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Using Compound-Specific Carbon Stable Isotope Analysis of Squalene to Establish Provenance and Ensure Sustainability for the Deep-Water Shark Liver Oil Industry

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Abstract: Deep-water dogfish (sharks) are caught on Australia's continental shelf as by-products to other deep-water species with revenue derived from fillets for human consumption and from the livers which are sold for their oil content. Deep-water dogfish utilise a large oil-rich liver for buoyancy, which may account for 20–25% of their body weight. An important constituent of certain dogfish liver oil is squalene, a highly unsaturated triterpenoid ($C_{30}H_{50}$) hydrocarbon which in some species can be up to 90% of the oil, though in the Australian commercial species it is typically around 50%. Squalene (and deep-water dogfish liver oil in general) has a long-standing high value in products, such as cosmetics and nutraceuticals. Manufacturers are increasingly required to demonstrate the sustainability of products, and this is integral to the importance of demonstrating product provenance. Australia's mid-slope deep-water dogfish fishery is recognised globally as well managed and sustainable; therefore, it is important to be able to distinguish products derived from these regions from other unregulated, unsustainable and cheaper sources in order to protect Australia's competitive advantages and ensure sustainability. In this study, we have sourced deep-water dogfish liver oil samples originating from Southeast Australia, New Zealand, India, Northeast Africa and the Arabian Sea. The squalene was isolated by commercial or laboratory processing. A compound-specific carbon stable isotope analysis of the derived squalene was then used to determine isotopic resolution and assign the likely origins of a variety of commonly available off-the-shelf nutraceuticals in Australian outlets. Squalene sourced and produced from Southeast Australian and New Zealand dogfish liver oils showed $\delta^{13}C$ values in the range of −22.1 to −24.2‰, with all other squalene samples distinguishable at −19.9 to −20.7‰. Many of the off-the-shelf squalene products claiming to be of Australian origin showed $\delta^{13}C$ values very distinct from the range of the genuine Southeast Australian- and New Zealand-produced squalene.

Keywords: dogfish; squalene; compound-specific stable isotope analysis (CSIA); isotopes; provenance

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1. Introduction

Shark liver oil is a generic term used to describe the marine lipids derived from the livers of certain deep-water dogfish (*Squalidae*, a family of sharks) which comprise diacylglyceryl ethers, triacylglycerols, and a major hydrocarbon, squalene [1]. Squalene is a triterpene hydrocarbon ($C_{30}H_{50}$; 2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (6E,10E,14E,18E)) first discovered in 1916 in shark liver oil from a deep-water dogfish (*Squalus* spp.) [2]. The livers from certain deep-water dogfish provide the largest natural source of squalene, with up to 700–800 mg/g of oil (70–80%) [1], while olive oil can contain 0.4–0.6% (of oil) [3]. Other natural sources include grape seed oil, soybean oil and peanut oil [4]

Approximately 90% of global squalene production is hydrogenated to form squalane and used largely as a moisturiser base in high-quality cosmetics, with around 9% used in the nutraceutical industry [5,6]. The nutraceutical use of squalene has suggested anti-cancer, anti-ageing and antioxidant properties, but there is limited published evidence for any direct health benefits [7]. Pharmaceutical interest in squalene is increasing with high purity squalene proving to be effective as an adjuvant in vaccines [8,9]. There is interest in developing alternative microbial or bio-synthetic sources for squalene [10]; however, deep-water dogfish liver oil and olive oil remain the two primary sources, with deep-water dogfish liver oil presently remaining the preferred source [3,9].

Deep-sea dogfish may generally be characterised by slow growth, late maturation and low levels of reproduction, with just under 20% of extant species of sharks (including selected deep-water dogfish) and rays listed as "threatened" [11]. Thus, it is essential that the catch and utilisation of these species is carefully managed to ensure sustainability. Although some of these species may be caught as by-products in Australia, they are also target species in all unregulated fisheries (e.g., African, Indian, Indonesian and Filipino dogfish fisheries), resulting in an unconstrained and under-reported harvest in these regions [12].

