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# Experimental evidence shows no fractionation of strontium isotopes ($^{87}Sr/^{86}Sr$) among soil, plants, and herbivores: implications for tracking wildlife and forensic science

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Strontium isotopes ($^{87}Sr/^{86}Sr$) can be useful biological markers for a wide range of forensic science applications, including wildlife tracking. However, one of the main advantages of using $^{87}Sr/^{86}Sr$ values, that there is no fractionation from geological bedrock sources through the food web, also happens to be a critical assumption that has never been tested experimentally. We test this assumption by measuring $^{87}Sr/^{86}Sr$ values across three trophic levels in a controlled greenhouse experiment. Adult monarch butterflies were raised on obligate larval host milkweed plants that were, in turn, grown on seven different soil types collected across Canada. We found no significant differences between $^{87}Sr/^{86}Sr$ values in leachable Sr from soil minerals, organic soil, milkweed leaves, and monarch butterfly wings. Our results suggest that strontium isoscapes developed from $^{87}Sr/^{86}Sr$ values in bedrock or soil may serve as a reliable biological marker in forensic science for a range of taxa and across large geographic areas.

**Keywords:** *Danaus plexippus*; geographic assignment; isoscape; isotope ecology; migration; migratory connectivity; monarch butterfly; plant; soil; strontium-86; strontium-87

## 1. Introduction

Isotopes can be powerful tools for assigning tissues or other organic substances of unknown origin to a specific geographic location. For example, isotopes have been used in applications of provenance in counterfeit pharmaceutical investigation [1], archaeology [2], food security [3], wildlife migration [4–6], illegal trade [7], and narcotics [8]. By relating stable isotopes of focal tissue to expected isotopic values across large geographic gradients, termed isoscapes [9], we can assign the statistical probability to a specific location as the origin of the focal material [10,11]. This probabilistic assignment requires information on the change in isotopic values between the source of the material used to develop the isoscape and the focal tissue (fractionation) as well as the variability in the isotope values of focal tissue grown at a single location [11].

Strontium isotopes ($^{87}Sr/^{86}Sr$) are one intrinsic marker that has shown significant promise for use in forensic science [2,8,12]. Natural variations in isotopic values of bedrock are determined by bedrock type and age that are derived from predictable rates of radioactive decays of isotopes of rubidium ($^{87}Rb$) to the stable isotope of strontium ($^{87}Sr$; [13]). Recent attempts to model

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these multiple processes have provided landscape-scale predictive $^{87}Sr/^{86}Sr$ surfaces that can be applied to studies of provenance [14–16]. $^{87}Sr/^{86}Sr$ values are potentially very useful for assigning geographic origins for two primary reasons. First, because $^{87}Sr/^{86}Sr$ values are influenced by fundamentally different processes compared to light stable isotopes, such as stable hydrogen ($\delta^2H$) and carbon ($\delta^{13}C$) isotopes, they can act as complimentary markers to increase the spatial resolution for assigning tissues of unknown origin to a specific location [17]. Second, because it is assumed that there is little or no fractionation in $^{87}Sr/^{86}Sr$ values from geological bedrock sources through food webs and ultimately into animal tissues (in other words, there is a 1:1 linear relationship [18]), predictive $^{87}Sr/^{86}Sr$ isoscapes based on bedrock or soil values can accurately reflect potential areas of origin for tissues and apply to a wide range of tissues that are grown at different trophic levels [19]. However, the transfer of Sr isotopes from the geosphere into the biosphere without any fractionation has never been tested. Previous studies have shown that tissues collected across different trophic levels from the same location show variance in $^{87}Sr/^{86}Sr$ values both between and among trophic levels [20,21], but no study has examined $^{87}Sr/^{86}Sr$ in multiple trophic levels while controlling for bedrock or soil type. Validating the assumption of no fractionation in $^{87}Sr/^{86}Sr$ values across trophic levels, and deriving estimates of the variation among all trophic levels, is paramount for the successful development of strontium isoscapes as a robust marker for applications in forensic science.

