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Published

2019

Journal Title

Science of The Total Environment

Version

Accepted Manuscript (AM)

DOI

[10.1016/j.scitotenv.2018.10.092](https://doi.org/10.1016/j.scitotenv.2018.10.092)

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PII: S0048-9697(18)33965-2
DOI: doi:[10.1016/j.scitotenv.2018.10.092](https://doi.org/10.1016/j.scitotenv.2018.10.092)
Reference: STOTEN 28995
To appear in: *Science of the Total Environment*
Received date: 27 July 2018
Revised date: 1 October 2018
Accepted date: 7 October 2018

Please cite this article as: C.A. Villa, I. Bell, C. Madden Hof, C.J. Limpus, C. Gaus , Elucidating temporal trends in trace element exposure of green turtles (*Chelonia mydas*) using the toxicokinetic differences of blood and scute samples. *Stoten* (2018), doi:[10.1016/j.scitotenv.2018.10.092](https://doi.org/10.1016/j.scitotenv.2018.10.092)

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Elucidating temporal trends in trace element exposure of green turtles (*Chelonia mydas*) using the toxicokinetic differences of blood and scute samples

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1.1. Abstract

Blood is considered a suitable biomonitoring matrix for evaluating relatively recent exposure to environmental contaminants since abrupt changes in exposure regimes are rapidly reflected in blood. On the other hand, keratinized tissues, such as turtle scutes, are known to integrate trace element exposure over relatively long time periods. This study aimed to test the use of the differences in blood and scute to inform on the historical trace element exposure of green turtles. We propose a blood-scute kinetic model to predict how an increase in exposure would affect the concentrations in these two matrices over time. We then tested the relationship between blood and scute concentrations for 19 trace elements in two green turtle populations presumed to experience relatively constant exposure conditions. Significant log-log and linear correlations were observed between blood and scute

concentrations for Co, As, Mo, Sb, and Cd. We then analysed blood-scute ratios in turtles from two coastal sites with known elevated exposure to various trace elements from previous studies. Deviations from the steady-state were clearly evident in these coastal turtles (for Co and Cd) and were consistent with the model prediction of changes in exposure. These field data provide evidence that blood-scute ratios can provide a valuable tool for examining the historical trace element exposure of turtles. We further present a method by which the general model may be refined and validated, by using data from individual turtles that had been recaptured across multiple years. Although the timeframe and number of recaptured samples available for this study were limited, the temporal changes in blood-scute ratios in these animals were generally consistent with those suggested by the model. Thus, the ratio between paired blood and scute trace element concentrations could be used to establish a temporal exposure index in turtles.

1.2. Introduction

Green turtle (*Chelonia mydas*) population numbers are robustly increasing in regions such as Queensland, Australia and Hawaii, USA in recent decades (Balazs et al., 2015; Chaloupka et al., 2008a; Limpus et al., 2013), with a corresponding increase in the number of reported strandings. In Queensland, green turtle strandings have been steadily rising since 1996 with a surge from 2009 through 2011 (Meager and Limpus, 2012). Far from being a regional issue, turtle strandings in the Hawaiian Archipelago have increased over the past 20 years or more where green turtles represented 97% of all reported turtle strandings (Chaloupka et al., 2008b). In both cases, investigators indicated that about half of these reports had no discernable stranding cause with disease as the second most common cause (Chaloupka et al., 2008b; Flint et al., 2017).

Understanding the health and prevalence of diseases in marine turtle populations provides a critical link between ecosystem health and turtle health (Jones et al., 2016). Diseases such as fibropapillomatosis as well as clinical markers of poor health in green turtles have often been reported to correlate with poor water quality (Adnyana et al., 1997; Ariel et al., 2017; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1994; Van Houtan et al., 2010; Villa et al., 2017). For green turtles that forage in shallow coastal embayments adjacent to urban and industrial activity, elevated exposure to pollutants, including non-metals, metals and metalloids and their compounds (trace elements), can occur as a result of both natural and anthropogenic impacts (e.g. floods, agricultural and industrial runoff, urbanisation, coastal dredging). It is therefore essential that we explore the links between contaminant exposure and declines in green turtle health. Baseline data, established

using carefully selected reference populations, will allow us to explore the links between trace element exposure and declines in green turtle health.

Trace element reference intervals for green turtle blood have only recently been developed to help identify elevated concentrations of elements in blood (Villa et al., 2017). However, depending on their blood elimination rates, trace element biomonitoring using blood is sensitive to the time at which samples are collected (Villa et al., 2017). On the other hand, marine turtle scutes, the outermost keratinized layers of the carapace, have been shown to provide long-term exposure information whereby each scute layer contains a snapshot of the blood concentrations from when it formed. Although the exact time of exposure is uncertain, scute is assumed to integrate trace element exposures over the past ~1.4-2.8 years (i.e. the estimated lifetime prior to shedding; (Vander Zanden et al., 2013)). There are currently no reported methods on ageing individual scute layers in green turtles which would be required to discern exposure at particular times. Nonetheless, time-integrated information can be obtained from the entire scute depth providing an accumulated trace element profile. Unlike blood, there are no trace element reference intervals or baselines for green turtle scute, limiting the usefulness of analytical data. One novel approach, however, is to use the exposure information from both blood (short-term signal) and scute (long-term signal) to inform on the temporal aspects of trace element exposure (Bezerra et al., 2012).

The distribution of trace elements from blood to all other tissues is governed by toxicokinetic parameters. Under relatively constant exposure conditions turtle blood and scute concentrations should approach steady-state conditions (Grillitsch and Schiesari, 2010).

Green turtles have a complex life cycle, in which adult phase includes long-distance migrations, metabolic shifts and maternal trace element transfer associated with breeding. Conversely, the oceanic juvenile turtles are omnivores where internal trace element concentrations are most likely to reflect opportunistic oceanic foraging at multiple trophic levels. Most of the key inter-individual and life-history factors that may significantly deviate from constant exposure condition can be minimised by selecting sexually immature individuals. Among these, subadults represent the oldest non-migrating and non-breeding class which reside within a small foraging range (few km²) over several decades (Chaloupka et al., 2008a; Hazel et al., 2013; Limpus, 2008; Shimada et al., 2016).

The steady-state relationship of trace element concentrations between blood and scute can be identified using paired blood and scute from subadult green turtle populations that forage in areas expected to experience relatively stable natural and anthropogenic trace element exposure. This includes, for example, foraging grounds distant from point sources and relatively remote to anthropogenic disturbances, which receive minimal deposition from terrestrial, atmospheric, and aquatic sources, as well as from nearshore protected areas that receive little riverine input and where the catchment is free from most anthropogenic activity. The following conceptual models detail how knowledge on the steady-state relationship may be used to inform on the temporal trace element exposure in other populations.

1.3. Conceptual model

The following model (Figure 1, top) illustrates our understanding of how trace elements are internally distributed in turtles, using a 2-tissue compartment (blood and scute) toxicokinetic approach (Nordberg et al., 2015) for turtles experiencing a

relatively constant exposure. For this kinetic blood-scute model, we assumed a relatively constant exposure (background) to a hypothetical element with a moderately short blood elimination half-life (see SI for model parameters).

At the onset of increased exposure, blood concentrations rise relatively rapidly (Figure 1, ①), and are deposited into the newest forming scute layer. As a result, the element concentration across the entire scute thickness increases slowly with each scute layer formed during elevated exposure. When exposure ceases, blood concentrations return to near background levels relatively fast (Figure 1, ②), and new scute layers reflect this lower exposure regime. In contrast, the scute layers formed during elevated exposure maintain the accumulated element concentration of that time. As a result, the scute over its entire thickness remains elevated compared to background. As old scute layers shed, the layers with high trace element concentrations are transported closer to the surface until they are ultimately eliminated. Long after blood has returned to baseline levels, the high element concentration locked within the scute persist until it gradually decreases with each shedding scute layer.

