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Within-wing isotopic (δ2H, δ13C, δ15N) variation of monarch butterflies: implications for studies of migratory origins and diet

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Within-wing isotopic (δ^2H, $\delta^{13}C$, $\delta^{15}N$) variation of monarch butterflies: implications for studies of migratory origins and diet

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Abstract: Increasingly, stable isotope measurements are being used to assign individuals to broad geographic origins based on established relationships between animal tissues and tissue-specific isoscapes. In particular, the eastern North American population of the monarch butterfly (*Danaus plexippus*) has been the subject of several studies using established δ^2H and $\delta^{13}C$ wing-tissue isoscapes to infer natal origins of migrating and overwintering individuals. However, there has been no study investigating potential variance that can derive from subsampling different regions of the wings, especially those regions differing in pigmentation (orange versus black). Within-wing isotopic (δ^2H, $\delta^{13}C$, $\delta^{15}N$) variance of 40 monarch butterflies collected from natural overwinter mortality on Mexican roost sites were split evenly into two groups: unwashed samples and those washed in a 2:1 chloroform:methanol solvent. Isotopic variance in δ^2H and $\delta^{13}C$ was related to pigment (within-wing range 5‰ and 0.5‰, respectively), but not region of subsampling. This variance was reduced 3 to 4 fold through solvent washing that removed pigmented surface scales and any adhered oils. Wing $\delta^{15}N$ was similarly influenced by pigment (range 0.3‰), but this effect was not reduced through washing. We recommend future isotopic studies of monarchs and other butterflies for migration research to use the same region for subsampling consistently and to wash samples with solvent to reduce isotopic variance related to uncontrolled variance in discrimination (δ^2H, $\delta^{13}C$, $\delta^{15}N$) and/or adsorbed water vapor (δ^2H). These data also need to be included in description of methods.

Keywords: carbon-13; deuterium; isoscapes; migration; monarch butterfly; nitrogen-15; stable isotopes

1 Introduction

The measurement of naturally occurring stable isotope ratios in animal tissues can provide valuable insights into the ecology of individuals and populations. Stable-carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in particular have been used to investigate dietary inputs, sources of feeding and trophic positions of a broad spectrum of taxa (Kelly 2000, Inger and Bearhop 2008, Hobson 2011, Hyodo 2015). More recently, measurements of stable hydrogen isotope ratios (δ^2H) have been used successfully to infer origins in numerous species of migratory birds and insects (Hobson and Wassenaar 2008, Hobson et al. 2012, Stefanescu et al. 2016). Among insects, the greatest focus has been on tracing origins and movements of the eastern North American population of monarch butterflies (*Danaus plexippus*). In fact, the first application of δ^2H measurements to track any animal was performed on monarchs to decipher natal origins of monarchs overwintering at their roost sites in central Mexico (Wassenaar and Hobson 1998, Hobson et al. 1999). Since then, the approach has been used by other researchers interested in monarch patterns of spring recolonization in North America,(Miller et al. 2012, Flockhart et al. 2013),

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the effects of natal origin on parasite loads (Altizer et al. 2015), the role of wing coloration in flight distance (Hanley et al. 2013) and general conservation concerns related to where most individuals are being produced (Flockhart et al. 2017). Such tracking studies have used continental patterns, or isoscapes, of both precipitation δ^2H and milkweed $\delta^{13}C$.

Despite the demonstrated value of stable isotope measurements of butterfly wings to investigate migration, to our knowledge, there has been no previous investigation into the isotopic variance among different regions of wing material (primarily chitin) despite the fact that monarch wings, as with those of many lepidopterans, are highly variable in pigmentation. Through the process of isotopic discrimination, products of biogeochemical reactions can differ from the substrate(s) used for their synthesis. So, even though all larval tissues are derived from the same host plant material, not all compounds in the larvae are isotopically identical. This is well established among macromolecules with, for example, lipids being isotopically lighter than proteins for ^{13}C and ^{2}H (Soto et al. 2013) but is expected to occur among broad classes of compounds. Monarch wing pigments are synthesized de novo during the last two days of metamorphosis, and their differing structure and metabolic pathways can potentially contribute to ultimate isotopic differences. Previous studies of bird feathers have shown stable isotope effects ($\delta^{13}C$, $\delta^{15}N$) of melanin content (Michalik et al. 2010), but no studies have investigated the effect of pigmentation on insect wing δ^2H. Monarch butterflies have wings that contrast strongly between orange and black pigments and the relative contribution of each varies between regions of the forewing and hindwing. The wing scales themselves obtain their color from either structural properties (related to interference patterns) or the presence of pigment molecules. The black scales of the monarch are colored by melanin pigments, while the orange are probably a combination of ommochromes and carotenoids (Rothshild et al. 1986, Janssen et al. 2001). Should there be inherent isotopic differences among these pigmented regions, then within- and between-study isotopic variance would be expected. Such isotopic differences could lead to potential errors or discontinuities in estimates of migratory origins of this and other butterfly species.

