

Environmental Toxicology

Manganese Exacerbates Seasonal Health Declines in a Suicidally Breeding Mammal

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Abstract: Reproductive costs must be balanced with survival to maximize lifetime reproductive rates; however, some organisms invest in a single, suicidal bout of breeding known as semelparity. The northern quoll (*Dasyurus hallucatus*) is an endangered marsupial in which males, but not females, are semelparous. Northern quolls living near mining sites on Groote Eylandt, Northern Territory, Australia, accumulate manganese (Mn) in their brains, testes, and hair, and elevated Mn impacts motor performance. Whether Mn is associated with other health declines is yet unknown. In the present study we show that male and female northern quolls with higher Mn accumulation had a 20% reduction in immune function and a trend toward reduced cortisol concentrations in hair. The telomere lengths of male quolls did not change pre- to postbreeding, but those with higher Mn levels had longer telomeres; in contrast, the telomeres of females shortened during the breeding season but recovered between the first year and second year of breeding. In addition, the telomeres of quolls that were recaptured declined at significantly higher rates in quolls with higher Mn between prebreeding, breeding, and/or postbreeding seasons. Future research should determine whether changes in cortisol, immune function, or telomere length affect reproductive output or survival—particularly for semelparous males. *Environ Toxicol Chem* 2024;43:74–86. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Reproduction is so costly that organisms typically distribute their efforts and offspring over time to accommodate the competing energetic demands of growth and survival, and delay breeding during periods of poor health or environmental stress (Koons et al., 2008; Tuljapurkar, 1990). However, not all species hold back for the future—some

invest all resources into a single (usually promiscuous) bout of breeding and then die. This life history strategy, known as semelparity or suicidal breeding, is rare among mammals, found only in the families Didelphidae and Dasyuridae—small marsupial insectivores such as *Antechinus* spp., *Phascogale* spp., and northern quolls (*Dasyurus hallucatus*), and sometimes in only males of those species (Dickman & Braithwaite, 1992; Oakwood et al., 2001). After a 2 to 3-week rut, semelparous male dasyurids physically deteriorate and die, often as a result of infection, anemia, or organ failure (McDonald et al., 1981; Naylor et al., 2008).

In the present study, we explored the interactive effects of sex and environmental contamination on physiological traits related to stress in northern quolls. We aimed to determine whether chronic exposure to manganese (Mn) dust associated with ongoing mining activities on Groote Eylandt, Australia, differentially affected the health of male quolls

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(semelparous and living for 1 year) versus female quolls (not semelparous and living for 2–3 years) due to their different life spans and breeding strategies. The island, recognized by the Northern Territory Government as a site of International Conservation Significance due to its outstanding conservation value, is crucially important for Australian mammal conservation because the widespread decline of small and medium-sized mammals occurring across Australia has not yet affected populations on the island (Anindilyakwa Land Council, 2023).

The island is also the site of one of the largest Mn producers in the world, which harvests approximately 3 to 4 million tonnes of Mn ore annually (South32, 2023; US Geological Survey, 2023). Covering an area of approximately 50 km² on the western side of the island, the mining operations liberate vast quantities of ore dust into the environment as Mn is extracted from open pits and then crushed onsite before it is transported in open trailers to the port for open-air storage and shipping (Figure 1). We found that the levels of airborne Mn were higher near Mn mining facilities on Groote Eylandt (Figure 1, Sites 1 and 2) than they were 20 km from the mine (Figure 1, Site 12). Furthermore, northern quolls living near the mine and ore storage areas had significantly higher concentrations of Mn in their hair, brains, and testes than did quolls living in the center of the island, 20 km from the mine (Amir Abdul Nasir, Cameron, von Hippel, et al., 2018). We also found that both sexes were compromised in motor agility associated with Mn concentration measured in hair; these findings are

consistent with cerebellar impairment (Amir Abdul Nasir, Cameron, Niehaus, et al., 2018).

Although Mn is an essential trace element, only small amounts are required in all living organisms, and these needs are met through dietary intake (Bouchard et al., 2011). Homeostatic mechanisms that regulate this Mn intake may, however, be bypassed when airborne Mn particles, particularly <1 µm in size, are inhaled, resulting in higher levels of Mn delivery into the brain (Agency for Toxic Substances and Diseases Registry, 2012; Mergler et al., 1994). Due to their size, Mn particles may enter the body via the respiratory tract, and are likely to travel further into the alveoli, where they can be transported to the brain via the bloodstream (Antonini et al., 2006; Lucchini et al., 2012). In addition, these particles may directly enter the brain through the nasal passages (Aschner, 2000; Dorman et al., 2004). In the nasal cavity, inhaled Mn may be absorbed by olfactory neurons or nerve receptor cells found within the olfactory epithelium, and transported along the olfactory nerve into the olfactory bulb of the brain (Tjalve & Henriksson, 1999). Studies on laboratory animals and people show that excessive Mn exposure impairs cognition, fertility, and motor and immune function (Josephs et al., 2005; Klos et al., 2006; Laohaudomchok et al., 2011; Levy & Nassetta, 2003; Liu et al., 2013).

The exertions of breeding cause visible aging of male quolls, including emaciation, hair loss, and poor wound healing, but the proximate cause of death is still unknown for this species (Oakwood et al., 2001). Because little is known