1. In Southern Australia, deep-water dogfish have historically been caught on the slopes of the continental shelf with the catch divided into two groups which are sustainably managed by the Australian Fisheries Management Authority (AFMA) [13,14]: Upper-slope (shallower) species comprise the gulper sharks (*Centrophorus* spp.) and green eye species (*Squalus* spp.). Individuals in this group typically have life histories with slow maturity and low fecundity. Three species (*Centrophorus harrissoni*, *Centrophorus moluccensis* and *Centrophorus zeehaani*) from this group are listed as conservation-dependent under the Australian Environmental Protection and Biodiversity Conservation (EPBC) Act and several have poor (threatened) International Union for the Conservation of Nature (IUCN) listings. The AFMA has in place a rebuilding strategy for several of these sharks.
2. The mid-slope (deeper) species are managed by the AFMA as the 'Deep-water Shark Basket' (DWSs). DWSs are a management basket of 18 species from five families. More than 90% of those harvested are a single species, *Deania calcea* (aka 'Birdbeak Dogfish'/'Brier Shark'/'Shovelnose Spiny Dogfish'), which has an IUCN listing of "least concern", is not EPBC listed and is not subject to a rebuilding plan. DWSs have significantly higher productive life histories than gulper sharks.

Deep-water dogfish in category #2 above are consistently caught as an element of both mixed and target species fisheries (such as blue grenadier (hoki), ling and others). Their trunks are utilised for fillets for human consumption and the livers for squalene production, which provide approximately 40% and 60% of revenue, respectively, to the industry, though this will vary with market fluctuations. The main harvest species, *Deania calcea*, has a lower squalene content (c.a. 50%) than some other deep-water dogfish [1] thus production costs are higher. Squalene has regular, long-standing, and reliable markets around the world. This makes the supply of squalene from a sustainably managed resource vulnerable to a high supply of cheaply produced oil from poorly regulated/unregulated and unsustainable fisheries.

To ensure the viability of a sustainable deep-water dogfish resource, it is imperative to be able to demonstrate provenance of supply. Stable isotope analysis has become an increasingly important tool in determining the authenticity and traceability of agricultural products, particularly for terrestrial systems (e.g., [15,16]), and has been successfully used to assign regions of origin to commercially important products, such as beef [17], wine [18] and honey [19]. This technique relies on the fact that the isotopic ratios of many elements vary by geographic region due to a combination of geology and hydrology [16,20]. In the marine environment, the application of stable isotopes to establish provenance may be more problematic since both water masses and animals are generally not static. To date, there are few reported marine applications of the technique being used for determining

provenance compared with terrestrial environments, although stable isotope analysis has demonstrated some success in the task of distinguishing wild caught versus aquaculture production [21,22]. It has also been possible to assign geographical origins to prawn production [23,24]. To our knowledge, no techniques have previously existed or been applied for the separation of the source of origin of squalene from the different regions of the world oceans.

An important requirement for this technique to be successful is the use of material free from contamination to establish reference libraries for the point of origin and, when required, to ensure only the ingredient of interest is being measured. To this end, in recent years, there has been an increase in the use of compound-specific isotope analysis (CSIA) in which compounds in a mixture are separated and their individual isotopic ratio determined. This technique has the ability to introduce an extra level of specificity to the analysis by targeting one or more individual compounds known to be endemic to the product of interest. CSIA to date has generally involved the use of carbon isotopes of volatiles and fatty acids in products, such as scotch whiskey [25], cocoa butter [26] and olive oil [27]. Relevant to this study, this technique was successfully utilised to distinguish between squalene derived from olive oil and deep-water dogfish liver oil [3].

There are well established gradients in the carbon isotopes of marine phytoplankton with temperature, and therefore latitude, in the southern hemisphere (e.g., [28]), but this relationship is less obvious in the northern hemisphere (e.g., [29]). Given deep-sea dogfish are not generally regarded to be highly mobile, we hypothesised that there is the potential for these latitudinal differences in phytoplankton isotopes to be reflected in deep-water dogfish liver oil and therefore their squalene. These differences would allow differentiation between squalene derived from deep-water dogfish caught in southern latitudes (Australia and New Zealand) and those caught in less well-regulated fisheries within the warmer waters of the Indian Ocean.