Monarchs, and presumably other butterfly species, differ in pigment concentrations due to amount and quality of larval diet (Davis 2014) and, if pigment type and concentration is related to wing stable isotope values, this will add to the isotopic variance among individuals from the same geographical origin. Sources of isotopic variance within monarch wings is particularly important because more and more, retrospective studies based on meta analyses rely on comparability of isotopic measurements among laboratories to infer changes in migration, origins or sites of productivity of monarchs in North America (Flockhart et al. 2017). Moreover, to our knowledge, few researchers routinely homogenize wing material and instead rely on small subsections cut from wings for stable isotope analyses (but see Yang et al. 2016). Studies specifically linking butterfly wing coloration with natal origin or migration distance based on isotopic models in particular (e.g. Hanley et al. 2013) need to establish how wing pigmentation and subsampling of wings in general for isotope analysis may affect stable isotope compositions. To this end, we investigated isotopic variance ($\delta^{13}C$, $\delta^{15}N$, δ^2H) within monarch butterfly wings with an emphasis on testing differences between orange and black pigmented regions and evaluated how standard sampling pretreatment methods such as solvent rinses that can remove surface pigmented scales might contribute to such variation.

2 Methods

2.1 The sample

Forty specimens (21 male and 19 female) were randomly selected from a collection taken under permit (SEMARNAT-Permit No. SGPA/DGVS/06299/15) for other investigations from natural overwinter mortality at two colonies (El Rosario and Sierra Chincua) in Michoacan, Mexico, during 2014 and 2015. All wings appeared clean and free of any adhered material and were stored individually in paper envelopes and frozen following collection. At the laboratory, wings were separated from the body and subject to one of two different preparatory methods.

Wings of 20 individuals were treated with a solution of 2:1 chloroform:methanol by soaking at room temperature for approximately 1 hour with only very gentle agitation at the beginning end of this period. After decanting the solvent, wings were dried at room temperature in a fumehood. The wings of another 20 individuals underwent no preparatory work prior to isotopic analysis. The wings of each butterfly were isolated in individual glass scintillation vials and stored in a desiccator for 3 to 10 days prior to weighing subsections for isotopic analyses.

A scalpel was used to remove four samples each from left and right wing pairs, for a total of eight samples per individual (Figure 1). Care was taken to remove only wing membrane and to avoid wing veins in the sample. Thus, our sample was designed to test for isotopic measurement

variation associated primarily with pigment (orange vs. black) and location (quadrants of left and right forewing vs. hindwing) of subsamples within individuals. We repeated the process on separate individuals for (1) δ^2H and (2) $\delta^{13}C$ and $\delta^{15}N$ analyses to allow for smaller subsampling (i.e. we were constrained by sample size requirements for mass spectrometric analysis).

We expected isotopic compositions among butterflies to be highly variable, given their diverse origins, and so restricted our analyses to within-wing variance for all isotopic measurements. General linear models were used for each of the two isotope groupings with the isotopic compositions as the dependent variable, wing quadrant and pigment as fixed factors and individual as a random factor. We did not anticipate differences between sexes, but each sample set was split approximately equally between males and females.

Figure 1. Top: Monarch butterfly from Sierra Chincua reserve in Mexico. Bottom: Sampling regions of monarch butterfly wings used in this study. We considered 4 quadrants (left upper, right upper, left lower, right lower) and eight subsections representing primarily black and orange pigmented regions.

2.2 Isotope measurement

2.2.1 δ^2H

Samples of 0.35 ± 0.02 mg of wing membrane were weighed into pressed silver 3.5 x 5 mm capsules, and analyzed using a Eurovector Uniprep autosampler (Milan, Italy) carousel attached to a Eurovector 3000 Elemental Analyzer, coupled to a Thermo Delta V Plus isotope ratio mass spectrometer (Bremen, Germany) in continuous flow mode with helium carrier gas. After the samples were loaded, the Uniprep autosampler (heated to 60°C) was vacuum evacuated and subsequently flushed with dry helium twice to remove adsorbed atmospheric moisture from the crushed silver capsules. Two USGS keratin standards, EC-01 (formerly CBS: Caribou Hoof Standard) and EC-02 (KHS: Kudu Horn Standard of Environment Canada) were included every ten samples. An internal laboratory standard, powdered keratin (MP Biomedicals Inc., Cat No. 90211, Lot No.9966H) was included to monitor instrument drift and provide a check on accuracy over the course of each analytical session. Samples were combusted at 1350°C using glassy carbon. Values of δ^2H of non-exchangeable hydrogen were derived using the comparative equilibration approach of Wassenaar and Hobson (2003) and calibrated to VSMOW using EC-01 (± 1.9 ‰ 1 SD, n = 18, accepted δ^2H = −197.0 ‰) and EC-02 (± 1.6 ‰, n = 17, accepted δ^2H = –54.1 ‰). Overall measurement error for EC-01 and EC-02 δ^2H was ± 2.7 and ± 2.6 ‰, respectively.