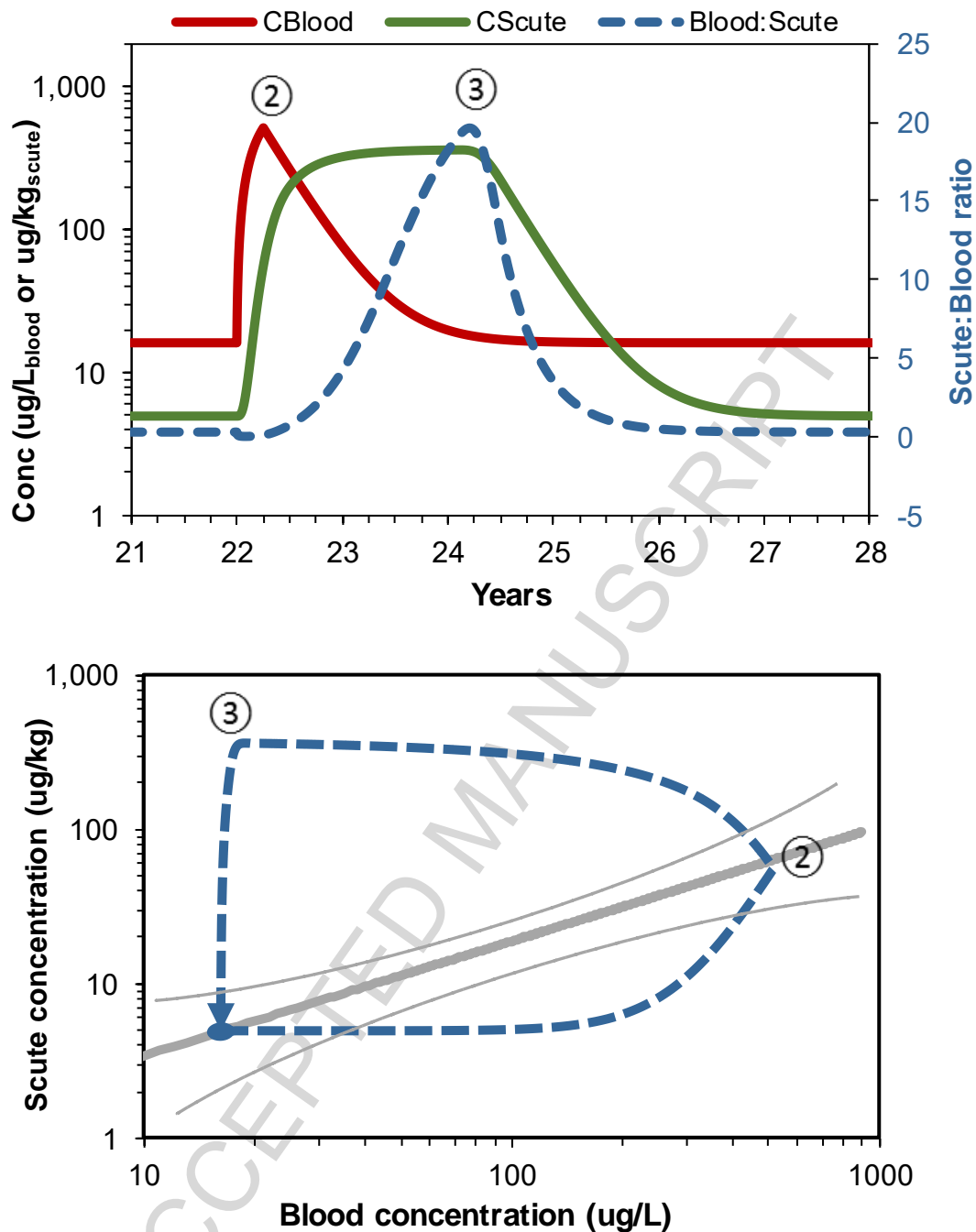


Figure 1. Top: Model of the temporal concentration course of a hypothetical trace element in blood and scute following a single elevated exposure period. Bottom: corresponding blood-scute ratios over time relative to the steady-state concentration (grey line and 90 percent prediction intervals; see results below); ① Baseline exposure and the onset of elevated exposure regime; ② peak trace element concentration in blood; ③ persistence phase in scute.

Theoretically, with steady-state exposure conditions, the blood-scute ratio remains constant over a range of exposure magnitudes (slope of Figure 1 bottom graph). When elevated exposure is encountered, the response in each matrix can be tracked for individual turtles against this steady-state relationship (Figure 2). At any given time, a blood-scute ratio which plots below the steady-state regression line is indicative of a recent exposure which is higher relative to historical levels (Figure 2, first panel). Conversely, when blood-scute ratios plot above the steady-state regression, scute concentrations are higher than predicted providing evidence of a historically elevated exposure relative to recent levels (Figure 2, last three panels). Of note are the very similar concentrations expected in the second and third panels of Figure 2 as scute layers continuously form and are shed.

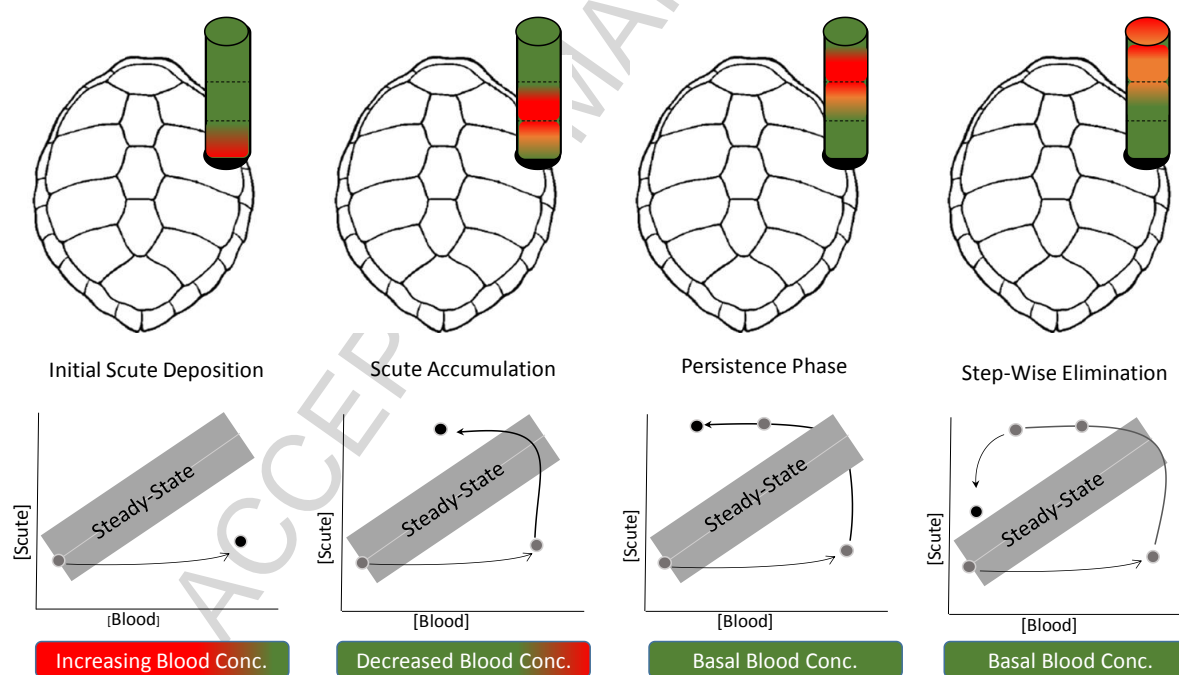


Figure 2. Time course of blood and scute concentrations following an increased exposure regime compared to steady-state. The line and shaded area in the lower graphs represent the steady state blood scute ratio. The dots (bottom graphs and bars) and scute illustrations (top

row) show the time course of blood and scute concentrations after an increased exposure regime.