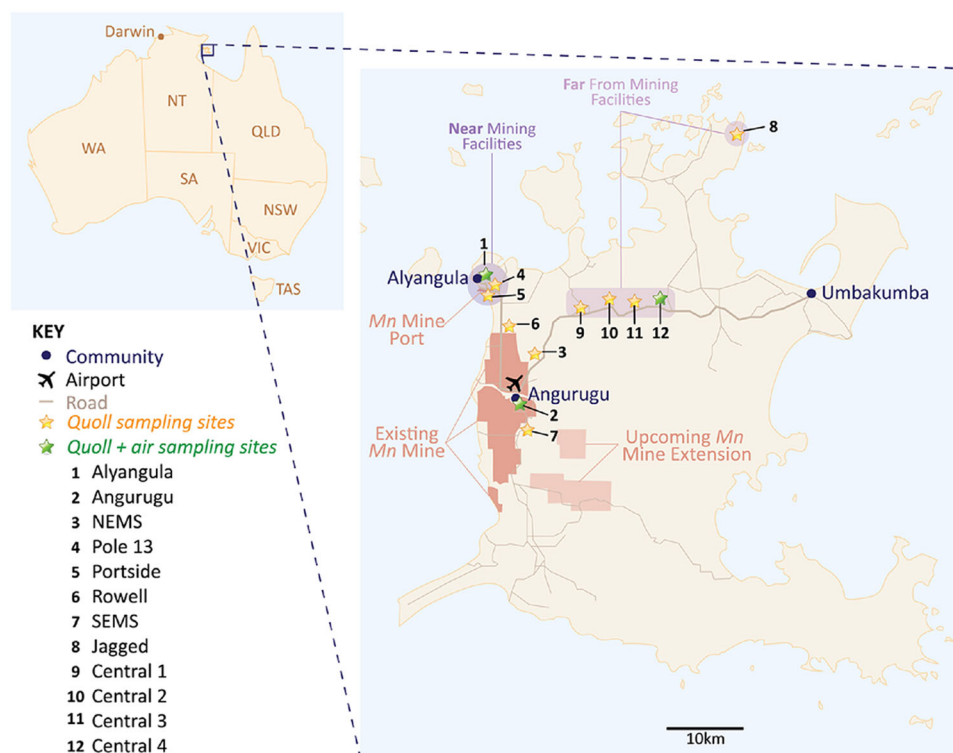


FIGURE 1: Study sites on Groote Eylandt (inset), located in the Gulf of Carpentaria, Northern Territory, Australia. The 12 study sites where northern quolls were captured (yellow stars) over 3 years were located at various distances from the Mn extraction and processing facilities (shaded orange). Out of 230 individual quolls measured for Mn bioaccumulation levels from 2013 to 2015, 138 captured in 2014 were also assessed for health declines in the present study. These quolls were captured in all the sampling sites shown in the figure except for Site 8 (Jagged). Adapted from Amir Abdul Nasir, Cameron, von Hippel, et al., 2018.

about how Mn manifests within wildlife in their natural environments, we assessed the impacts of Mn exposure on health in free-living wild northern quolls. Amir Abdul Nasir, Cameron, von Hippel, et al. (2018) demonstrated a gradient of decreasing Mn concentrations in the hair of northern quolls the further removed the animals were from the Mn mine and ore storage areas—based on 11 sampling sites located from 0.1 to 20 km away from the point sources (Figure 1). We hypothesized that animals living near the mine—and therefore exhibiting higher concentrations of Mn—would have altered cortisol concentrations, reduced immune function, and shortened telomere lengths, particularly in semelparous males. We show that Mn exposure diminishes the immune function of male and female quolls over the breeding season but affects concentrations of cortisol and telomere lengths differently between the sexes.

METHODS

Trapping and handling

We trapped wild northern quolls at 11 sites across Groote Eylandt, Northern Territory (13.9700°S, 136.5933°E), where we have studied the species since 2010: seven sites near Mn extraction, crushing, and open-air storage facilities and four sites located up to 20 km from mining facilities (Figure 1). Trapping occurred over three consecutive nights at each site between May and September 2014 during prebreeding (May 12–June 22), breeding (July 7–27), and postbreeding (August 11–September 14), and the order in which sites were trapped was randomized among these time periods (hereafter we refer to prebreeding, breeding, and postbreeding time periods as seasons). A minimum of 30 Tomahawk original series cage traps (20 × 20 × 60 cm; Tomahawk ID-103) were placed at 50-m intervals, baited with wet dog food, and left overnight. Early the following morning, we checked the traps and transferred captured quolls into individual cloth bags for later processing. The location of each captured quoll was recorded via GPS (Garmin e-Trex 20). The animals were in transit for at most 1 h following capture, and processing and sampling required approximately 1 h/individual quoll. We trapped only during the dry season (during which breeding takes place) when weather conditions and food resources are fairly stable.

Hair and blood sampling

Captured quolls ($n = 65$ males and 73 females) were immediately transferred to the Anindilyakwa Threatened Species Centre, where they were ear-tagged, measured, and sampled for blood and hair. The ear-tagging device was sterilized with alcohol after each use. Each quoll was microchipped with a passive integrated transponder (PIT tag; Trovan nano-transponder ID-100) inserted between the shoulder blades for later identification during recaptures. We recorded sex and breeding status, and we estimated age based on body mass (± 1 g; A & D HL200i), incisor wear, and reproductive condition (Oakwood, 2000).

Hair accumulates Mn over the growth period of the hair (i.e., weeks) and is an effective and minimally invasive tissue to

sample for the assessment of chronic Mn exposure (Amir Abdul Nasir, Cameron, von Hippel, et al., 2018). Approximately 200 hairs (underfur and guard hairs) were plucked from between the shoulder blades of each quoll for quantification of Mn and 14 other elements. This procedure was repeated for quolls captured during the prebreeding, breeding, and postbreeding seasons. In the case of recaptured individual quolls, the same amount of regrown hair was plucked from the same location. Quoll hair regrows quickly and is not punctuated by molts, and therefore hair is readily resampled after a few weeks. We only had 16 individual quolls that were recaptured among seasons, so most hair samples were taken from unique individuals. We did not resample within a season because we expected little difference in Mn exposure over such a short period.

Each quoll was restrained in a cloth bag and sampled for 2 mL of blood by venepuncture of the jugular with a 27-gauge needle. No anesthesia was required for this procedure. Blood was transferred into a lithium heparin blood tube (BD Biosciences) and placed on ice for 10 to 60 min. New needles and syringes were used for each individual quoll. Samples were centrifuged at 1300 rpm for 10 min at 4 °C after which the plasma was pipetted into a labelled 0.5-mL sterile tube (Eppendorf) and frozen at −20 °C. The remaining cellular component was stored in 70% ethanol at 4 °C and used for analysis of relative telomere length (RTL). Quolls were released in the evening of the capture day at the site of capture. If a quoll was recaptured within a season (e.g., prebreeding), it was not resampled and was released at the site of capture. All research methodologies were approved by the University of Queensland Animal Ethics Committee (SBS/541/12/ANINDILYAKWA/MYBRAINSC) and were conducted under a permit provided by the Northern Territory Parks and Wildlife Commission (permit number: 47603) and with the permission of the Traditional Owners of Groote Eylandt, the Anindilyakwa peoples.

Element concentrations in hair

Prior to analysis, external dust was removed from hair samples by washing once with deionized water, mixing thoroughly with 1% Triton® X100 nonionic detergent solution, and rinsing in deionized water 8x until detergent residues were removed. Each sample was dried for 1 h at 110 °C to measure dry mass, to estimate per-kilo element concentrations (procedure adapted from Filistowicz et al., 2011 and Kempson and Skinner, 2012). Hair samples were then mixed with nitric acid and hydrochloric acid solution (3:1 ratio) and closed-vessel digested using an Ethos 1 microwave digester (Milestone), diluted in triple deionized water, and analyzed using inductively coupled plasma–optical emission spectrometry (Varian) to determine concentrations of Mn and 14 other elements (Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mo, Ni, P, Pb, Se, and Zn; Amir Abdul Nasir, Cameron, von Hippel, et al., 2018). Analyses were conducted at the University of Queensland Diagnostics Laboratory, and all concentrations are reported in mg/kg. All Mn readings were well above the limit of detection of 0.03 mg/kg.

Cortisol level in hair

Keratinized tissues, such as baleen, feathers, shed reptile skins, spines, and hair, accumulate steroids and other hormones during the growth phase and thus can serve as a useful matrix from which these biomolecules can be extracted and quantified for retrospective assessment of life history and stress events (Branco et al., 2022; Dillon et al., 2021; Fabio-Braga et al., 2022; Hunt et al., 2017; Zena et al., 2021). Because hair accumulates cortisol over weeks or months (depending on the rate of growth), levels accumulated in hair at the time of capture are not influenced by the immediate stress of handling (Sheriff et al., 2011).