2.2.2 $\delta^{13}C$ and $\delta^{15}N$

Approximately 0.35 ± 0.02 mg of wing membrane was weighed into 4 x 3.2 mm tin pressed capsules. Concurrent $\delta^{13}C$ and $\delta^{15}N$ analyses were performed using a Costech Elemental Analyzer coupled to a Thermo Delta Plus XL isotope ratio mass spectrometer operated in continuous flow mode, with helium carrier gas. Two standards, USGS-40 and USGS-41 were included for every ten samples and two internal laboratory standards, powdered keratin (MP Biomedicals Inc., Cat No. 90211, Lot No.9966H) and IAEA-CH-6 were included to monitor instrument drift and provide a check on accuracy over the course of each analytical session. Values of $\delta^{13}C$ were calibrated to VPDB using USGS-40 (± 0.1 ‰ 1 SD, n = 8, accepted $\delta^{13}C$ = –26.4 ‰) and USGS-41 (± 0.1 ‰, n = 8, accepted $\delta^{13}C$ = +37.6 ‰).

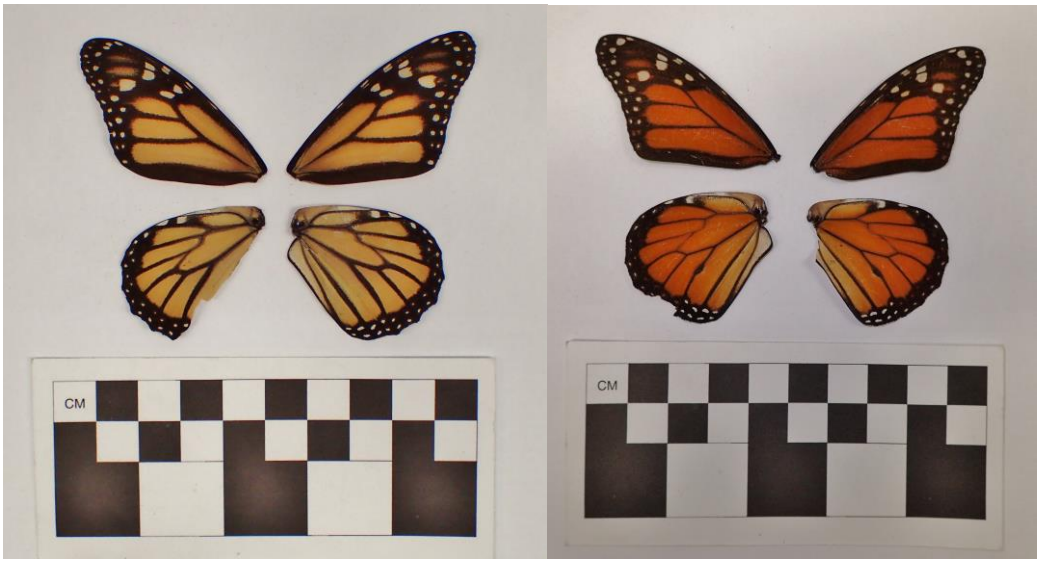

Figure 2. A demonstration of the reduction in color intensity due to washing monarch wings in 2:1 chloroform:methanol solvent. Individual on the left was treated with solvent and the individual on the right was not. While not the same individual, this color change was typical of all of the specimens we examined.

Values of $\delta^{15}N$ were calibrated to AIR using USGS-40 (± 0.0 ‰, n = 8, accepted $\delta^{15}N$ = –4.5 ‰) and USGS-41 (± 0.2 ‰, n = 8, accepted $\delta^{15}N$ = +47.6 ‰). Measurement error was ± 0.1 ‰ for $\delta^{13}C$, and ± 0.2 ‰ for $\delta^{15}N$.

3 Results

3.1 Variation in δ^2H

As expected, mean δ^2H among individual butterflies ranged from –150.4 ‰ to –98.7 ‰ reflecting expected broad natal latitudinal origins in North America.

For washed wings, both quadrant (df=3,27; F=2.213, p=0.110) and pigment (df=1,9; F=1.874; p=0.204) had no effect on wing δ^2H but there was a significant interaction between pigment and quadrant (df=3,27; F=3.070; p=0.048). For unwashed wings, quadrant had no effect on wing δ^2H (df=2,27; F=2.15; p=0.117) but pigment was significant (df=1,9; F=7.25; p=0.025) with black pigment generally more depleted of ^2H than orange pigment. We found a 3 to 4 fold increase in total range of within-wing isotopic variance for unwashed versus washed wings (Table 1). No effect of sex was found for differences in within-wing δ^2H values for washed (df=1,8; F=0.245; p=0.634) and unwashed (df=1,8; F=0.232; p=0.643).