Several studies have shown positive linear, as well as log-log correlations between trace elements (i.e. Zn, Mn, and Hg) in scutes and internal tissues of green turtles (Bezerra et al., 2012; Faust et al., 2014; Komoroske et al., 2011; van de Merwe, 2009), loggerheads (Bezerra et al., 2012), Kemps Ridley's (Innis et al., 2008), and other estuarine and freshwater turtles (Dyc et al., 2016; Schneider et al., 2015; Smith et al., 2016). The use of paired blood and scute to elucidate temporal exposure in marine turtles has to date only been investigated by Day et al. (2005), specifically for Hg in loggerhead turtles. The authors used blood and scute to demonstrate that recent exposure of Hg correlated with the proximity of the capture sites to highly industrialised watersheds. Elucidating temporal exposure using a ratio between blood and keratinised tissues (hair, nails, feathers, skin, or scute) has not been described for any other species or element presumably due to the need to first describe the steady-state relationship between these matrices for each element. Such a relationship may be difficult, if not impossible, to determine experimentally or to extrapolate from the literature since the majority of those studies relied on moribund or stranded turtles, from populations close to point sources or unknown foraging areas, and were often composed of mixed age classes. In the interim, approximations for the chemical distribution between tissues under steady-state conditions can be inferred using turtle populations exposed to constant exposure conditions. This could include foraging turtles from pristine or impacted sites, as long as the exposure conditions do not significantly vary over time.

For over three-years the Rivers to Reefs to Turtles (RRT) project has explored the links between environmental contaminants and turtle health with results of novel

chemical analyses of water and sediments (Gallen et al., 2018 THIS SPECIAL ISSUE), green turtle forage (Thomas et al., 2018 THIS SPECIAL ISSUE), turtle tissues (herein and Heffernan et al. (2017); Vijayasarathy et al. (2018 THIS SPECIAL ISSUE); Villa et al. (2017)), as well as, genetic stock analysis Bell et al. (2018 THIS SPECIAL ISSUE), and clinical health metrics Brand et al. (2018 THIS SPECIAL ISSUE); Flint et al. (2018 THIS SPECIAL ISSUE) compiled within a special issue of Science of the Total Environment (THIS SPECIAL ISSUE). Thus, the aims of this study were first to examine the relationship between blood and scute for multiple elements using populations of turtles that are expected to experience close to constant exposure conditions, thus approximating a steady state between blood and scute. The resulting steady-state relationships were then used to test the use of blood-scute ratios to understand the exposure history for turtles from two case study sites, which included turtles repeatedly sampled across different years (recaptures).

Materials and Methods

1.3.1. Sampling Campaigns and Capture Details

Paired blood and scute samples were collected from 118 subadult green turtles (female curved carapace length (CCL) ≥ 60 cm to ≤ 100 cm; males ≥ 63 cm to ≤ 95 cm; unknown sex ≥ 65 cm to ≤ 90 cm) captured as part of the RRT collaborative research program on marine turtle ecology and health at different foraging grounds in Queensland, Australia from 2013 – 2017. Detailed descriptions of the geomorphology, chemistry, and physio-chemical properties of the sediments, water, and seagrasses at each site are detailed in Thomas et al. (2018 THIS SPECIAL ISSUE) and Bell et al. (2018 THIS SPECIAL ISSUE) as part of a special issue investigating organic and inorganic exposure to green turtles. Briefly, the Howick

Group of Islands (HWK), is a remote collection of sheltered mid-shelf, uninhabited reefs which lie within the remote northern Great Barrier Reef Marine Park (14.416695°S, 144.880484°E;). HWK is located approximately 30 km offshore from the Cape York region catchment, a remote coastal wetland with low pressures from nutrient, sediment, and pesticide loads, or water regime changes and habitat alterations (Bell et al., 2018 THIS SPECIAL ISSUE; Senior et al., 2015). Triangular Island in Shoalwater Bay (SHL; 2.370076°S, 150.515198°E), is a large embayment with no major rivers draining into the Bay. The catchments of these streams were predominantly grazing land prior to establishment of the Shoalwater Bay Military Training Area commencing in 1965. The majority of the area contained within the training area functions as a buffer zone that separates the public from the core activities of defence training (Limpus et al., 2015). Triangular Island is subject to higher rates of tidal flushing (>7m tidal range during spring tides) in comparison to any of the other study sites (Limpus et al., 2005; Limpus et al., 2015). In this context, Shoalwater Bay represents the largest area of relatively undisturbed marine embayment for eastern Australia. Cleveland Bay (CLV; 19.235428°S, 146.938284°E) is adjacent to the city of Townsville (population >175,000), which is home to a major port and major industries including metal processing and refining (Zn, Cu, Ni, and Co) (Esslemont, 2000). Upstart Bay (UPB; 19.767554°S, 147.702955°E), a rural coastal area approximately 100 km south of CLV and 50 km north of an international coal terminal, with a system of mostly ephemeral creeks receiving wet-season discharges from one of Queensland's largest catchments (130,000 km²) dominated by intensive agriculture, grazing and mostly legacy mining (Bartley et al., 2014).

Each of the foraging grounds was visited multiple times resulting in recaptured individuals (n=28) both within and across sampling years (Table 1), with the

exception of SHL which for logistical reasons was only visited once. All blood concentrations from 2014 were previously reported in Villa et al. (2017) with all other blood and scute samples reported here for the first time.

Table 1. Number of recaptured turtles analysed at each site.

	Captured		Recaptured		
	2013	2014	2015	2016	2017
Howick Island Group (Pristine/Offshore Control)	--	34	10	--	--
Shoalwater Bay (Military/Coastal Control)	--	28	--	--	--
Upstart Bay (Agriculture)	3	29	7	4	2
Cleveland Bay (Urban-Industrial)	--	27	1	4	--

All turtles were captured using rodeo techniques (Limpus and Reed, 1985) and returned to shore for processing (tagged, measured, weighed, visually assessed for health status (Thomson et al., 2009). Blood samples were collected and processed according to methods described in Villa et al. (2017): Briefly, whole blood was collected from the dorsal cervical sinus using disposable syringes fitted with 21-18 gauge needles. For trace element analyses, a total of 5 mL of whole blood was transferred into acid-rinsed, sodium heparin dosed (10 IU/ml whole blood), polyethylene tubes (1,000 IU/ml whole blood; DBL™ Hospira, Australia). Carapace scutes were scrubbed with seawater and briefly rinsed with methanol before collecting approximately a total of 1 gram (total scute fragments) subsampled from the posterior three marginal bridging scutes of each side of the carapace-plastron interface and the two supracaudal scutes (SI Figure 1). Further treatment of scute

fragments was performed in the laboratory to reduce residual exogenous contamination (SI Scute In-lab Processing).

1.3.2. Trace Element Analysis

Trace element analysis of blood followed methods previously described by Villa et al. (2017) with details provided in the supplement materials (SI Blood and Scute Digestion). Briefly, 1ml whole blood aliquots, in acid rinsed polyethylene tubes, was amended with high purity concentrated HNO₃ and H₂O₂ (1ml each) and heated on heating block until fully digested. Scute sample processing followed a similar methodology with the following modifications: Briefly, 0.5 mL concentrated HNO₃ and H₂O₂ was added to 250 mg of clean, dry scute fragments in acid rinsed polyethylene tubes and heated on a heating block until fully digested.

All samples were analyzed via inductively coupled mass spectrometry (ICP-MS ; Agilent 7900) and processed using Agilent's ChemStation software (ICP-MS MassHunter, version 4.3). Method detection limits (MDLs) were defined as three times the standard deviation of the blanks (SI Table 1).

1.3.3. Statistical Analysis

Trace element data distributions were initially assessed for normality using histograms and boxplots and evaluated statistically using the Anderson-Darling test. Outliers were identified visually and through ROUT (False Discovery Rate set to 1%) and Spearman Rank Correlations performed between blood and scute trace element concentrations. Between year comparisons of blood or scute samples were performed using the Wilcoxon matched-pairs signed rank test ($\alpha=0.05$). Data visualisation, outlier detection, normality tests, paired comparisons and correlations were performed using GraphPad Prism, version 7.00 for Windows (GraphPad

Software, San Diego California, USA). One-way analysis of variance comparing group means and subsequent posthoc tests were performed in using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY).

