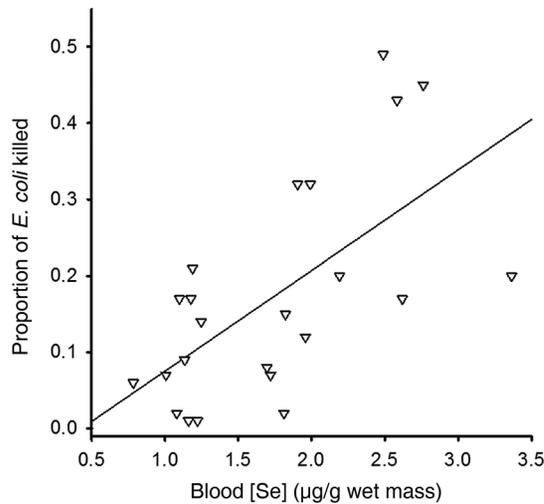


Figure 2: The relationship between Se exposure and bactericidal capacity in nestling tree swallows at the spill site. Higher Se concentrations were strongly associated with greater bactericidal capacity at this colony ($r^2 = 0.40$, d.f. = 21, $P = 0.001$). Analyses were performed with log-transformed Se concentration, but we show untransformed data for clarity.

model $r^2 = 0.10$, d.f. = 91, $P = 0.05$). Selenium concentrations remained in the final model and indicated a weak, but statistically significant positive relationship between blood Se concentrations and the bactericidal capacity of plasma (Table 4; final model, $r^2 = 0.07$, d.f. = 95, $P = 0.01$). We ran this model again using data from the spill site alone and found a strong positive relationship between bactericidal capacity and Se concentrations (Fig. 2; final model, $r^2 = 0.40$, d.f. = 21, $P = 0.001$). Our assay of cell-mediated immune response was unrelated to bactericidal capacity ($P = 0.24$).

Discussion

We examined the effects of exposure to elements from a recently remediated coal fly ash spill on the stress and immune responses of nestling tree swallows. We found that nestlings were exposed to elevated concentrations of some elements, particularly Se, at the spill site. However, we found no evidence of adverse effects on nestling physiology associated with the remediated fly ash spill. Element exposure was not related to the stress response of nestling tree swallows and was unrelated to a measure of the cell-mediated immune response. Only bactericidal capacity was affected by element exposure; greater exposure to Se was weakly associated with enhanced bactericidal capacity among all of the colonies, but strongly associated with Se exposure at the spill site. Overall, we suggest that remediation efforts and natural processes (e.g. offsite transport and dilution) in the years following the spill have left only modest concentrations of elements in the system and these low concentrations do not adversely affect the immune and stress physiology of swallows.

In 2011, we found that nestlings at the spill site were exposed to elevated concentrations of Cu, Hg, Se and Zn in comparison to nestlings in reference colonies, while in 2012, nestlings at the spill site were exposed to elevated Se only. Selenium is the primary driver of ecological risk in systems polluted by fly ash, and we found that even 4 years after the spill and massive remediation efforts, nestlings at the spill site were exposed to concentrations of Se elevated above those of reference colonies. However, most blood Se concentrations at the spill site were below concentrations typically associated with reduced survival or condition in avian species (Ohlendorf and Heinz, 2011). Blood Se concentration at the spill site ranged between 1.08 and 2.83 $\mu\text{g/g}$ wet mass in 2011 and between 0.78 and 3.36 $\mu\text{g/g}$ wet mass in 2012. Blood Se concentrations above 1.0 $\mu\text{g/g}$ wet mass are considered a threshold level for concern (reviewed by Ohlendorf and Heinz, 2011), but many studies detect effects on adult survival or body mass only at blood Se concentrations above 5.0 $\mu\text{g/g}$ wet mass (Heinz and Fitzgerald, 1993). However, blood Se concentrations within a range similar to those found in our study have been associated with reduced body mass in captive American kestrels (*Falco sparverius*) fed high-Se diets (Yamamoto and Santolo, 2000) and with signs of oxidative stress in emperor geese (*Chen canagica*, Franson *et al.*, 2002). All of these studies were conducted in laboratory animals that were exposed to Se in their diet for a minimum of 11 weeks (Heinz and Fitzgerald, 1993; Yamamoto and Santolo, 2000; Franson *et al.*, 2002). In our study, nestlings were exposed to elevated Se levels for only 13 days prior to sampling, and this may be why we found no negative physiological effects of these concentrations of Se when other researchers have.