2. Materials and Methods

Samples of deep-water dogfish liver oil of confirmed origin were sourced from Southeast Australia, New Zealand, Northeast Africa, the Arabian Sea and India (Figure 1; Table 1). Australia squalene is derived from dogfish raw materials available for harvest mostly from Southeast Australia, as outlined in Figure 1. Samples of raw oils containing squalene were supplied by traders who are active with local fishermen in their areas. Oil samples were large batches (typically 5–15 tonnes, representing many thousands of dogfish covering both sexes, seasons and age ranges). All oil samples were prepared by maceration of the livers, settling and separation of the oil followed by filtration. Molecular distillation and filtration over activated carbon was then used to obtain 99.85% purity squalene. In addition, we analysed a range of squalene capsules, commercially available and purchased in Australia, with a view to assessing their likely origin (Table 2).

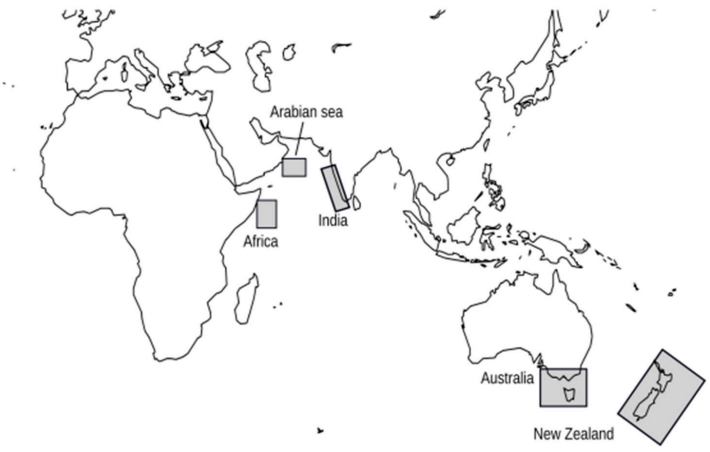

Figure 1. Approximate geographical areas of deep-sea dogfish fisheries sampled in this study.

Table 1. CSIA results for squalene isolated from deep-sea dogfish liver oil with a known origin. AUS denotes Southeast Australian, NZ denotes New Zealand. $\delta^{13}C$ values are means of duplicate or triplicate analyses, SD = standard deviation.

Oil Sample Origin	$\delta^{13}C$ (‰ vs. V-PDB [1])	SD
AUS	−22.6	0.3
AUS	−22.6	0.1
AUS	−22.5	0.1
AUS	−22.6	0.2
AUS	−22.5	0.1
AUS	−22.6	0.2
AUS	−22.3	0.2
NZ	−22.5	0.3
NZ	−24.2	0.1
NZ	−22.3	0.1
NZ	−22.4	0.1
NZ	−22.1	0.2
NZ	−22.4	0.2
NZ	−22.4	0.2
India	−20.4	0.2
Northeast Africa	−20.6	0.1
Northeast Africa	−20.7	0.1
Northeast Africa	−20.6	0.2
Arabian Sea	−19.9	0.2
Arabian Sea	−20.1	0.2

[1] The standard Vienna PeeDee Belemenite (V-PDB).

Table 2. CSIA results for squalene isolated from capsules commercially available in Australia. $\delta^{13}C$ values are means of duplicate or triplicate analyses, SD = standard deviation.

Capsule	Product Origin Information [1]		$\delta^{13}C$ (‰ vs. V-PDB [2])	SD
	Australian Made [3]	Australian Squalene [4]		
1	Y	n.s.	−21.1	0.1
2	Y	n.s.	−20.8	0.1
3	Y	n.s.	−21.1	0.1
4	Y	n.s.	−21.2	0.0
5	Y	n.s.	−22.0	0.1
6	n.s.	n.s.	−21.1	0.2
7	Y	n.s.	−21.3	0.5
8	Y	Y	−21.2	0.1
9	Y	Y	−21.6	0.3
10	Y	Y	−22.0	0.1
11	n.s.	n.s.	−20.5	0.1
12	n.s.	n.s.	−20.5	0.0

Table 2. *Cont.*

Capsule	Product Origin Information [1]		$\delta^{13}C$ (‰ vs. V-PDB [2])	SD
	Australian Made [3]	Australian Squalene [4]		
13	Y	n.s.	−21.1	0.1
14	n.s.	n.s.	−21.1	0.2
15	n.s.	n.s.	−20.8	0.2
16	Y	n.s.	−21.5	0.1
17	Y	n.s.	−21.1	0.1

[1] Obtained from manufacturer product website; [2] The standard Vienna PeeDee Belemenite (V-PDB); [3] Australian made does not currently have to include Australian-derived squalene; [4] Product website states oil is from dogfish caught in waters around Australia; Y = Specified as Australian made or sourced; n.s. = not specified.