For those elements with significant positive correlations, Model II regression (ordinary-least-squares [OLS] and Major Axis [MA]) models were used to evaluate the relationship between paired blood and scute trace elements and a 90% prediction interval calculated. Per the regression selection criteria outlined by Legendre and Legendre (2012), the OLS method was chosen when the error in the dependent variable (scute) is >3x that of the independent variable (blood). Similarities in regression variable estimates between HWK (off shore control) and SHL (coastal control) were evaluated using Akaike's Information Criterion in GraphPad Prism.

The regression variable estimates were evaluated using the D'Agostino & Pearson omnibus K2 test for normality of their residuals and a test for homoscedasticity. Regression estimates, evaluations and 90% prediction intervals (RPI₉₀) were determined using GraphPad Prism. Significance was defined as $p \leq 0.05$ for all of the above tests.

1.4. Results and Discussion

Blood and scute concentrations in all turtles were determined for 19 elements (Na, Mg, K, Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Ba, and Pb; SI Figure 2). In addition, concentrations of the elements Be, Al, Sn, Hg, Tl, Th, and U were quantified, but were mostly <MDL and thus excluded from further analyses. Box plots with individual turtle trace element concentrations in blood are compared by year in SI Figure 2 for each site.

1.4.1. Constant Exposure and Steady-State Blood Scute ratios

Turtles sampled in 2014 from two sites with expected relatively stable and constant exposure regimes (Table 1) were used to evaluate steady-state relationships between blood and scute trace element concentrations: HWK (pristine/offshore control) and SHL (military/coastal control). No temporal trend data is available for the SHL turtles. The assumption of constant exposure is based on the distance of this foraging site from known major point sources, the high flushing rate for the Bay, the absence of significant riverine inputs, and a previous investigation in this region indicating low persistent organic contaminants in green turtle blood (Hermanussen, 2009). In addition, turtles from the SHL region were previously described by Flint et al. (2010) as a healthy cohort and used to establish reference ranges for clinical health markers and haematology.

Out of the 19 trace elements tested, significant positive correlations (SI Table 2), and normally distributed standardised regression residuals were obtained between paired blood and scute concentrations of Co, As, Mo, Cd, and Sb (Figure 3) indicating that these elements would be good candidates for blood:scute steady-state regression described in our conceptual model (Figure 1 bottom). No significant differences for these elements were found in scute concentrations between 2014 or 2015 HWK populations and all blood concentrations for 2015 HWK turtles were within baseline reference interval limits (SI Figure 3 top) with the exception of Sb and Cd which had either blood or scute values for 2015 all <MDL (SI Figure 3 Top). This supports our original hypothesis that the HWK subadult population is generally subject to low and relatively stable trace element exposure for these elements. To be certain that these populations could be combined, we explored possible blood and scute concentration differences between HWK and SHL for each element. Although the absolute trace

element concentrations between these populations differ (for example Co; SHL > HWK), regression variables (slopes and intercepts) for 2014 HWK and 2014 SHL populations did not significantly differ from the global (HWK and SHL combined dataset) slope and intercept. It is, therefore, reasonable to combine both data sets into a single group, which has the advantage to provide a wider range of exposure magnitudes and thus steady-state blood-scute trace element concentrations than either population could produce alone (particularly for Co, Mo, and Sb; SI Figure 3).

Significant log-log regression statistics were obtained for Co, As, Mo, and Sb concentrations in blood and scute, with linear regression for untransformed Cd concentrations. The log-log and linear regressions from the combined blood and scute concentrations for Co, As, Mo, Cd, and Sb of HWK and SHL are expected to approximate a steady-state ratio between these two matrices (as depicted in Figure 1). Normality of Cd blood and scute concentration distributions could not be achieved through transformation (Logarithmic or Box-Cox) which precluded the use of Model II regression methods other than OLS, even though the measurement error ratio (scute to blood) was < 3. No transformation was necessary prior to OLS regression to achieve normality of the Cd residuals. Thus it is the only element plotted using untransformed concentration data (Figure 3).

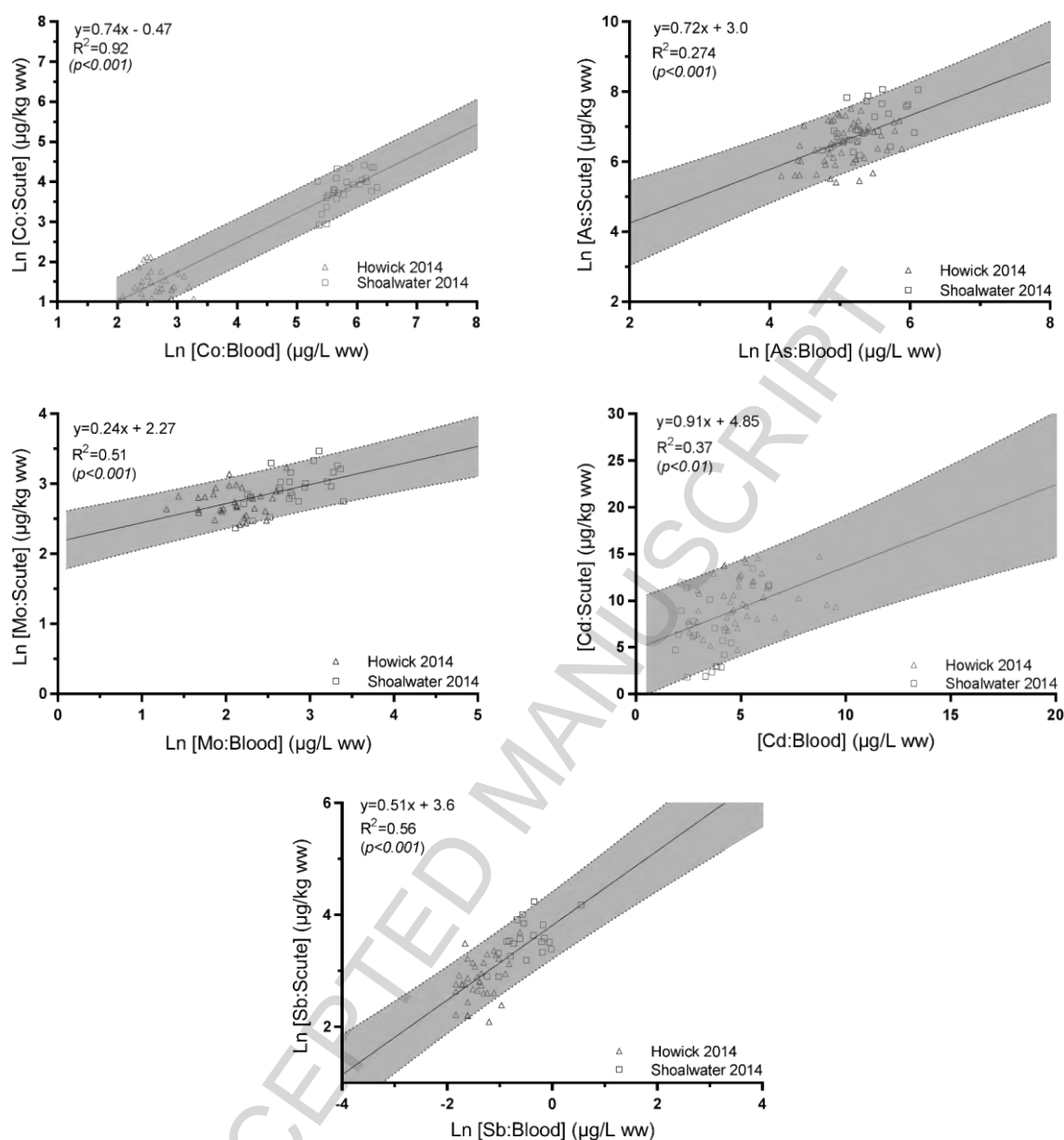


Figure 3. Regression between blood and scute (2014 HWK open triangles and SHL open squares) showing a 90% prediction interval (grey area; RPI_{90}) for natural log-transformed Co, As, Mo and Sb concentrations. Concentrations and regression parameter estimates for Cd are based on non-transformed data.