In this paper, we use a controlled experimental design to examine $^{87}Sr/^{86}Sr$ values across three trophic levels with monarch butterflies (*Danaus plexippus*) as the top-level consumer. Monarch butterflies in eastern North America have a complex migratory system where individuals travel between three different countries over the course of multiple breeding generations during the annual cycle [21–24]. Since threats to the population viability of monarchs vary across space and time, accurate and precise assignment of migratory connectivity is fundamental to protecting this species [25]. Because monarchs are small and short-lived, approaches such as mark–recapture and radio-tracking are largely unsuitable for delineating migratory connectivity across the annual cycle [26]. In contrast, monarchs are an excellent model organism for studying and applying stable isotopes to understand patterns of long-distance migration [23,24,27,28]. Larvae feed on a single family of obligate host plants (*Asclepias* spp.) and they are relatively immobile during their larval development moving only metres between plants [29], so that stable isotope values from inert wing tissue of the adults reliably reflect the location of their larval development no matter how far an individual has migrated [28].

Here, we investigate the hypothesis that strontium isotopes are suitable forensic markers to determine origins by testing the assumption that there is no fractionation of $^{87}Sr/^{86}Sr$ among different environmental samples that contribute to the bioavailability of strontium isotopes during development in monarch butterflies. Our objective was to measure $^{87}Sr/^{86}Sr$ isotopes among leachable Sr from soil minerals (hereafter, mineral soil), organic soil, milkweed plants, and monarch butterfly wing tissue and use linear models to test if these relationships differed from a predicted 1:1 relationship.

## 2. Methods

### 2.1. *Soil*

We collected soil from seven locations across Canada that represented a range of soil characteristics and underlying geology (Table 1). The soil was collected from natural, relatively undisturbed sites with natural vegetation to minimize the possibility of soil inputs from outside the focal area. After removing surface vegetation, a garden spade was used to remove approximately $0.3 \times 0.3 \times 0.3$ m of soil (approximately 25 kg) at each location. Soil was put into rubber totes

Table 1. Description and summary statistics including main bedrock, soil type, and the $^{87}Sr/^{86}Sr$ value of leachable Sr from soil minerals ('Mineral'), organic soil ('Organic'), milkweed plants ('Milkweed'), and mean value of monarch butterfly wing tissue ('Monarch') from seven locations across Canada. The standard deviation and sample size are included because multiple butterflies were raised on plants grown in soil from each location.

| Location | Coordinates | Main bedrock [30] | Soil type [31] | Mineral | Organic | Milkweed | Monarch (SD) |
|---|---|---|---|---|---|---|---|
| Coquitlam, BC[a] | 49.3°N, 122.8°W | Mesozoic intrusive | Ferro-humic podzolic | 0.70817 | 0.70789 | 0.70808 | 0.70836 (0.00012) $n = 4$ |
| St. Albert, AB | 53.6°N, 113.6°W | Mesozoic sedimentary | Black chernozemic | 0.70945 | 0.70956 | 0.70959 | 0.70969 (0.00042) $n = 8$ |
| Saskatoon, SK | 52.1°N, 106.7°W | Mesozoic sedimentary | Dark brown chernozemic | 0.70837 | 0.70870 | 0.70869 | 0.70872 (0.00012) $n = 8$ |
| Thunder Bay, ON | 48.4°N, 89.3°W | Paleoproterozoic sedimentary | Dystric brunisolic | 0.71010 | 0.70931 | 0.70945 | 0.70958 (0.00031) $n = 12$ |
| Guelph, ON | 43.5°N, 80.2°W | Paleozoic sedimentary | Gray brown luvisolic | 0.70860 | 0.70866 | 0.70868 | 0.70913 (0.00021) $n = 4$ |
| Kingston, ON | 44.2°N, 76.5°W | Paleozoic sedimentary | Gray luvisolic | 0.70882 | 0.70890 | 0.70896 | 0.70923 (0.00050) $n = 6$ |
| St. John's, NL | 47.6°N, 52.7°W | Neoproterozoic sedimentary | Humo-ferric podzolic | 0.70864 | 0.70864 | 0.70878 | 0.70877 (0.00004) $n = 8$ |