Hair samples were prepared as described by Macbeth et al. (2010). To remove surface contaminants, hair samples were washed 5x with 0.04 mL laboratory-grade methanol/mg of hair for a 3-min/wash on a slow rotator. Between washes, hair samples were gently blotted and rewashed with methanol to ensure thorough cleaning. Washed samples were placed in plastic dishes to air-dry on a laboratory bench at room temperature for 3 days. Dried samples (10–15 mg) were pulverized for 5 min in rubber-capped 1.8-mL stainless steel vials with a Mini-Beadbeater-24 (Biospec Products). Powdered samples were weighed, transferred into 5-mL screwtop glass conical vials (Fisher Scientific), immersed in 0.5-mL high-performance liquid chromatography-grade methanol, and gently vortexed for 10 s before being placed on a Nutator mixer for 24 h. Tubes were centrifuged at 1900 g (2912 rpm) at room temperature for 15 min, and the supernatant from each sample was collected into individual glass test tubes. This procedure was repeated twice, each time adding to the collected supernatant. The collected supernatant was blown to dryness at 38 °C using a nitrogen manifold. Once dried, the samples were resuspended with 200 μ L stock phosphate buffer (0.2 M, pH 7.4) and stored at 4 °C overnight (in at least four 25- μ L aliquots) until they were assayed.

Individual hair samples were run in duplicate, using a commercially available cortisol enzyme immunoassay kit (Salimetrics Cortisol EIA Kit, Item No. 1-3002, [Single] 96-Well Kit) according to the manufacturer's protocol. This kit was validated for use with quoll hair extracts using tests of parallelism and standard addition (Supporting Information, Figure S1; Hunt et al., 2017). An internal laboratory control was run on every plate and used to adjust all cortisol level measurements (in pg/mg unit) to compensate for any interassay variation. The controls included two manufacturer controls from the Salimetrics Cortisol EIA Kit and one laboratory control (extracted mouse hair pool). Inter- and intra-assay variations were 15.55% and 6.38%, respectively.

Bacterial killing capacity by plasma

We used bacterial killing assays (BKAs) to assess immune function in quolls before, during, and after breeding; these values were then assessed in relation to Mn concentration in hair. We used the protocol of French and Neuman-Lee (2012) for ex vivo BKA with modifications to assess the microbicidal activity of plasma. *Staphylococcus aureus* (ATCC no. 6538) was reconstituted from a lyophilized pellet (Epower Assayed Microorganism Preparation; Microbiologics) in prewarmed

phosphate-buffered saline (PBS) to make a stock solution of 10^7 colony-forming units (CFU). For each BKA, a working solution of 10^6 CFU was prepared from the stock solution. The BKAs were conducted using 96-well plates and plasma samples that had not been previously thawed. Each plate included negative (24 μ L PBS) and positive controls (18 μ L PBS and 6 μ L 10^6 *S. aureus* CFU). For each sample, 6 μ L of plasma was added to 12 μ L of PBS and 6 μ L of bacteria working solution. Each sample was assayed in quadruplicate and randomly assigned to plates, with all samples from the same individual on the same plate. A 125-mL aliquot of tryptic soy broth (Sigma-Aldrich #T8907; 15 g broth/500 mL nanopure water) was added to all wells, and a background reading (300 nm) was obtained with a microplate reader (Multiskan GO; Thermo Scientific). All plates were incubated at 37 °C for 12 h to ensure that exponential growth was obtained, and then plates were reassayed. The bacterial killing capacity (BKC) toward *S. aureus* was assessed by: (1) subtracting the background reading from the final reading, (2) taking the mean of the positive controls and that of the quadruplicates for each sample, and (3) calculating the percentage of bacteria killed in each well as described by French and Neuman-Lee (2012).

Relative telomere length in leucocytes

Telomeres are DNA caps that protect genes from damage during cell replication (Blackburn, 2001) and are linked with aging because, in many species, they shorten over an individual's lifetime or as a result of stress-induced damage (Epel et al., 2004; Houben et al., 2008; Kotschal et al., 2007; Monaghan, 2010; Richter & von Zglinicki, 2007). In most mammals, the rate of shortening is also greater for species with a “fast” life history (characterized by rapid growth, high reproduction, and early death) such as the northern quoll. We used RTL to estimate the aging rates of male and female northern quolls, and to determine whether Mn exposure exacerbates telomere shortening.

Telomere restricted fragmentation is typically used to analyze telomere length, yet this technique cannot be used effectively with the DNA of dasyurid marsupials because the DNA-cleaving enzymes used in the process also cleave the telomere (Ingles & Deakin, 2016). Therefore, we used quantitative real-time polymerase chain reaction (qPCR) to estimate the relative number of telomere copies compared with the number of a single copy gene—in this case *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)—within leucocytes (Cawthon, 2002). Telomeres are built by addition of repetitive nucleotide sequences, for example, the motif of 5'-TTAGGG-3' being repeated hundreds to thousands of times. By quantifying how many copies of the telomere motif an individual has compared with the one copy of the *GAPDH* gene, and then comparing the relative copy numbers across individuals, one can estimate RTL differences (Cawthon, 2002).

In mammalian blood, only leukocytes are nucleated; therefore, DNA was isolated from the cellular component containing leukocytes by phenol–chloroform extraction. The concentration and purity of each sample was quantified using a NanoDrop 2000 Spectrophotometer (NanoDrop Technologies).

Telomere-specific primers were adapted from Bize et al. (2009): the forward and reverse telomere primers were 5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' (Tel1b) and 5'-GGCTTGCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel2b), respectively. Primers were designed by using the Primer3Plus website (Bioinformatics, 2023) and aligned with the GAPDH sequences of the Tasmanian devil (*Sarcophilus harrisii*; ENSSHAT0000009418), the tammar wallaby (*Macropus eugenii*; ENSMEUT00000015320), and the gray short-tailed opossum (*Monodelphis domestica*; ENSMODT00000044008): GAPDHF: 5'-TCTTGGGCTACACAGAGGAC-3'; GAPDHR: 5'-ATACCAAGA AATGAGCTTGACA-3'.