2.1. Squalene Isolation

One drop of each sample was transferred to a 1.8 mL vial and made up to 1.5 mL with dichloromethane (DCM). The sample was reduced under nitrogen gas, dissolved in hexane, then transferred to a 1 g silica chromatographic column and the hydrocarbon fraction eluted with 10 mL of hexane and collected into a 50 mL round bottom flask. Most of the solvent was removed under vacuum. The sample was transferred to a 12 mL test tube and then blown to dryness under a stream of nitrogen gas. To ensure adequate purity for the subsequent instrumental analysis, the samples were then saponified using 3 mL of methanolic potassium hydroxide (5% KOH in 80:20 methanol:Milli-q water) by heating for 2 h at 80 °C with vortex mixing every $\frac{1}{2}$ h for 5 s. Samples were then cooled and 1 mL of Milli-q water and 1.5 mL of hexane:DCM (4:1) was added to all samples, which were then vortexed for 5 s and centrifuged at 2500 rpm for 3 min. The top hexane layer was carefully removed and transferred to a 1.8 mL GC vial and the solvent was removed under a stream of nitrogen gas. This was repeated with a further two extractions and the solvent layers were combined. Samples were dissolved in 1.5 mL of DCM and then an aliquot of 200 µL was transferred to a second vial, dissolved in 1.5 mL of DCM and analysed for purity by gas chromatography (GC).

GC was performed on an Agilent Technologies 7890A GC (Santa Clara, CA, USA) equipped with a non-polar Equity-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 mm film thickness), flame ionisation detector and split/splitless injector. Samples were injected in splitless mode at an oven temperature of 120 °C, and after injection, the oven temperature was increased to 270 °C at 10 °C min^{-1} and then to 310 °C at 5 °C min^{-1}. Peaks were quantified with Agilent Technologies ChemStation software Rev. C.01.10 [201] (Santa Clara, CA, USA).

2.2. Compound-Specific Stable Isotope Analysis

Compound-specific isotope ratio mass spectrometry was performed using a Thermo Scientific Trace Ultra GC (Waltham, MA, USA), which was coupled via a Finnigan MAT GC combustion interface and Conflo IV to a Thermo Scientific Delta V plus isotope ratio mass spectrometer. The GC was equipped with a 60 m J&W HP-5, 0.32 mm i.d. column with He as the carrier gas. Samples (0.5 µL) were injected split (1:7) via a hot split/splitless injector (250 °C). The initial oven temperature of 45 °C was maintained for 1 min and then increased at 30 °C min^{-1} to 140 °C, then at 3 °C min^{-1} to 310 °C when it was held for 10 min. Combustion of individual peaks to CO_2 was achieved in a combustion reactor at 940 °C. Samples were analysed in either duplicate or triplicate depending on the agreement between analyses and were co-injected with a squalane internal standard with known $\delta^{13}C$ value. Squalane (Sigma, St. Louis, MO, USA, Lot 10K0167) was used in this instance, as it elutes close to squalene and was not present in the samples.

Isotope values are reported relative to the standard Vienna PeeDee Belemenite (V-PDB) and expressed as parts per thousand (‰) using:

$$\delta^{13}C = \left(\left[\frac{R_{sample}}{R_{standard}} \right] - 1 \right) \times 10^3$$

R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratio in the sample and standard, respectively. Average standard deviation for triplicate analyses was 0.2‰. The standard deviation across all co-injected squalane analyses was 0.5‰ (n = 60).

2.3. Statistical Analysis

Data were transferred to the R statistics package (ver. 3.4.0) operating within RStudio (ver. 1.0.153) and plotted using the ggplot2 package (Vienna, Austria). A linear regression comparing Southeast Australia, New Zealand, the north-west Indian Ocean/Arabian Sea (Arabian Sea, India, Northeast Africa) samples and capsules was applied with region/capsules as a categorical factor.