[a]Lime was added to this soil to improve plant growth and therefore Sr values may not represent those of natural soils found at this location.

and transported to the laboratory where we homogenized the sample by removing large organic components and breaking mineralized components (except aggregates) to approximately 1 $cm^3$ with a trowel and mixed it repeatedly before we collected a single soil sample of approximately 500 g from each geographic location to analyse for strontium isotopes. Strontium isotopes were measured for both mineral soil material and organic soil material after we sieved the soil sample into organic ($>180\,\mu m$) and mineral ($<180\,\mu m$) fractions and homogenized them. Generally, mineral soil material is $< 2$ mm in diameter and contains $< 20\%$ organic carbon by weight typical of the subterranean A and B soil horizons, whereas organic soil material contains $> 20\%$ organic carbon by weight typical of decomposing leaf litter or the O horizon nearest the soil surface. Because the soil from BC was acidic (pH 4.0) and initially stunted the growth of milkweed, we added lime (0.011 g $g_{soil}^{-1}$) individually to each pot with BC soil (pH 6.5) to improve milkweed growth. We then replanted seeds and continued with the experiment leaving the collection of the BC soil sample to the end of the experiment after we combined the used potted soil together and homogenizing with a trowel as indicated above.

### 2.2. *Plants*

We grew approximately 50 tropical milkweed (*Asclepias curassavica*) in a homogeneous sample of each soil type. Milkweed seeds were sprouted in growth chambers (28°C, 80% relative humidity, 18 h light:6 h dark) until bearing 2–4 leaves and approximately 5 cm tall, at which time single plants were transferred to 10 cm-wide pots. The pots were labelled with the soil collection location and moved to a glasshouse maintained under ambient light conditions at 29°C during the day and 23°C at night. Milkweed was watered daily with distilled water and fertilized (High Nitrate 20-8-20, Plant-Prod Solutions; http://www.plantprod.com/Portals/1/WSF%27s.pdf) approximately weekly. During the period when we were raising monarch butterfly larvae, we randomly collected 1–3 milkweed leaves from plants of each soil type daily and air-dried leaves to analyse for strontium isotopes. Since caterpillars fed on various leaves from various plants (see below), we pooled leaves grown from each soil location which resulted in a single plant sample for each geographic location.

### 2.3. *Monarch butterflies*

Wild monarch butterflies can migrate long distances [23,24,27] but we required butterflies that developed from the egg stage that were restricted to food plants grown from a single soil source. To do so, wild-caught monarch butterflies were placed in netted enclosures with five host plants from each location and were left to lay eggs over several hours; butterflies that developed from these eggs were used in the isotopic analysis. For each soil type, we collected the leaves containing eggs and placed them in plastic containers with fine mesh lids. Larvae were fed *ad libitum* with leaves plucked from milkweed grown in the appropriate soil type until approximately third instar when they were moved individually to plastic containers to prevent competition for plant material and aggressive interactions with conspecifics [32]. Larvae pupated on the mesh lids and were transferred to a large netted enclosure for each soil type until they enclosed. After butterflies were allowed to dry their wings for 24 h, we transferred the butterflies to glassine envelopes and placed them in $-15$°C freezers until they were analysed for strontium isotopes.

### 2.4. *Strontium isotope analysis*

Soil samples were prepared for Sr isotope analyses using a dilute acid leach procedure to extract only the Sr that is easily dissolved in soil waters. Soil samples are weighed (ca. 0.5 g) into clean

leach tubes and 5 ml of 2% $HNO_3$ added. The samples are covered and put in an ultrasonic bath for 2 h, centrifuged and 1 ml of the liquid weighed into a clean LDPE bottle, evaporated to dryness and re-dissolved in acid suitable for chromatography on Sr Spec (Eichrom). The separated Sr collected from the columns are dried on a hot plate at 70°C and dissolved in 2% $HNO_3$ for measurement of isotope ratios. NIST 2710 and NIST 987 carbonate were used as reference materials. The Sr isotope ratios are measured using a ThermoFinnigan Neptune MC-ICP-MS, with all ratios normalized to an $^{86}Sr/^{88}Sr$ ratio of 0.1194, thereby eliminating any mass-dependent fractionation effects that may have occurred. The external analytical uncertainty in the $^{87}Sr/^{86}Sr$ ratio is 0.0001 ($2\sigma$) based on results for NIST 987 ($n = 23$) and duplicate samples ($n = 5$).