The qPCR reactions were performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) in 20-μL total volume, containing 10 μL Bio-Rad SsoAdvanced Universal SYBR Green Supermix (2x), 0.2 μM and 0.75 μM forward and reverse primers (telomere and GAPDH, respectively), and 2 μL of genomic DNA (0.01 ng/μL concentration). A standard sample of 2 μL of a composite was made by pooling a few μL from every sample. This standard was run on every plate at a concentration of 0.02 ng/μL. Quantitative polymerase chain reaction conditions were established according to the manufacturer's protocol: 95 °C for 30 s (denaturation), followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s (annealing temperature [AT]). The fluorescence signal was acquired at the AT. To evaluate the specific amplification, a final melting curve analysis (from AT up to 95 °C) was added under continuous fluorescence measurements. Standard curves were generated using serial 1:5 dilutions of a composite sample containing equal parts of DNA from all blood extracts. Standard curves had $R^2 = 0.991$ (GADPH) and $R^2 = 0.996$ (telomere) and PCR efficiencies of 126% (GAPDH) and 119% (telomere). The standard curves contained five dilutions from the dilution series with a linear dynamic range of at least 3 orders of magnitude. All samples and standards were run in triplicate, and all quantification cycle values for unknowns fell within the linear quantifiable range of the appropriate standard curve. The normalized fold change of the target gene compared with the reference gene

was calculated by a Pair Wise Fixed Reallocation Randomisation Test[®] as described by Pfaffl et al. (2002).

Statistical analyses

In nature, elements do not occur in isolation, and other elements liberated in mining dust may augment or counteract the effects of Mn on wildlife. Therefore, we statistically accounted for concentrations of Mn and 14 covarying elements: Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mo, Ni, P, Pb, Se, and Zn. Elements detected in quoll hair were condensed into a composite measure of elemental load, using principal components analyses (PCA; Figure 2 and Supporting Information, Table S1). All concentrations were log₁₀-transformed prior to PCA, to normalize the data.

Cortisol concentrations were also log₁₀-transformed prior to analysis to normalize the distribution. We applied an information-theoretic approach to develop and select the most likely model to predict variation in cortisol, immune function (BKC), and telomere length (RTL) among wild northern quolls relative to our three Mn measures, two of which accounted for covarying elements (PC_{1Load} and PC_{2Load}; see the Results section), and one that did not (mean Mn concentration in hair, hereafter termed [Mn]hair). For each Mn measure, we constructed a global model with four main variables (Sex + Season + Mn measure + Body mass) and 5 s order interactions, or "submodels" (Sex:Body mass + Sex:Season + Sex:Mn measure + Body mass:Season + Mn measure:Season).

We calculated Akaike's Information Criterion using R package MuMIn (Bartoń, 2016; R Core Team, 2016) with small-sample adjustment, and we ranked submodels according to their Akaike weights (w_i). The most parsimonious submodel (with the highest w_i) was further examined using analyses of variance (ANOVAs). If multiple submodels had similar Akaike weights—indicating uncertainty in model selection—we averaged all submodels to obtain conditional and unconditional parameter estimates and relative importance values for each

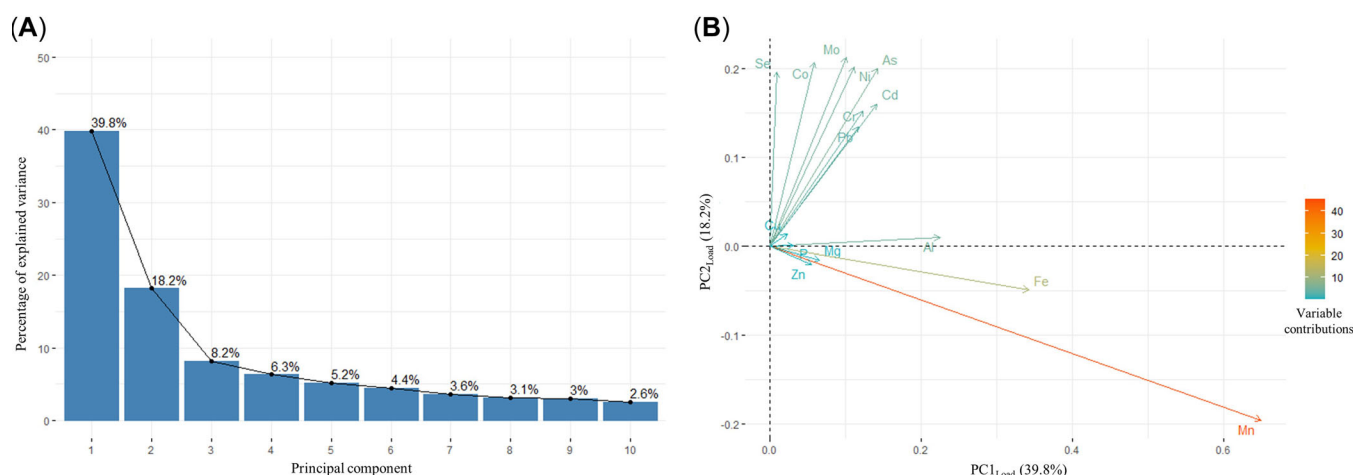


FIGURE 2: The principal component (PC) analysis on 15 log₁₀-transformed element concentrations measured in hair samples of northern quolls ($n = 138$; 73 females, 65 males). **(A)** The percentage of explained variances in the first 10 components, and **(B)** the contributions of each element in the first 2 components, PC_{1Load} and PC_{2Load}.

variable. The (1) mean and standard deviation of all elements and health indicators, and (2) the 10 most parsimonious submodels, along with relative importance values and parameter estimates obtained from model averaging relevant to each physiological trait, are provided in the Supporting Information, Tables S2–S3.

A longitudinal analysis of RTL was conducted. To assess whether high-Mn-exposed quolls would degrade at faster rates during breeding in quolls that were recaptured, we used a mixed model regression with gap in capture days as the random effect (R package lmerTest; R Core Team, 2016) and analyzed the changes in RTL in individuals between the two captures against their mean Mn concentration in hair, averaged from the first and second captures.

RESULTS

Element concentrations in hair

Metal concentrations in hair did not vary by sex, with the exception of As, Cu, Mg, and Pb, with female quolls having significantly higher concentrations of these metals (metals adjusted $p = 0.003, 0.012, 0.018, 0.041$, respectively; post hoc Tukey's test). To account for covarying element concentrations, we performed a PCA. The PC1_{Load} accounted for 39.8% of the variance in element load, and although all elements positively covaried, Mn had the strongest loading (0.78), followed by Fe (0.41; Figure 2 and Supporting Information, Table S1). Therefore, PC1_{Load} can be thought of as a measure of Mn load. The PC2_{Load} accounted for a further 18.2% of the variance in element load. In this case, Mo, Co, As, Ni, and Se loads varied in opposition to that of Mn.

Cortisol level

Cortisol levels did not differ between first- ($n = 50$) and second-year ($n = 9$) female quolls (adjusted $p = 0.968$, post hoc Tukey's test), but were significantly higher in males than in females ($n = 61$; $p < 0.001$; one-way ANOVA; Figure 3). When Mn levels in hair were evaluated on their own ([Mn]hair), without controlling for other, covarying elements, the most parsimonious model predicting cortisol level included [Mn]hair + Mass + Season + Sex + ([Mn]hair:Season) + (Mass:Season), with a $w_i = 0.10$ and adjusted $R^2 = 0.441$ (Supporting Information, Table S3A). In this model, the interaction between body mass and season (Mass:Season) was significantly associated with cortisol level ($F_{(2,110)} = 3.802$, $p = 0.025$)—heavier quolls had lower cortisol levels prior to the breeding season than did lighter quolls ($F_{(1,113)} = 6.391$, $p < 0.05$), regardless of sex. The interaction between [Mn]hair and season ([Mn]hair:Season) approached significance ($F_{(2,110)} = 2.830$, $p = 0.063$)—higher concentrations of Mn in the hair tended to be associated with lower cortisol concentrations during the breeding season ($F_{(1,112)} = 4.937$, $p = 0.085$).