Day et al. (2005) calculated blood and scute regression residuals for a group of apparently healthy live captured turtles. The residuals from each individual blood and

scute point were calculated as Euclidean distances from the line of best fit and their sign (positive or negative) was used to identify departures from their regression model, but the authors did not indicate how large a residual they considered adequate before it is no longer considered to be within the steady-state. We reason that the upper and lower bounds of the 90% prediction intervals (RPI_{90}) define a conservative threshold to identify points that do not lay within a steady-state. Hence, paired blood and scute ratios below the RPI_{90} indicate higher concentrations in blood compared to those in scute under steady-state conditions (or vice versa), and thus elevated exposure that occurred relatively recently. In contrast, ratios that fall above the RPI_{90} indicate lower concentrations in blood compared to those in scute under steady-state conditions, and thus elevated past exposure.

An example of this can be seen on the HWK 2015 ratio of blood to scute concentrations for Co, As, Mo, Sb and Cd which the majority of all points lay within their respective RPI_{90} further indicating no major alterations in exposure conditions over the approximately 1-year sampling interval (SI Figure 4). This further supports the notion that stable and low exposure profiles persist for the HWK turtles.

1.4.2. Case Studies

Individual paired blood and scute concentrations of Co, As, Mo, Sb and Cd from turtles foraging at two coastal sites in Queensland, Australia (CLV and UPB) were compared against the steady-state relationships developed from the HWK and SHL 2014 populations. The recapture data further allows tracking of the short-term (blood) and long-term (scute) signals as exposure conditions change over time, offering a unique opportunity for validating the conceptual model of this study (as depicted in Figure 2).

Each of the coastal sites is influenced by unique mixtures of trace elements and organic contaminants which reflects their respective land use (water and sediments (Gallen et al., 2018 in-press), green turtle forage (Thomas et al., 2018 in-press), turtle tissues (Heffernan et al. (2017); Vijayasathy et al. (2018 in-press); Villa et al. (2017)). Thus, each of these case studies offers a different perspective for temporal exposure investigations using field data. The first, UPB, is a case where turtles likely have experienced acute exposure to Co, Sb, and Mo (Villa et al., 2017). The second case, CLV, is an example of a turtle population with previously described haematological indicators of poor health and elevated exposure to Sb and Co based on 2014 comparative blood data (Villa et al., 2017), although no reports of mass mortality have been reported. Of the four subsequently recaptured turtles (2014 and 2016 n=3 and, 2015 and 2016 n=1), paired blood and scute were available for three individuals.

1.4.2.1. Case-Study 1: Upstart Bay

The green turtle population at UPB was subject to an unusual mass stranding and mortality event in 2012 (2-years prior to the first sampling campaign of this study), where abnormal neurological symptoms were observed closely followed by death, and with recurring isolated episodes. Semiquantitative trace element blood analysis for one of the 2012 euthanised turtles identified V, Co, Ni, Sb, TI, and U as potential monitoring targets (Villa et al., 2015). In 2014, UPB turtles were confirmed to have high Co (470 ± 190 $\mu\text{g/L}$; range 130-840 $\mu\text{g/L}$), Sb (2.5 ± 1.2 $\mu\text{g/L}$; range 0.81-6.4 $\mu\text{g/L}$), and Mo (25 ± 15 $\mu\text{g/L}$; range 3.3-68 $\mu\text{g/L}$) blood concentrations relative to reference intervals (Villa et al., 2017) leading to the hypothesis that these elements may have posed an acute threat to turtles during the mass stranding event.

Additional clinical findings for the 2014 UPB turtles identified markers of systemic

stressors including liver dysfunction, which correlated in particular with Co and Sb concentrations in blood (Villa et al., 2017).

Recapture data demonstrated that elevated blood concentrations of Co, Mo, and Sb for UPB turtles remained elevated until 2017 (SI Figure 2). While there are no reference intervals for trace elements in scute, we can utilise the blood-scute steady-state regression to identify a range of predicted trace element concentrations in scute or blood that would be representative of a constant exposure scenario. At the measured Co blood concentrations, Co scute concentrations in UPB turtles were significantly higher in a large proportion (70%) of animals sampled in 2014 as well as the 2015, 2016 and 2017 recaptures, compared to the 90% steady-state prediction interval (Figure 4). This suggests Co exposure was highest prior to the first sampling event in 2013.

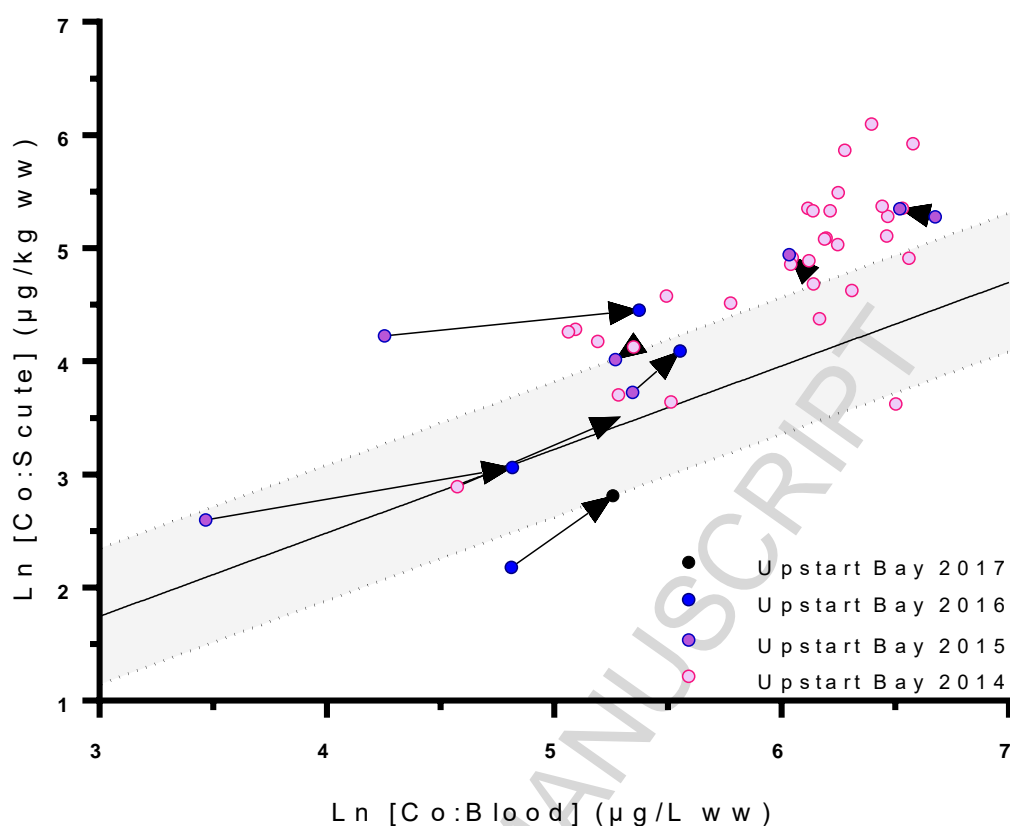


Figure 4. Paired blood and scute cobalt concentrations for UPB turtles sampled in 2014-2017 (Arrows indicate the temporal changes in recaptured individuals). Steady-state regression model (HWK and SHL) shown with 90% prediction intervals (grey area).

Although the exact time of exposure is uncertain, scute is assumed to integrate trace element exposures over the past ~1.4-2.8 years (Vander Zanden et al. (2013). While it may be tempting to extrapolate the historical peak blood concentrations (approx. $>5,000 \mu\text{g}/\text{L}$) based on the observed scute concentration, the toxicokinetic behaviours of Co in green turtles or the saturation concentration limits for any particular biological matrix are still unknown. This is particularly relevant for essential trace elements like Co where various metabolic processes are believed to be responsible for maintaining Co levels within different compartments of the body (Leggett, 2008). Thus, the steady-state log-log relationship may not hold at either

extreme of the model where we expect increased rates of detoxification (i.e. excretion or protein binding). Overall, the blood-scute ratios observed for Co are consistent with the previous hypothesis of acute cobalt exposure in 2012 (Villa et al., 2017), and indicate persisting elevated blood concentrations in line with the expected slow Co blood elimination rates observed in other vertebrates (Ayala-Fierro et al., 1999).