Mercury, copper and zinc are other important elements found in some sources of fly ash, depending on the parent coal composition and combustion procedures used at power plants (Rowe *et al.*, 2002; NRC, 2006). In 2011 at the spill site, Hg concentrations in swallow blood ranged from 0.004 to 0.025 $\mu\text{g/g}$ wet mass. These concentrations are one-14th of the concentrations that caused physiological effects in a study by Wada *et al.* (2009) at a highly Hg-contaminated site in Virginia. Indeed, our highest Hg concentration is comparable to those found in blood samples at the reference colonies, 0.017 $\mu\text{g/g}$ wet mass, in the study by Wada *et al.* (2009). Concentrations of Cu and Zn at the spill site are largely similar to concentrations of these elements found in white storks, in studies that detected few effects of these elements on physiology (Baos *et al.*, 2006a, b).

Consistent with the low observed exposure to elements experienced by swallows in our remediated study system, we found no evidence of adverse physiological effects on the HPA axis. Basal and stress-induced corticosterone concentrations of swallows in this system were not related to element exposure. Indeed, corticosterone concentrations of swallows from impacted colonies were similar to the corticosterone concentrations found at reference sites in other studies (Franceschini *et al.*, 2008, 2009; Wada *et al.*, 2009). We did

find subtle differences in basal and induced corticosterone concentrations among colony types, but not between the spill site and reference colonies, indicating these differences are not related to the fly ash spill. Downstream colonies had significantly higher basal corticosterone concentrations than reference colonies, and induced corticosterone concentrations were significantly higher at downstream colonies than those at the spill site and reference colonies. It is likely that the slight differences in corticosterone concentrations at downstream colonies are related to subtle ecological differences among colonies, such as resource availability, rather than contaminant exposure.

Likewise, we found few effects of elements from the fly ash spill on the immune response of nestling tree swallows. We found no differences among colonies in either the response to PHA injection or bactericidal capacity. We expected that blood Se concentration would affect the immune response, because adequate dietary concentrations of Se are necessary for robust innate and acquired immune responses (Maggini *et al.*, 2007; Wintergerst *et al.*, 2007). However, we found no relationship between the blood Se concentrations and the nestling response to PHA injection. Adult common eiders fed a high-Se diet had blood Se concentrations $>8 \mu\text{g/g}$ wet mass and produced smaller swellings in response to PHA injection than control birds (Franson *et al.*, 2007). Thus, higher Se concentrations and more prolonged exposure than found in our study may influence this aspect of immunity. In contrast, we found that the bactericidal capacity of nestling plasma, an assay of innate immunity, showed a strong, positive relationship to their blood Se concentration at the spill site, and there was a weak association when all colonies were included in the analysis. The bacteria-killing capacity of plasma reflects several aspects of the innate immune response, including the ability of complement enzymes and lysozyme to destroy cell walls and lyse cell membranes of bacteria (reviewed by Matson *et al.*, 2006). The selenoenzyme thioredoxin reductase influences the regulation and expression of genes involved in the innate and adaptive immune responses (reviewed by Maggini *et al.*, 2007). It is possible that exposure to slightly elevated concentrations of Se enhanced the expression of genes associated with innate immunity and enhanced bactericidal capacity. In fish fed supplemental Se, lysozyme activity is enhanced (Lin and Shiau, 2007), and this may have contributed to the enhancement of bactericidal capacity that we detected.

Overall, our results indicated that nestling tree swallows near the spill site were exposed to modest increases in element concentrations from a recently remediated fly ash spill. Exposure to low element concentrations was largely unrelated to several aspects of physiology in nestling tree swallows. It is currently unknown whether exposure to elements during development has long-term effects on physiology in adulthood or how chronic exposure to low element concentration may be related to physiology. Future studies should address the long-term effects of element exposure on physiology, particularly aspects of physiology that could ultimately affect

recruitment and survival of young or future reproductive success. Additionally, concentrations of similar combinations of elements would be much higher in active fly ash settling basins (Bryan *et al.*, 2012), potentially putting swallows and other taxa attracted to these sites at greater risk of exposure and physiological effects. In the near future, concentrations of Hg and other heavy metals in fly ash are expected to increase as new clean air regulations reduce air emissions by coal-burning power plants and increase the concentration of these elements in the solid waste stream (USEPA, 2012). Thus, the hazards posed by fly ash are projected to increase in the future, warranting disposal procedures that minimize its potential to contaminate ground and surface water in order to prevent exposure and adverse effects in wildlife.

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