Plant materials are washed in vials in an ultrasonic bath for 10 min with DI water ($> 18$ Mohm) followed by drying the plant material at 70°C. The plant along with certified reference materials (NIST1547A and NIST 1571A) were weighed into clean Savillex beakers to which 1 ml of concentrated $HNO_3$ was added. The samples were digested on a 70°C hotplate with addition of 1 ml aliquots of concentrated $HNO_3$ until the solid is dissolved followed by 0.5 ml aliquots of 30% $H_2O_2$ (Seastar Baseline) until a clear, uncoloured solution resulted. The samples are split and one aliquot was prepared for Sr isotope analysis using the same method as the soils.

Two left-side wings of each butterfly are washed in DI water ($> 18$ Mohm), dried, weighed in clean Savillex beakers, and dissolved, capped, in 1 ml of concentrated $HNO_3$ on a 120°C hotplate. Additional concentrated $HNO_3$ is added until the solid is fully digested, followed by addition of 30% $H_2O_2$ (Seastar Baseline) similar to plants. The liquid is evaporated on a 70°C hotplate, the samples are diluted, and one aliquot was used for Sr isotope analysis using the same procedure as the soils.

### 2.5. *Statistical analysis*

Since we had only one soil and milkweed sample per geographic location, this would have resulted in pseudo-replication had we compared these single values to multiple butterfly samples. Thus, we used the mean monarch value for each geographic location in the regressions. We used linear regression models and applied an offset of the dependent variable ($y \sim x + \text{offset}(x)$) to test the relationships between $^{87}Sr/^{86}Sr$ values among soil, milkweed, and monarchs. The offset term allows us to test the null hypothesis that the slope was equal to 1 and that the intercept was equal to 0 [33] and we therefore report the *t*-value statistic and *p*-value associated with each estimated model parameter. Model fit was assessed using the coefficient of determination ($r^2$) to compare between the different analyses. All statistical analyses were run using program R v. 3.1 [34].

## 3. Results

We raised a total of 50 butterflies from the 7 soil types (Table 1). The range of the $^{87}Sr/^{86}Sr$ values was 0.70817–0.71010 in mineral soil, 0.70789–0.70956 in organic soil, 0.70808–0.70959 in milkweed plants, and ranged between 0.70821–0.71046 in wing tissue of individual monarch butterflies (Table 1). There was a correlation of $^{87}Sr/^{86}Sr$ among all environmental material (all $p < 0.017$). In all relationships, there was no significant difference between an intercept of zero and a slope of 1 (Table 2). All mineral soil, organic soil, and plant material reliably predicted $^{87}Sr/^{86}Sr$ values in the butterfly wing tissue (Figure 1) but the coefficient of determination was the lowest when

Table 2. Linear model (SE of parameter estimate) of $^{87}Sr/^{86}Sr$ between leachable Sr from soil minerals ('Mineral'), organic soil ('Organic'), milkweed plants ('Milkweed'), and mean monarch butterfly wing tissue ('Monarch') derived from seven locations across Canada. The test statistics report whether the intercept is significantly different from zero and whether the slope is significantly different from one.