In two separate model analyses in which both PCs of element loading were independently accounted for, cortisol levels were related to sex, body mass, and season, as well as the

interaction between body mass and season ($F_{(2,113)} = 3.431$, $p < 0.05$) in the most parsimonious submodels (PC1_{Load}: 0.12 w_i , PC2_{Load}: 0.17 w_i ; adjusted $R^2 = 0.421$, and F and p values were the same for both submodels; Supporting Information, Table S3A). Similar to the [Mn]hair model, the interaction between body mass and season (Mass:Season) was significantly associated with cortisol concentration in these models.

Bacterial killing capacity

Bacterial killing capacity did not differ between first- ($n = 44$) and second-year females ($n = 11$; $p = 0.999$), nor between females ($n = 55$) and males ($n = 45$; $p > 0.05$; one-way ANOVA; Figure 3). However, quolls with higher concentrations of all 15 elements had lower BKC values in the most parsimonious submodel including PC1_{Load} (adjusted $R^2 = 0.058$, 0.15 w_i , $F_{(1,97)} = 6.212$, $p < 0.05$; Supporting Information, Table S3B and Figure 4D). Because Mn and Fe had the two highest loadings in PC1_{Load}, a simple linear regression was then used to test whether each metal, on its own, significantly predicted BKC; Mn did not, and Fe was almost significant ($p = 0.3$ and 0.06, respectively; Figure 5).

Season was a significant predictor of BKC in the most parsimonious submodels including PC2_{Load} (adjusted $R^2 = 0.076$, 0.16 w_i , $F_{(2,96)} = 3.923$, $p < 0.05$) and hair[Mn] (adjusted $R^2 = 0.061$, 0.18 w_i , $F_{(2,96)} = 3.773$, $p < 0.05$), indicating that quolls had significantly higher capacity to kill bacteria before the breeding season compared with after it (adjusted $p < 0.05$, post hoc Tukey's test; Figure 3).

Relative telomere length

Male quolls ($n = 49$) had longer telomeres than females ($n = 56$; $p < 0.001$; One-way ANOVA), and the RTL of first- ($n = 45$) and second-year females ($n = 11$) were indistinguishable (adjusted $p = 0.837$; post hoc Tukey's test). In the most parsimonious submodel with PC1_{Load} (adjusted $R^2 = 0.301$, 0.09 w_i), RTL was significantly associated with season ($F_{(2,98)} = 6.574$, $p < 0.01$), PC1_{Load} ($F_{(1,98)} = 4.776$, $p < 0.05$), sex ($F_{(1,98)} = 25.315$, $p < 0.001$), and the interaction between PC1_{Load} and sex ($F_{(1,98)} = 4.805$, $p < 0.05$; Supporting Information, Table S3C).

Overall, quolls had significantly shorter RTLs after breeding than before (adjusted $p = 0.003$) or during it (adjusted $p = 0.002$; post hoc Tukey's test; Figure 3). Using F -tests, we found that males with higher elemental loads (particularly Mn) had longer telomeres ($F_{(1,98)} = 5.418$, $p < 0.05$) but females did not ($F_{(1,98)} = 0.187$, $p = 0.666$; Figure 4G). Again, a simple linear regression was then used to test whether Mn and Fe, on their own, significantly predicted RTL. None of the metals did ($p = 0.36$ and 0.16, respectively; Figure 5).

When RTL was assessed against hair[Mn], the most parsimonious submodel (adjusted $R^2 = 0.332$, 0.10 w_i) also showed that RTL displayed a significant interaction between sex and hair[Mn] ($F_{(1,96)} = 5.156$, $p < 0.05$): the higher the hair[Mn], the longer the RTL of males (F -tests; $F_{(1,96)} = 9.993$, $p < 0.01$; Figure 4I), but not females (F -tests; $F_{(1,96)} = 0.111$, $p = 0.739$).