Concentrations of Cd in UPB turtle blood were within reference intervals (2.0-9.1 $\mu\text{g/L}$; Villa et al. (2017)) in 2013 and 2014 but were elevated above reference interval upper limits in half of the recaptures (n=2) in 2015 and 2016, and once again within reference interval limits for 2017. Blood-scute ratios for the recaptured turtles (Figure 5) are consistent with the kinetic model for a previous elevated exposure.

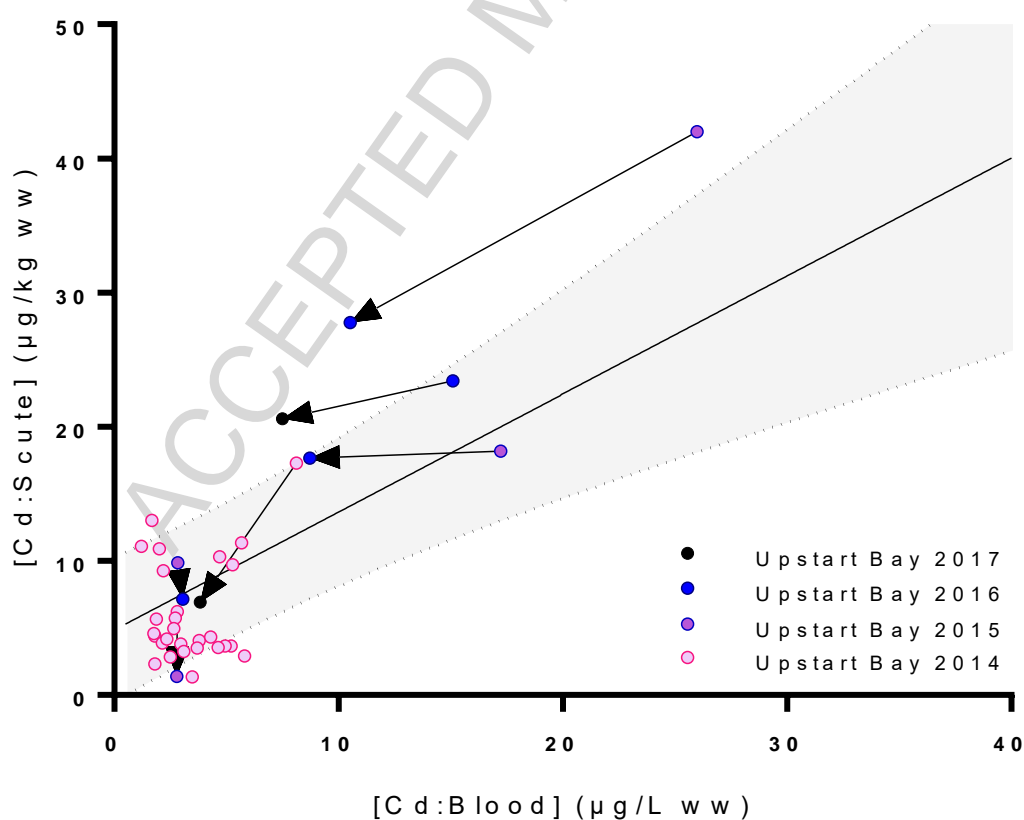


Figure 5. Paired blood and scute cadmium concentrations for UPB turtles sampled in 2014-2017 (Arrows indicate the temporal changes in recaptured individuals). Steady-state regression model (HWK and SHL) shown with 90% prediction intervals (grey area).

Among most vertebrates, Cd is reported to accumulate with age, primarily in liver and kidney tissues, with relatively long biological half-lives (7-10 years; (ATSDR, 2008)). The exception is blood, where clearance rates can be very fast due to a high binding affinity to transport proteins (primarily metallothioneins). A Cd feeding study of red-eared slider turtles (*Trachemys scripta elegans*) demonstrated that even with highly contaminated diets, blood levels remained low making it a poor biomonitoring matrix for elevated exposure (Guirlet and Das, 2012). In a similar study using painted turtles (*Chrysemys picta*) <10% of total Cd dose remained in blood 8-days post exposure with rapid accumulation in carapace and plastron over that time period (Rie et al., 2001). Interestingly, the toxicological information available for Cd in other vertebrates indicates that an elevated concentration in blood would be an unlikely observation given the exceedingly fast blood half-life. In 2015 and 2016 we observed elevated blood Cd concentrations with one case of an accompanying scute concentration indicative of an elevated past exposure. The recapture data consistently show a large decrease in blood concentrations with some evidence of decreasing scute concentrations, becoming more pronounced over greater time intervals. This suggests an elevated past exposure as well as a persisting exposure occurring at the time of sampling in 2015 and 2016.

Paired blood and scute concentrations of Mo in UPB turtles indicate historical exposure for some 2014 individuals (above the RPI_{90}), however, all recaptured turtles analysed in subsequent sampling years were within the RPI_{90} (SI Figure 5). Although no toxicokinetic information is available for Mo in green turtles, studies on

its distribution in other vertebrates indicate rapid accumulation 1-24 hrs after dietary exposure with a return to near basal blood levels after 1-hr in humans and near complete excretion within 2-weeks for guinea pigs, rats, goats, and pigs (Tallkvist and Oskarsson, 2015). Even if the toxicokinetics of Mo is rapid in green turtles, increases in blood Mo observed in recaptured turtles are shown to generally correspond with increases in scute as predicted by the kinetic model. While blood-scute Mo temporal patterns appear consistent with a past acute exposure for a few individuals, the recapture data indicates a more recent exposure also occurred in 2016 and 2017.

Blood - scute ratios for As from several UPB turtles indicate recent exposure in each sampling year (SI Figure 6). Blood and scute As concentrations in recaptures appear to be changing in a manner that is inconsistent with the conceptual model of this study. This is likely due to the expected higher variability in As accumulation as a result of the naturally high abundance of organoarsenical compounds in seagrasses (Kilminster, 2013) and algae (Agusa et al., 2008; Kunito et al., 2008) which make up the bulk of the green turtle diet. Studies on the kinetics of As have demonstrated that organoarsenicals (the dominant and relatively low toxic As form in marine systems) are readily absorbed in the gastrointestinal tracts of both animals and humans and can be rapidly excreted in urine and faeces (Fowler et al., 2015). Considering this and the relatively low As concentrations in UPB turtles, the random temporal patterns may simply reflect recent feeding behaviour or a change in diet composition favouring one source (i.e. algae) over the other.

While elevated blood Sb concentrations in 2013 have been declining until 2017, all turtles sampled remain above the Sb reference interval (0.071-0.94 $\mu\text{g/L}$; Villa et al. (2017)). Blood-scute ratios of Sb in 2014 individuals were mostly within the RPI_{90} limits with a few exceptions. In subsequent years, all blood-scute Sb concentration ratios were below the RPI_{90} indicating a potential recent or on-going exposure. Unfortunately, the relatively large analytical error for Sb data makes it difficult to interpret the results with a great degree of confidence. This is apparent in the recapture data (SI Figure 7) where a within-year recapture had similar blood concentrations but scute concentrations differed by an order of magnitude. Nevertheless, the consistently elevated blood concentration, above the reference interval upper limits, still warrants concern. Sb blood elimination kinetics in other vertebrates is very rapid (70%-90% of dose eliminated within 48-hours; (ATSDR, 1992; Sundar and Chakravarty, 2010), making it difficult to make any inferences regarding Sb exposure condition during the 2012 mass mortality event at UPB, particularly since we did not observe blood-scute ratios above the RPI_{90} in 2014 turtles that would indicate elevated past exposure.