3. Results and Discussion

Carbon utilised by primary producers (microalgae and phytoplankton) in marine ecosystems is primarily derived from dissolved inorganic carbon that has a $\delta^{13}C$ value around 0‰ [30]. In comparison, primary producers in terrestrial ecosystems primarily derive carbon from atmospheric CO_2 with an approximate $\delta^{13}C$ value of −8‰ [31]. During photosynthesis further isotope C fractionation occurs, resulting in clear differentiation between terrestrial plants (−9 to −38‰) [32,33] and marine phytoplankton and microalgae (−15 to −22‰) [30,33].

As carbon moves through the marine food chain to higher trophic levels, further isotopic fractionation occurs, with an approximately 0.8 to 1.5‰ increase in ^{13}C value per trophic level [30]. The difference in carbon sources as well as trophic discrimination in the dogfish results in an isotope distinction between squalene derived from olive oil (−26.8 to −29.3‰) [3] and from deep-sea dogfish liver oil (−19.9 to −24.2‰, Table 1).

Compound-specific carbon isotope values for squalene isolated from deep-water dogfish liver oils with a known origin and contained within commercially available capsules are shown in Tables 1 and 2 and Figure 2. Squalene originating from deep-water dogfish caught in Southeast Australia and New Zealand are clearly separated from that originating from other fisheries by >1‰. This is also well above the average analytical error of ±0.2‰ for within sample replicates (n = 3) and ±0.5‰ for the total number of squalane standard co-injections (n = 60). A linear regression showed that the ^{13}C values were significantly higher in the Arabian Sea, Northeast Africa and India region compared to Southeast Australia ($p < 0.0001$). New Zealand was not significantly different from Southeast Australia ($p = 0.73$). An average carbon isotope value by CSIA for olive oil-derived squalene of −27.9‰ (±0.5‰) was reported [3], which, as expected, is significantly more depleted in ^{13}C compared to marine-derived squalene and would be easily distinguishable.

The isotopic separation observed between the southern high latitudes squalene samples (−22.1 to −24.2‰) sourced from Australia and New Zealand from those closer to the equator and those in the northern hemisphere (−19.9 to −20.7‰) is most likely primarily driven by the well-established relationship between southern hemisphere water temperature and CO_2 solubility and subsequent planktonic isotope fractionation [28]; phytoplankton from high southern latitudes have significantly more negative $\delta^{13}C$ values. However, it is worth noting that this relationship is not as strong in the northern hemisphere, where other factors probably also play a part. Thus, while the isotopic differences exhibited here are strong, we have little information on how consistent they might be over longer time periods due to seasonal effects or possible small shifts in the location of where the dogfish are caught within a "region". While it seems apparent that, based on the samples analysed to date, the exact region of origin for squalene is difficult to assign on a global scale, there is very strong evidence that it is possible to ascertain whether

marine-derived squalene is sourced from within the Southeast Australian/New Zealand region currently and therefore from a well-regulated and sustainable fishery.