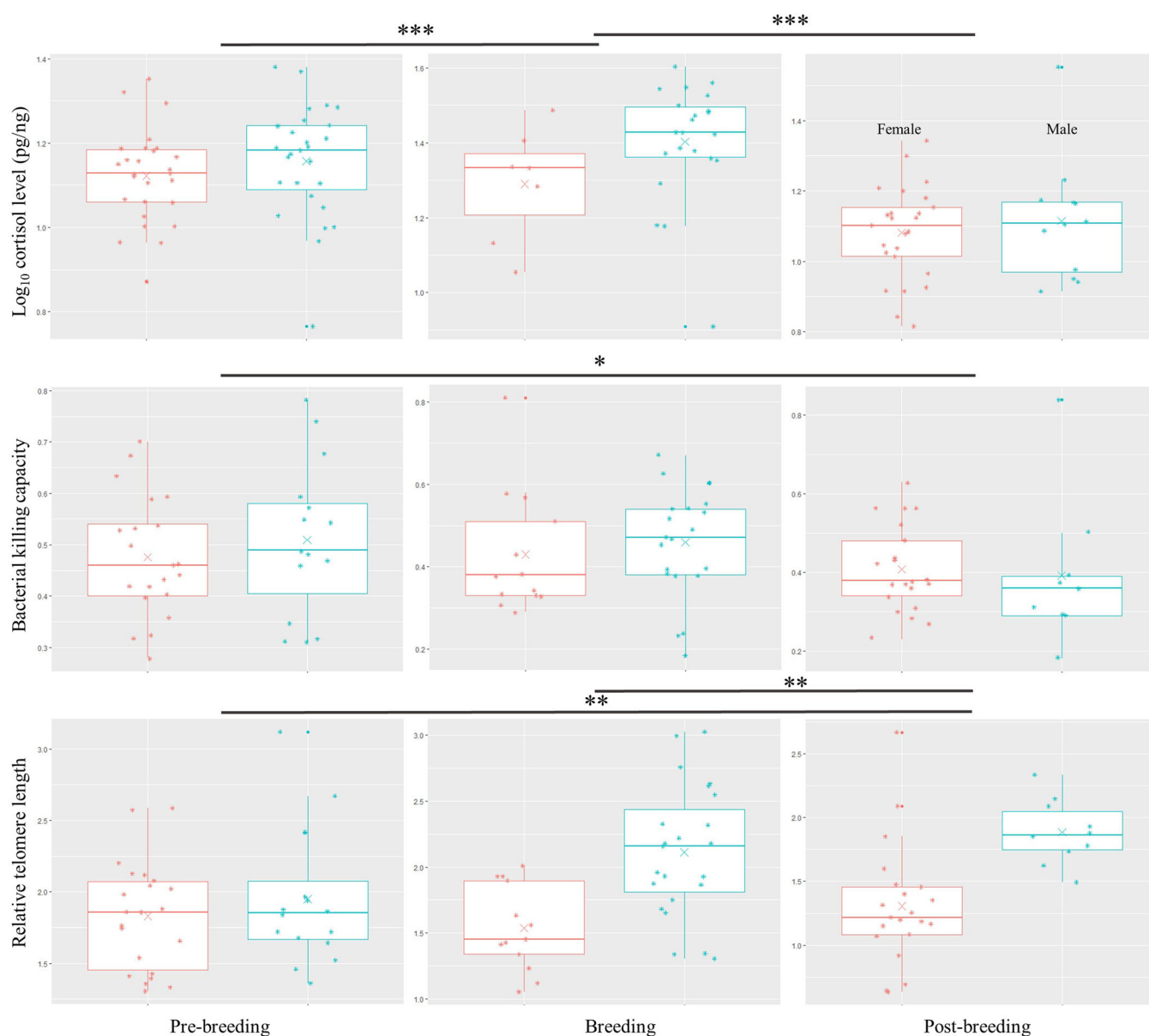


FIGURE 3: The variation in cortisol concentration ($n=120$; 59 females, 61 males), bacterial killing capacity ($n=100$; 55 females, 45 males), and relative telomere length ($n=105$; 56 females, 49 males) in male and female northern quolls captured during prebreeding, breeding, and postbreeding seasons. The differences between seasons (regardless of sex) were analyzed using post hoc Tukey's tests ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). The model mean value for each sex within each season is indicated by an X.

The most parsimonious submodel when RTL was assessed against PC2_{Load} (adjusted $R^2 = 0.298$, 0.13 w_i) showed that RTL differed between the sexes ($F_{(1,98)} = 23.074$, $p < 0.001$) and among seasons ($F_{(2,98)} = 8.899$, $p < 0.001$), and that the model contained a significant interaction between sex and season ($F_{(2,98)} = 3.200$, $p < 0.05$). The RTL of female quolls declined between pre- and postbreeding (adjusted $p < 0.001$; post hoc Tukey's test), whereas that of males remained unchanged (all adjusted $p > 0.05$). As in previous models, females had shorter telomeres than males but only during ($p < 0.001$) and after the breeding season ($p < 0.01$).

A total of 16 individual quolls (13 females, 3 males) were captured twice throughout the study and were used for the

longitudinal analysis of RTL. A significant negative relationship existed between the change in RTL and the mean hair[Mn] over the two captures ($df = 14$, $t\text{-value} = -2.467$, $p = 0.0271$; mixed model regression).

DISCUSSION

Environmental stress compounds the energetic costs associated with reproduction, but should affect semelparous organisms—characterized by a single reproductive episode before death—more severely than it does iteroparous organisms, which extend reproduction over a longer proportion of their lives. Although iteroparity provides a species with

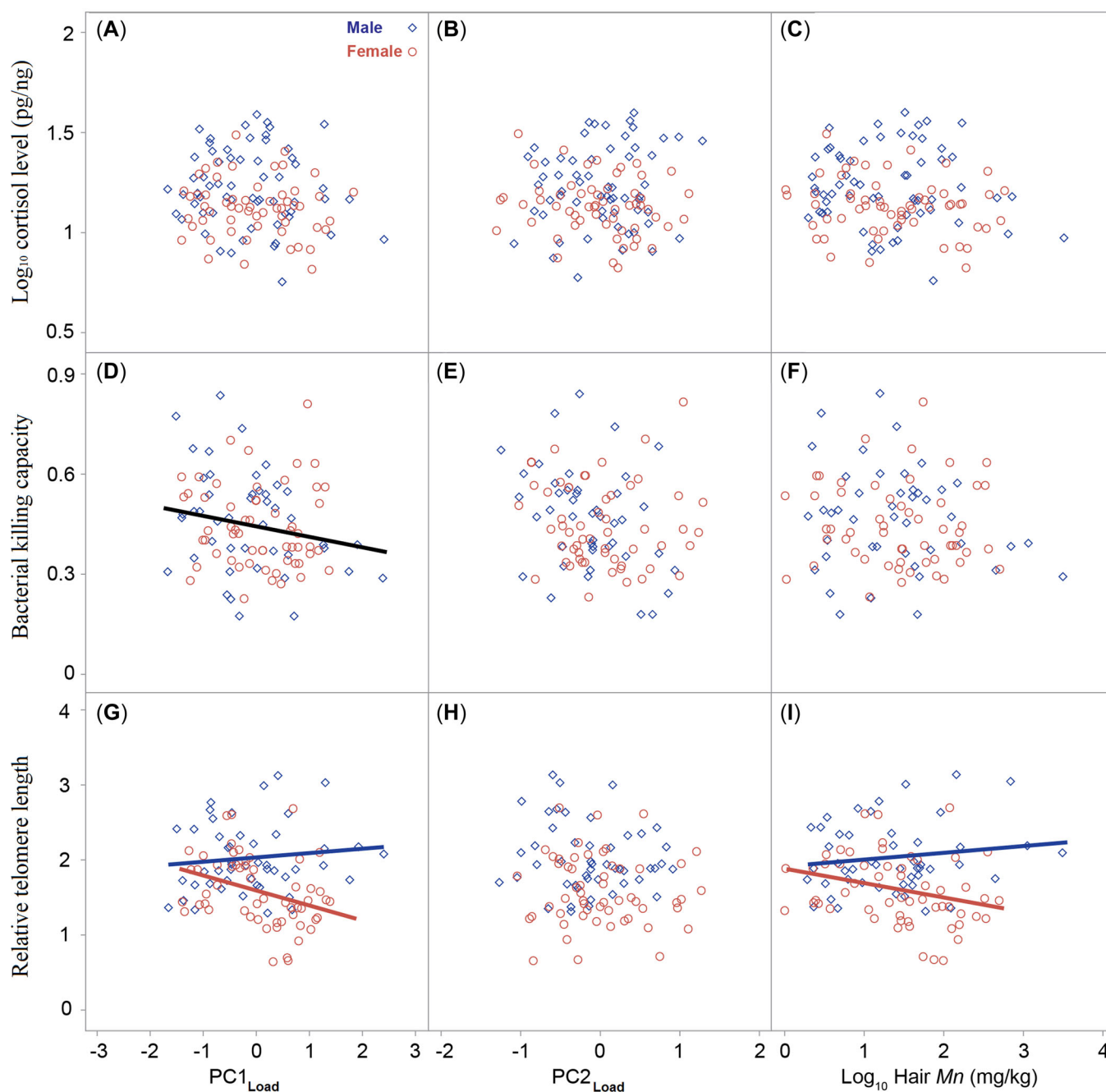


FIGURE 4: (A–I) The variation in log10-transformed cortisol concentration ($n = 120$), bacterial killing capacity ($n = 100$), and relative telomere length ($n = 105$) relative to hair $PC1_{Load}$, hair $PC2_{Load}$, and log10-transformed hair Mn (hair[Mn]) concentration in northern quolls. Solid black line in (D) indicates the significant correlation regardless of sex. Solid blue lines in (G) and (I) indicate the correlation in only the male northern quolls (significant), in comparison with that of females, in red lines (not significant).

numerous chances to reproduce as a response to adversity, their semelparous counterparts only get a single chance with no opportunity to postpone reproduction when under stressful conditions (Bell, 1976; Minelli & Fusco, 2019). However, because semelparity is so rare among mammals, many of the physiological mechanisms underlying this extreme life history are unknown.