The objective of the RRT programme was to test the hypothesis that acute exposures of trace elements (particularly Co, As, Cd, Mo, and Sb) played a role in the 2012 UPB turtle mass stranding event. The key limitation was that no paired blood and scute samples were collected during or immediately after the mass stranding. A resulting 2-year gap between suspected exposure and first sampling was too long for some elements to have remained within the available biomonitoring matrices to provide insight into potentially elevated exposures. This is especially true for those elements suspected of having rapid blood elimination half-lives (i.e. Mo and Sb) in green turtles. Since it is currently not possible to ascertain the timing of the

initial exposure, it is possible that enough time has elapsed such that the peak exposure period recorded in the scute has already shed (i.e. Figure 2 final panel). Nevertheless, elements with very long blood elimination rates in most vertebrates (i.e. Co or Cd) would be expected to result in a persistent body burden.

1.4.2.2. Case-Study 2: Cleveland Bay

Elevated concentration of Co, Sb, As, and Cd were found in the blood of 2014 CLV turtles relative to the upper limits of green turtle reference intervals (Villa et al., 2017). Additional haematological findings suggested poor health (low albumin:globulin ratio and elevated creatinine kinase) in the 2014 CLV population due to a yet to be identified systemic stressor. The correlations previously described between trace elements and the haematology (poor health indicators positively correlating with elevated blood Co, Sb, As, and Cd) suggested that turtles from CLV faced chronic trace element exposure conditions. As with the previous case study, the aim here is to utilise the blood-scute trace element ratios to help elucidate temporal trends in trace element exposure for a wild population of green turtles.

Blood Co concentrations in CLV were elevated above reference interval limits (6.3-40 $\mu\text{g/L}$; Villa et al. (2017)) for 2014 through to 2016. Blood-scute ratios from the 2014 sampling event indicate that 44% of those turtles were above the RPI_{90} (elevated past exposure). The recaptured turtles analysed in subsequent years all showed an increase in scute cobalt levels which is consistent with the modelled scenario of a past elevated exposure.

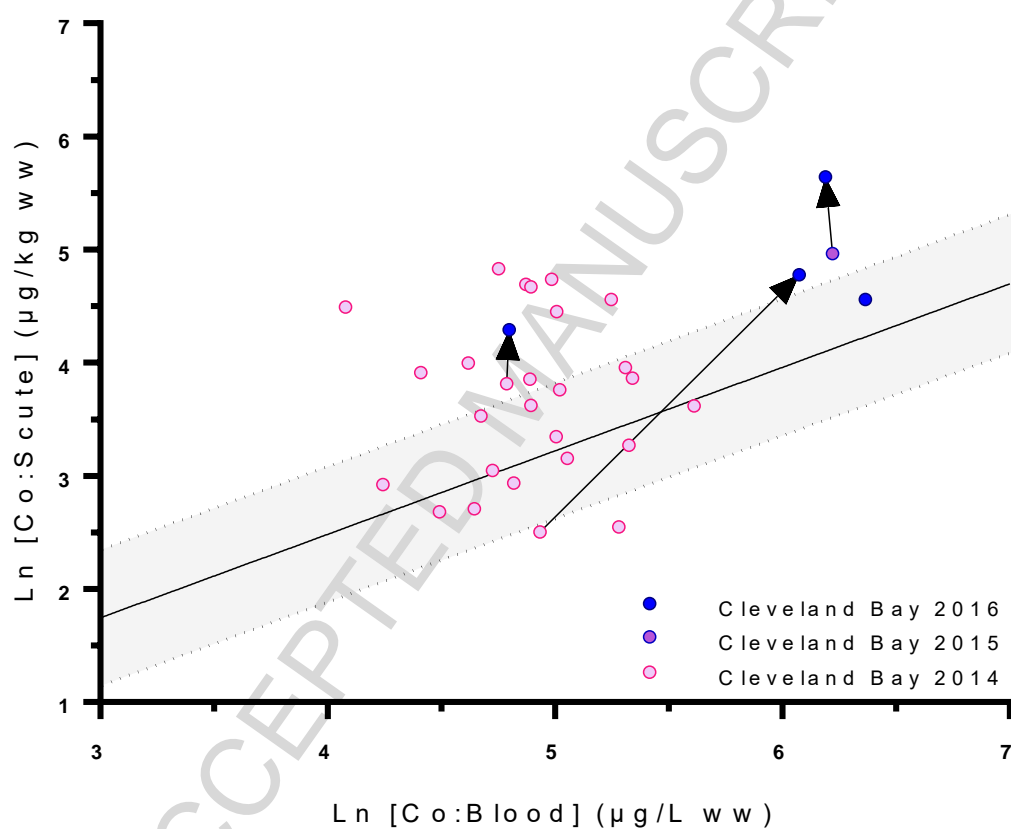


Figure 6. Paired blood and scute cobalt concentrations for CLV turtles sampled in 2014-2016 (Arrows indicate the temporal changes in recaptured individuals). Steady-state regression model (HWK and SHL) shown with 90% prediction intervals (grey area).

Concentrations of Cd in green turtle blood are predicted to be low based on toxicological data from most other vertebrates which demonstrate a very fast blood elimination half-life. Interestingly, we observe a single CLV turtle with Cd in blood 8-

times greater than the upper reference interval limit (Figure 7). This individual also had a blood and scute concentration below the RPI_{90} indicating a recent exposure in 2014. Upon being recaptured in 2016, blood and scute concentrations decreased with blood still remaining over 2-times the upper reference interval limit. In order to adequately interpret these data, additional analyses would be required for turtles after the 2014 campaign.

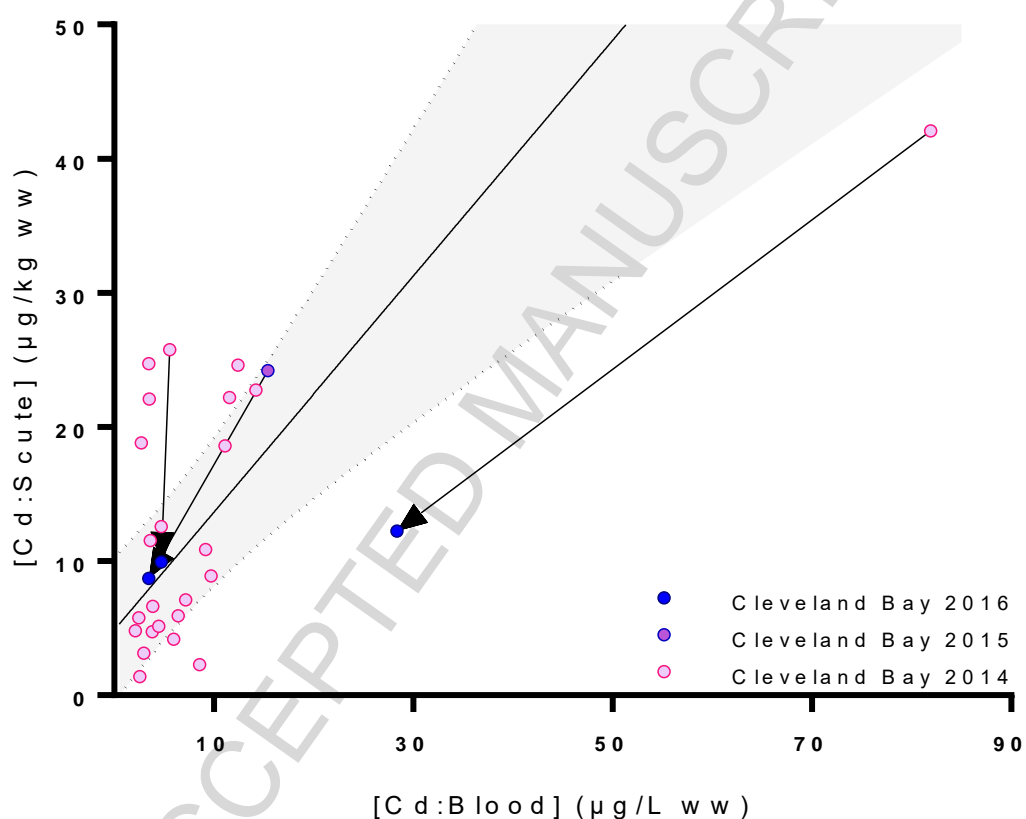


Figure 7. Paired blood and scute cadmium concentrations for CLV turtles sampled in 2014-2016 (Arrows indicate the temporal changes in recaptured individuals). Steady-state regression model (HWK and SHL) shown with 90% prediction intervals (grey area).