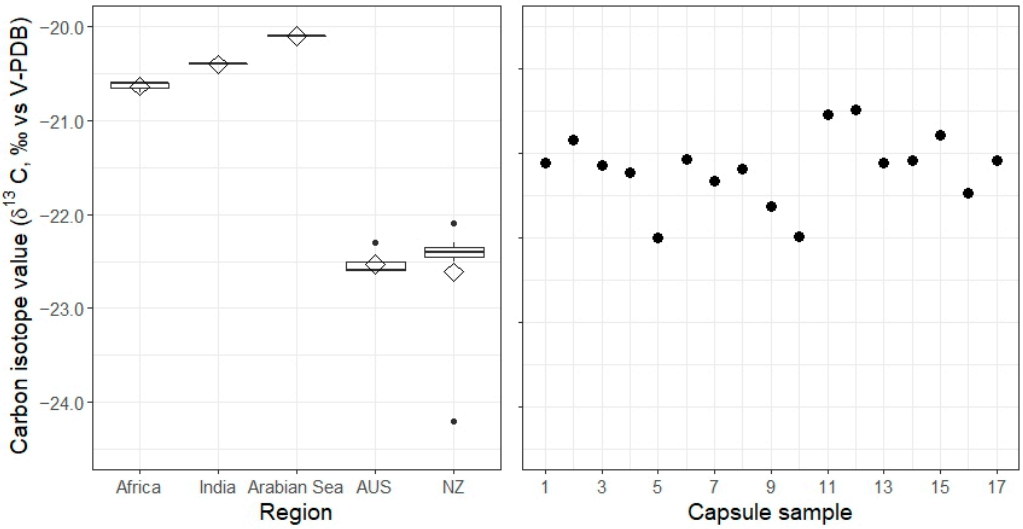

Figure 2. Compound-specific carbon isotope values for squalene isolated from deep-sea dogfish liver oil with a known origin (**left**) and samples from a range of commercially available squalene capsules (**right**). AUS denotes Southeast Australia, NZ denotes New Zealand. Boxplot: Boxes = upper and lower quartiles; horizontal line = median; vertical lines = value range; ◇ = mean; gong = outliers.

Stable isotopes are a conservative tracer (e.g., [34]); thus, for example, squalene which is derived from a mix of both Southeast Australian and alternatively sourced deep-water dogfish liver oils will possess a δ^{13}C value which reflects the relative proportion of each source. Based on the above, it would appear that only two of the capsule samples analysed (numbers 5 and 10) are potentially using squalene primarily derived from the Southeast Australia or New Zealand regions. Of the three products claiming to be sourced from Australian waters (as opposed to Australian made; numbers 8, 9 and 10), only one (number 10) appears to be using oil from Southeast Australia. The average isotopic value of the capsules is different than the Australian ($p < 0.00001$), New Zealand ($p < 0.00001$) and the Northeast Africa, India and Arabian Sea ($p < 0.0006$) squalene. The results strongly indicate that the majority of capsules analysed are either using squalene derived from other regions or from a mixture of sources. The less negative the δ^{13}C value, the lower the possible proportion of squalene obtained from the Australian and/or New Zealand regions. Clearly, none of the capsules analysed here are using squalene derived from olive oil (−26.8 to −29.3‰) [3].

Compound-specific isotope analysis is more technical and expensive compared to the more traditional bulk material methods utilising an elemental analyser coupled to the isotope ratio mass spectrometer (EA-IRMS). Both types of analyses were performed on samples and clearly demonstrated the potential effect of impurities with values obtained by EA-IRMS varying from those using CSIA on the same sample by up to 1‰ [3]. Thus, for analysing relatively pure squalene samples (for example, capsules), results from EA-IRMS may provide sufficient resolution to distinguish olive oil-derived squalene from marine sources. At this stage, the use of EA-IRMS is unlikely to be sufficient for distinguishing between Australian/New Zealand sources and those from other fisheries. To use EA-IRMS it would be necessary to ensure >90% purity of the squalene and even then, based on the data from [3], confidence limits would still be reduced. The analytical preparation used in this study routinely achieves a high level of purity which then ensures optimal compound-specific carbon isotope results by GC-IRMS. In addition, GC-IRMS requires several orders of magnitude less material than EA-IRMS, allowing for a much smaller scale of analytical preparation, particularly when analysing raw deep-water dogfish liver oil. In some instances (e.g., cosmetics), GC-IRMS alone may be sufficient with no sample

pre-treatment following a solvent extraction [3]. Other complementary approaches might also be used to assist in determining where the current tested products originate from.

4. Conclusions

The CSIA technique appears particularly promising with respect to assigning a South-east Australian/New Zealand origin for squalene which is beneficial in ensuring deep-water dogfish-derived squalene is being sourced from robustly managed and sustainable fisheries. Similarly, as new and emerging sources for squalene are developed (e.g., olive oil, microalgae, synthetic production), there will continue to be a need to ensure only sustainable sources are being utilised. Compound-specific carbon isotope analysis would appear to be an ideal tool in that it facilitates the analysis of squalene in a wide range of matrices, from raw deep-water dogfish liver oil and other potential sources of squalene to final products, such as those used in cosmetics and nutraceuticals.

Author Contributions: P.D.N., R.S. and A.T.R. conceived the study; A.T.R. and E.A.B. refined and validated the methodology and undertook sample and data analysis; R.S. provided samples and partial funding; A.T.R. led the writing of the manuscript. All authors substantially contributed to critical assessment and editing of drafts. All authors have read and agreed to the published version of the manuscript.

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