| Model | $r^2$ | Intercept | Slope |
|---|---|---|---|
| Organic $\sim$ 0.6714 Mineral (0.1929) + 0.2329 (0.1368) | 0.65 | $t = 1.703, p = 0.15$ | $t = -1.703, p = 0.15$ |
| Milkweed $\sim$ 0.6773 Mineral (0.1521) + 0.2288 (0.1078) | 0.76 | $t = 2.121, p = 0.09$ | $t = -2.121, p = 0.09$ |
| Milkweed $\sim$ 0.9412 Organic (0.0568) + 0.0418 (0.0403) | 0.98 | $t = 1.038, p = 0.35$ | $t = -1.036, p = 0.35$ |
| Monarch $\sim$ 0.6283 Mineral (0.1547) + 0.2637 (0.1096) | 0.72 | $t = 2.405, p = 0.06$ | $t = -2.405, p = 0.06$ |
| Monarch $\sim$ 0.8502 Organic (0.1301) + 0.1064 (0.0922) | 0.87 | $t = 1.154, p = 0.30$ | $t = -1.151, p = 0.30$ |
| Monarch $\sim$ 0.8964 Milkweed (0.1355) + 0.0736 (0.0961) | 0.88 | $t = 0.766, p = 0.48$ | $t = -0.765, p = 0.48$ |

considering mineral soil and the highest among organic soil, milkweed, and monarchs (Table 2).

## 4. Discussion

Our experimental results support the hypothesis that there is minimal change in strontium isotopes between associated trophic levels. All environmental materials predicted mean isotopic values in the butterfly wing tissue, suggesting that geographic variation in $^{87}Sr/^{86}Sr$ of soil or plants may be used as a forensic marker to estimate the larval natal origin of migratory monarch butterflies. Given the correspondence between $^{87}Sr/^{86}Sr$ and the multiple sources of bioavailable strontium that we tested, a strontium isoscape for monarch butterflies in North America could be constructed using bedrock-based geomorphology approaches [15,16,35] and validated using $^{87}Sr/^{86}Sr$ values derived from milkweed samples collected across the breeding distribution of monarch butterflies [22,28].

Although we found no evidence of fractionation of Sr isotopes in our controlled experiment, it is possible that our results may not always transfer to natural conditions. For example, studies have shown that Sr concentrations and isotope ratios in plant material are influenced by specific factors during uptake, such as root depth and plant-specific cycling [36]. Localized differences in mineral weathering may also influence assignment accuracy across large geographic spatial scales [36,37]. Therefore, a remaining challenge to fully apply Sr isotopes in provenance studies requires quantifying the variation of the mixing processes among whole soil, leachable Sr from soils, plants, and herbivores tissues from multiple samples within close proximity [38] to account for within-site variation in the fractionation process that increase certainty in the assignment of origin [39].

Strontium isotopes in bedrock and water show a general east–west gradient in North America [15,35], a pattern that has also been shown to occur in known-origin bird feathers [17]. In this sense, strontium isotopes are complementary to light isotopes (especially O, H) which tend to vary in north–south gradients in North America. The complementarity aspect is important given that using multiple isotopes can improve geographic assignment [10] that has been applied to estimate the origin of migratory birds across large spatial areas [12,17]. Admittedly, the range in $^{87}Sr/^{86}Sr$ values in our study did not cover the full range of values expected across North America (e.g. [15]), but our results show that the measured values directly relate to local $^{87}Sr/^{86}Sr$ values and that variation within the predominant isotope values across North America could be discerned after including the between-individual variance of $^{87}Sr/^{86}Sr$ necessary in studies of provenance [11,39]. Therefore, given small variation of between-individual values measured at a common site, geographic assignment that involves values at the extremes of the range can be