Blood concentrations of Mo for the majority of 2014 CLV turtles fell within the reference intervals (3.5-15 $\mu\text{g/L}$; Villa et al. (2017)). Similarly, the majority of blood-scute concentration ratios fell within the RPI_{90} (SI Figure 8). Although the blood-scute

ratio for one turtle was well below the RPI_{90} for Mo, its blood concentration was within the Mo reference interval, indicating that this is likely not an elevated exposure of concern. Two of the three recaptured turtles remain within the RPI_{90} 2-years later. Additional recapture data for each of the years past 2014 are needed to provide a better indication of exposure trend.

In 2014, 40% of CLV turtles had blood As concentrations above the reference interval limits (63-420 $\mu\text{g/L}$; Villa et al. (2017)) with an equal number of turtles with blood-scute ratios below the RPI_{90} indicating that above baseline exposure had occurred recently (SI Figure 9). There is insufficient data from subsequent years to interpret a trend with any confidence, but blood levels in subsequent years have decreased to reference interval levels for the three recaptures. A corresponding increase in scute concentrations of As was not observed for these recaptures. As described in the UPB case-study, concentrations of As can fluctuate with dietary habit and are therefore difficult to interpret without corresponding dietary information.

Nearly half of CLV turtles had Sb blood concentrations above reference interval limits in 2014. Nevertheless, blood-scute concentrations ratios for 40% of 2014 CLV turtles were within the RPI_{90} (SI Figure 10). The analytical error for Sb was large enough to warrant caution in interpreting the blood-scute results. The most conservative interpretation for 2014 would place all the blood-scute ratios within the RPI_{90} . This suggests a relatively constant exposure to Sb above background levels.

1.5. Summary and Conclusion

Trace element concentrations found in green turtle blood can provide valuable information on recent environmental exposures, whereas scute data can reveal signals of elevated past exposures long after they have been eliminated from other

tissues. We show here, using a kinetic model of trace element distribution between blood and scute, how these two tissues (which can be obtained with minimally invasive methods) can be used to elucidate temporal exposure patterns. The field data collected from a foraging ground within the Great Barrier Reef (Howick Island group) as well as from Shoalwater Bay, provided robust proxies of steady-state exposure conditions which provided the basis for comparing blood-scute concentration ratios from turtle populations from 2 additional coastal sites. Of the two coastal sites, recapture data from Upstart bay in particular provided the opportunity to validate the model where acute exposure to Co was suspected of playing a role in a mass-stranding event 2-years prior to this investigation. In this example, when compared to the steady-state exposure log-log regression plot for Co, recaptured turtles followed a pattern that the kinetic model predicted for elevated past exposures.

Our ability to see the changes illustrated in the blood-scute model are suspected to be strongly influenced by internal distribution mechanisms dictated by the toxicokinetics of each element. Conversely, elimination from the scute is largely a physical process (shedding of layers) and we would, therefore, expect it affects all elements equally. This approach for marine turtle biomonitoring illustrates how our described methods can be utilized as a powerful tool for investigating suspected trace element exposures (i.e. mass strandings) as well as a proactive tool for monitoring changes in accumulation in response to forecasted environmental disruptions (i.e. inclement weather, climate change, or coastal development). Better toxicokinetic data, specific to green turtles and trace elements, will provide blood-scute ratio models of greater precision with the opportunity for model validation through recaptured individuals. Additional blood and scute data from control

populations from other foraging grounds could be used to develop a globally relevant steady-state kinetic model until better toxicokinetic data becomes available. Thus, we strongly promote the incorporation of blood and scute sampling in marine turtle biomonitoring programs with an emphasis on collecting paired blood and scute from recaptured turtles from appropriate control as well as study sites.

1.6. Acknowledgements

Study components related to HWK, UPB and CLV were funded by the World Wide Fund for Nature (WWF) Australia, through philanthropic donations by Banrock Station Environmental Trust. Fieldwork was supported by The Great Barrier Reef Marine Park Authority, The QLD Parks and Wildlife Services, QLD Department of Environment and Heritage Protection, and QLD Department of Defense. We thank all members of the 2014-2017 field teams, and Kristina Dunn-Johnston, Gabriel Oliveira de Carvalho and Marjolijn Kock for assistance with sampling logistics, archiving and processing. The Queensland Alliance for Environmental Health, which incorporates the National Research Centre for Environmental Toxicology (Entox) at the University of Queensland, is co-funded by Queensland Health. These investigations were completed under AEC Approval Number: NRCET/147/14/APA/WWF.

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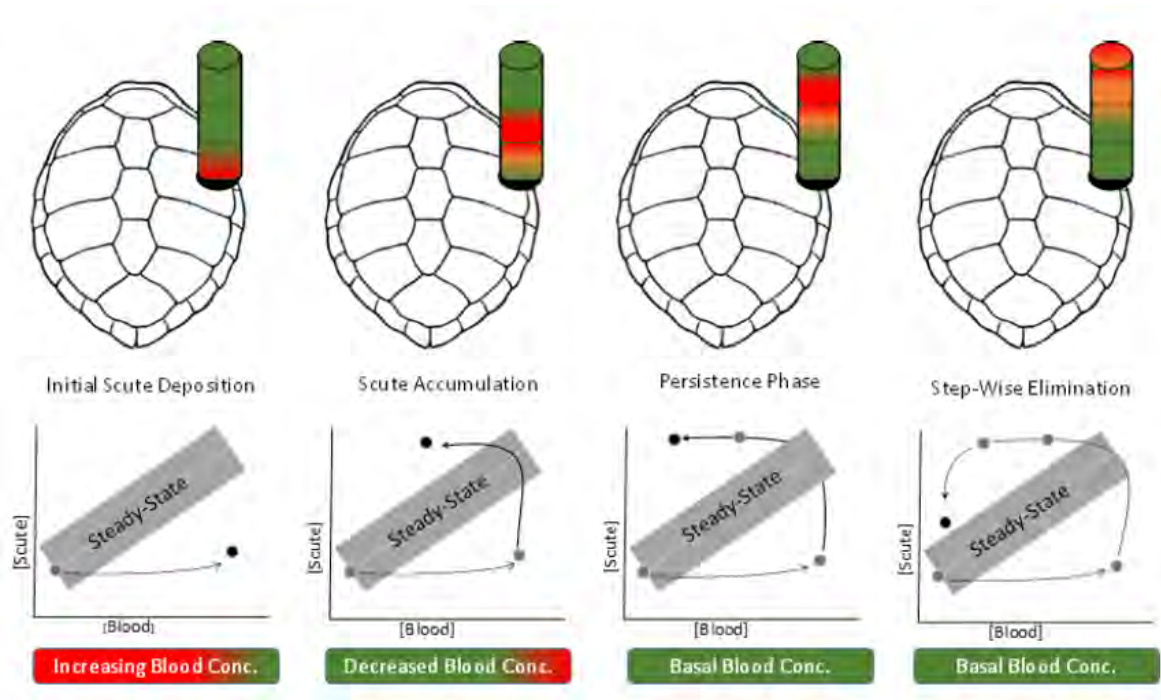
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Graphical abstract



Highlights

- A general kinetic model is described for trace elements in turtle blood and scute
- Great Barrier Reef turtles used to approximate steady-state between blood & scute
- Recaptured turtles over successive years provide initial model validation
- Turtles from impacted sites provide case-studies of elevated past exposure to Co

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