It is unknown how impacts of exposure to environmental stressors manifest in semelparous species—with only a single chance to maximize fitness—in their natural habitat, although

laboratory studies provide some insights. For example, reproduction can be impaired in salmon under simulated conditions of ecological stress (*Oncorhynchus gorbuscha*, McConnachie et al., 2012; *Oncorhynchus nerka*, Jeffries et al., 2012). Working with a free-living population of wild northern quolls, we examined associations between Mn exposure and a set of health measures, considering the effects of season and sex, among other parameters. Historically, studies of stress in wildlife have been hampered by the inclusion of only one marker (e.g., glucocorticoids; Romero et al., 2015) or

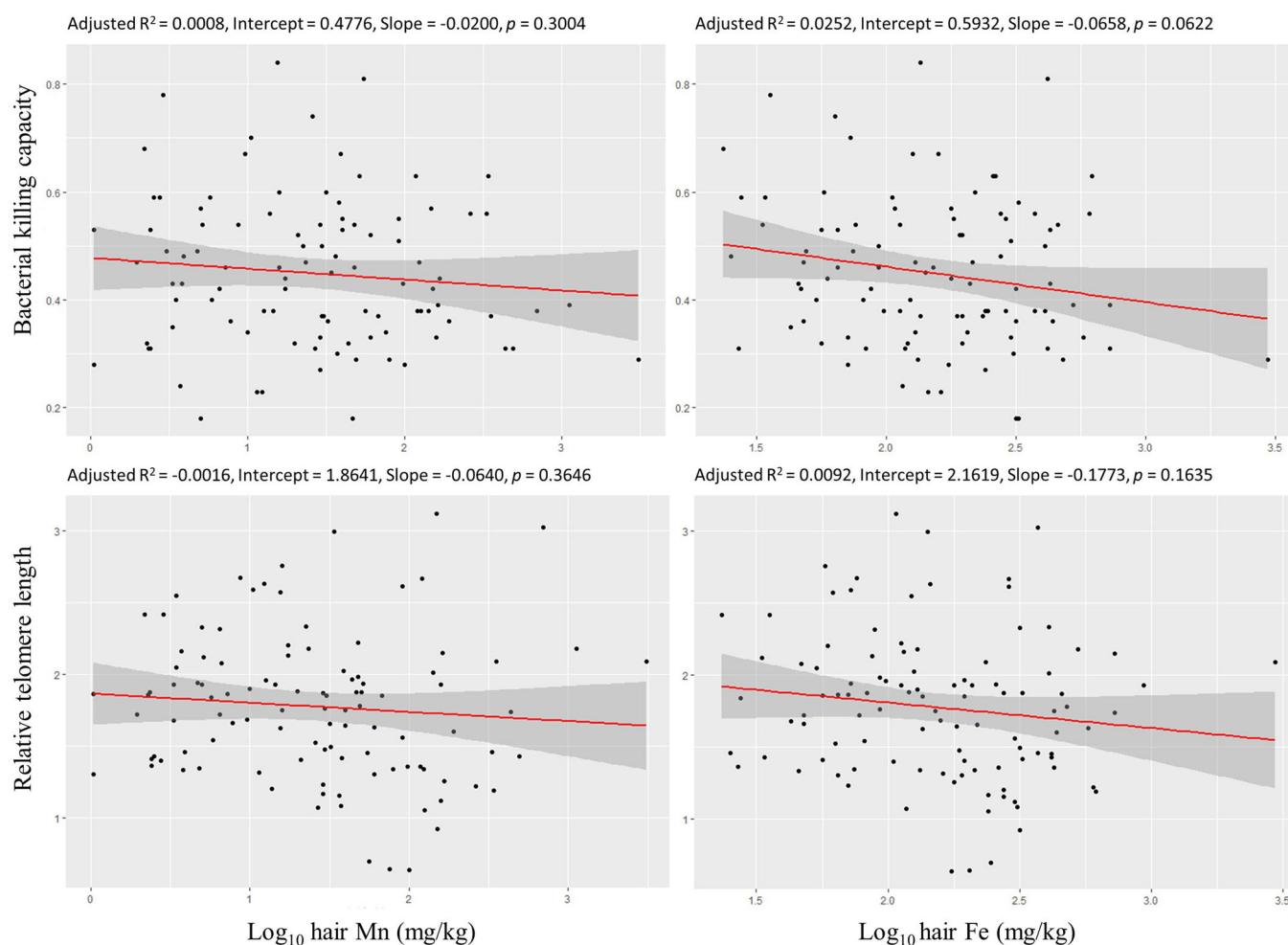


FIGURE 5: Simple linear regressions were used to test whether log_{10} -transformed hair Mn and Fe, on their own, significantly predicted bacterial killing capacity and relative telomere length. All correlations were not significant, and the R^2 , slope, intercept, and p value were as reported for each interaction.

consideration of one of a myriad of biological mediators (Del Giudice et al., 2018; see review by Gil-Jiménez et al., 2021). In the present study, we examined two health measures in addition to glucocorticoid concentrations: immune function as determined by BKC and aging as determined by RTL.

We found that sublethal levels of contamination, driven primarily by Mn exposure, and by Fe to some extent (based on metal loadings on PC1; Figure 2 and Supporting Information, Table S1), impose physiological costs on wild northern quolls. Accumulation of Mn in male and female quolls was associated with reduced immune activity—a trait associated with increased infection risk, fatigue, and weight loss in mammals (Barker et al., 1978; Bradley, 1987, 2003; Speakman, 2008). We also found a trend toward lower cortisol concentrations in the hair of quolls with higher Mn accumulation; elevated contaminant exposure is often associated with reduced ability to mount a normal post stress response (Franceschini et al., 2008, 2009; Gendron et al., 1997; Lorenzen et al., 1999; Love et al., 2003; Martinovic et al., 2003; Tartu et al., 2015). Accumulation of Mn was positively correlated with telomere length in males, but not in females, indicating potential differential effects of Mn on male and female aging.