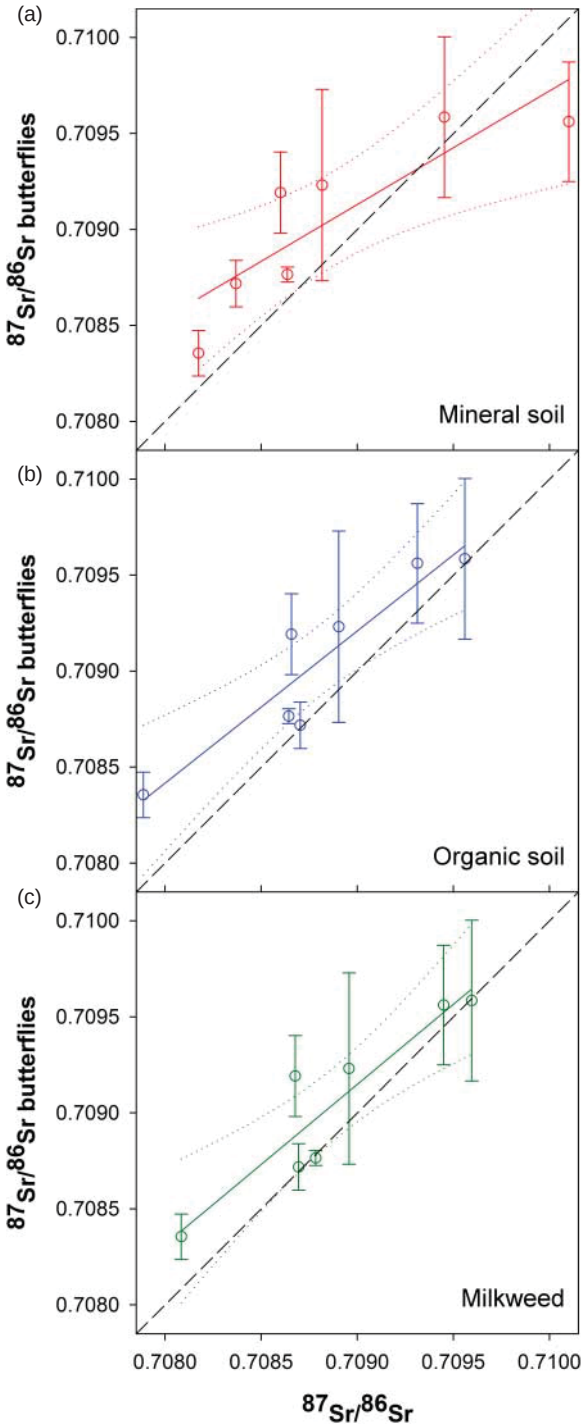


Figure 1. The relationship between strontium isotopes ($^{87}Sr/^{86}Sr$) in monarch butterfly wing tissue and $^{87}Sr/^{86}Sr$ isotopes in (a) leachable Sr from soil minerals (mineral soil), (b) organic soil, and (c) milkweed plants. Adult monarch butterflies were raised on obligate larval host milkweed plants that were, in turn, grown on seven different soil types collected from unique bedrock areas across Canada. Points represent mean monarch values for a given soil or milkweed treatment and error bars are $\pm 1$ standard deviation. In all cases, the linear model estimated mean (solid line) and 95% CI (dotted line) of the fractionation between the materials were not significantly different from the 1:1 reference line (dashed black).

dealt with easily, which is analogous to previous studies that have attempted to assign the region of origin between two areas that have vastly different expected values [38].

Strontium isotopes incorporated into soil, plants, and herbivores should primarily reflect large-scale underlying geology [13] with secondary inputs from localized sources of atmospheric aerosol, fertilizers, and other inputs [20,40–42]. Small-scale variation in $^{87}Sr/^{86}Sr$ values from localized or recent anthropogenic inputs may be one reason why some studies have found differences among associated trophic levels [20] while others have found close correspondence among some, but not all, trophic levels [38]. However, in the case of Blum et al. [38], variation in $^{87}Sr/^{86}Sr$ values derived from large samples of bones tissue of migratory wood warblers likely reflected signatures from both North American breeding grounds and Caribbean wintering grounds because of the relatively slow turnover rate of isotopes in bone [18]. However, recent analytical advances that require smaller amounts of focal tissue mean that $^{87}Sr/^{86}Sr$ values can be analysed from a wider range of faster growing tissues, such as feathers [17] and insect wings (our study), that are more likely to reflect a single geographic location. This, coupled with advanced modelling that can account for several sources of Sr inputs [16] and the ability to allocate multiple sources of variation when assigning probabilistic surfaces of origin [11], provide the necessary tools to fully apply Sr isoscapes for understanding provenance. Given the consistency in values between materials that we measured, it appears that these relationships are robust over the majority of $^{87}Sr/^{86}Sr$ isotopic values that would be found in monarch butterflies, and likely, across a wide range of taxa.



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## Disclosure statement

No potential conflict of interest was reported by the authors.

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