Glucocorticoid synthesis and secretion are modulated by the hypothalamic–pituitary–adrenal (HPA) axis of vertebrates. Cortisol, among other functions, serves to mobilize energy for essential processes associated with survival. During the adaptive stress response, protein is catabolized, blood glucose levels rise, and immunity and inflammation are altered. Under chronic elevated glucocorticoid conditions, the stress response becomes pathological and may diminish health (Bradley, 1990). In fully semelparous dasyurids, such as *Antechinus* spp. and *Phascogale* spp., both males and females experience a spike in cortisol secretion during the mating season; this spike is higher for males and is thought to be directly linked with their death soon after breeding (Bradley, 1987; Bradley et al., 1980; McDonald et al., 1981). Our results suggest that the HPA axis in northern quolls behaves similarly: cortisol concentrations in hair are higher for males than for females, and are highest for both sexes during breeding; after breeding, cortisol returns to pre-breeding concentrations (Figure 3).

High Mn levels tended to be associated with low cortisol levels during breeding ($p = 0.08$); a study with a larger sample size is needed to confirm this effect and further characterize the potentially suppressive effect of Mn on adrenocortical activity.

The adrenal gland—the site of cortisol production—becomes hypertrophic during breeding in male northern quolls in response to chronic stress (Dickman & Braithwaite, 1992). Hypertrophy is characterized by increased organ size and reduced production of hormones including cortisol (Schoenfeld, 2020). The adrenals are vulnerable to environmental toxicity and may become hypertrophic in response to toxicants (see review by Harvey & Sutcliffe, 2010), indicating that Mn and other toxic elements could disrupt the HPA axis. Further research is needed to establish whether disrupted HPA activity reduces fitness in northern quolls.

Long-term elevation of cortisol and sex hormones are thought to be immunosuppressive in dasyurids. Most studies have not measured immune activity directly, but have recorded lesions (Oakwood et al., 2001), gut ulcerations (Barker et al., 1978; Bradley, 2003; McDonald et al., 1981), or bacteria or parasite loads in tissues (Barker et al., 1978; Oakwood et al., 2001). Regardless, declines in immune activity seem to be prevalent in semelparous marsupials and may be linked to a higher incidence of cancer in dasyurids (Canfield et al., 1990). We assessed immune activity using BKAs, which quantify the microbicidal activity of plasma (French & Neuman-Lee, 2012), and found that BKC decreased for both sexes across the breeding season. However, quolls with higher PC1_{Load}—and therefore quolls with a higher metal load, especially Mn and Fe—had on average 20% lower BKC, indicating that Mn and/or Fe contamination is immunosuppressive in northern quolls. The mechanism for reduced immune function could be related to Fe metabolism. Dietary Fe and Mn are both important to normal immune function (Cassat & Skaar, 2013). However, Mn competes for Fe binding sites, thus decreasing Fe absorption and ultimately causing anemia (Aschner et al., 2007). Because anemia is also characteristic of postbreeding physiology among other dasyurids (Bradley, 1987, 1997; Lee & Cockburn, 1985; McDonald et al., 1986), interactions between Mn exposure and breeding physiology on the development of anemia and impairment of immune function warrants further investigation.

In most mammals, telomeres shorten over the lifetime, and the rate of shortening is greater for species with a “fast” life history (characterized by rapid growth, high reproduction, and early death) and for individuals experiencing environmental stress (Monaghan & Haussmann, 2006). For this reason, we expected that the telomeres of semelparous male quolls would shorten more rapidly than those of nonsemelparous females. However, the telomeres of male quolls did not shorten. Furthermore, the telomeres of 1- and 2-year-old females did not significantly differ in length, indicating that female quolls recover telomere length between their first and second breeding seasons. On top of breeding stress, we expected to find that Mn accumulation would hasten the shortening of telomere length. Instead, we found that Mn was associated with increased telomere length in male quolls (PC1_{Load} and hair[Mn] models). This finding suggests several possibilities: (1) male quolls in high-Mn areas may be born with relatively longer telomeres than those in low-Mn areas, although this seems unlikely; (2) Mn may protect telomeres in males but not females,

which again seems unlikely; or (3) males with short telomeres and elevated Mn may die prior to reproduction, and therefore are not represented in our samples.

If high Mn exposure compounds the stress of reproduction, we would expect that the telomeres of high-Mn exposed quolls would degrade at faster rates during breeding. We tested this idea by examining longitudinal changes in RTL within quolls that were recaptured and found that RTL declined at significantly higher rates in quolls with higher Mn among prebreeding, breeding, and/or postbreeding seasons. Unfortunately, our recapture rate of individuals from sites near mining operations was low (suggestive of high mortality in individuals with higher Mn exposure); nevertheless, this preliminary test suggests that Mn increases the rate of telomere loss in northern quolls. Thus, the relationship between Mn concentration and telomere length may be complex. At the population level, Mn appears to be associated with increased telomere length among males only, yet recaptured animals indicate that high Mn exposure accelerates the rate of telomere shortening. Our finding warrants further investigation into these dynamics and the differences between the sexes. Although rare, a human study that observed telomere lengthening in healthy adults exposed to low doses of persistent organic pollutants suggested that low-dose exposure to carcinogenic chemicals may act as a tumor promoter at the very early stage of carcinogenesis in humans (Shin et al., 2010). Whether that might also be the case for the male northern quolls should be explored.

Our study is one of the first to characterize seasonal changes in the telomeres of dasyurids, which have unusual telomere dynamics (Bender et al., 2012). The technique we used—qPCR—is conservative, and averages telomere length across chromosomes. Studies using fluorescent in situ hybridization show that dasyurids appear to alter telomere length in their sperm and eggs—males lengthening and females shortening telomeres on each chromosome (Bender et al., 2012; Ingles & Deakin, 2016). Telomeric lengthening occurs via recombination or the enzyme telomerase (Rubtsova et al., 2012). In some mammals, such as humans, this only happens during embryonic development, within stem cells, or in a state of cancer (Blackburn, 2001; Nabetani & Ishikawa, 2011). In adult small mammals such as mice, however, telomerase is active even within somatic cells (Gomes et al., 2011; Ingles & Deakin, 2016; Seluanov et al., 2007). Further research into the telomere dynamics of dasyurids is warranted.

CONCLUSIONS

Sublethal environmental stressors such as exposure to toxic metals may compound the costs of breeding and shorten the already brief life spans of semelparous mammals, including the northern quoll and other threatened species. We have demonstrated an interaction between reproductive and environmental stress in quolls, one of only a small number of mammalian species with a suicidal breeding strategy. The roles of stress and environmental toxicants on reproduction and aging of

semelparous marsupials remain important areas of investigation. Beyond this, the long-term health of Australian ecosystems relies on the ability to minimize the environmental costs of mining, which is a key component of the Australian economy (Australian Bureau of Statistics, 2023). Understanding the impacts of contaminants on the biology and conservation of wildlife—especially those native to mining landscapes—is a relatively new but essential priority in Australia (Kendall, 2010; Penrose et al., 2022).

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (r.wilson@uq.edu.au) and uploaded in The University of Queensland's institutional repository, UQ eSpace.

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