3.2 $\delta^{13}C$ and $\delta^{15}N$

As with δ^2H measurements, we found a broad range in wing $\delta^{13}C$ (–29.9 ‰ to –24.9 ‰) and $\delta^{15}N$ (+1.7 to +12.0 ‰) among individuals. This reflects a broad range in geographic origins (**$\delta^{13}C$**) and also the potential for host-plant (milkweed) utilizing different sources of bioavailable nitrogen in different regions.

For unwashed wings, pigment had a strong effect on $\delta^{13}C$ (df=1,9; F=157.87; p<0.001) but quadrant did not

Table 1. Mean (±SD in ‰) of the within-wing range in stable isotope compositions (i.e. within-wing isotopic variance) for the monarch butterfly sample. The treated group were soaked and rinsed in a 2:1 chloroform:methanol and air dried. Wings from ten individuals were used per treatment and each sample consisted of 8 subsamples taken from each wing set as shown in Figure 1. Evident is a dramatic reduction in within-wing isotopic variance following treatment, especially for δ^2H values and pervasive differences in $\delta^{13}C$ related to pigment.

Pigment	Treated			Untreated		
	δ^2H	$\delta^{13}C$	$\delta^{15}N$	δ^2H	$\delta^{13}C$	$\delta^{15}N$
Orange	2.1±1.0	0.17±0.07	0.16±0.05	8.8±10.4	0.29±0.2	0.31±0.2
Black	2.8±1.0	0.15±0.08	0.31±0.15	8.8±2.8	0.27±0.1	0.33±0.1
Overall	2.5±1.1	0.16±0.08	0.24±0.14	8.8±7.4	0.28±0.2	0.32±0.2
Black-Orange	0.25±1.9	-0.54±0.08	0.06±0.14	-4.9±6.1	-0.45±0.11	0.37±0.17

(df=3,27; F=1.195; p=0.330). Black pigments were found to be more depleted of ^{13}C than orange (Table 1). This trend held after washing (quadrant: df=3,27; F=0.320; p=0.811; pigment: df=1,9; F=426.7, p<0.001) with a similar difference in $\delta^{13}C$ between pigments (Table 1). No interaction terms were significant. No effect of sex on differences between pigment $\delta^{13}C$ within wings was found for either treatments (washed; df=1,8; F=1.04; p=0.337; unwashed: df=1,8; F=1.06; p=0.33).

For $\delta^{15}N$, unwashed wing samples showed a similar strong effect of pigment (df=1,9; F= 50.855; p<0.001) where black pigments were generally more enriched in ^{15}N than orange. Quadrant had no effect on wing $\delta^{15}N$ (df=3,27; F=0.344; p=0.793). This differed from washed samples for which $\delta^{15}N$ was affected by quadrant (df=1,9; F=5.513; p=0.004) but not pigment (df=1,9; F=1.846; p=0.207). In both washed and unwashed samples, interaction between pigment and quadrant were significant (unwashed: df=3,27; F=31.642; p<0.001; washed: df=3,27; F= 9.643; p<0.001). No effect of sex on differences between pigment $\delta^{15}N$ within wings was found for both treatments (washed; df=1,8; F=0.00; p=0.983; unwashed: df=1,8; F=1.06; p=0.33).

4 Discussion

We found that the stable isotope measurements of individual monarch butterflies varied by the relative amounts of black and orange pigments, but not by wing quadrant. These effects were reduced considerably through washing the wings in a solvent (2:1 chloroform;methanol), which clearly removed pigments as well as adhered oils. Wing scales and associated pigments apparently contribute greatly to the isotopic variance measured in butterfly wings. In addition or alternatively, the presence of any oils on wings is also expected to contribute to wing δ^2H and $\delta^{13}C$ variance because lipids are well known to be depleted in the heavier isotope in each case. The remaining wing membrane by contrast, is relatively isotopically homogenous. Our results have important ramifications for studies that attempt to assign migratory monarchs and other butterflies to natal origin using combined δ^2H and $\delta^{13}C$ wing isoscapes because different sampling approaches and wing pretreatments among laboratories could result in error of the order of 5 ‰ for δ^2H and 0.5 ‰ for $\delta^{13}C$. Although, to our knowledge, $\delta^{15}N$ has not been used to assign monarch butterflies to origin, this isotope has been used to assist with assignment to molt origins of birds in Africa (Hobson et al. 2014). Future studies using this isotope for provenance or dietary studies of monarchs and other insects should be aware that the effect of pigment can be of the order of 0.4 ‰.

While the magnitude of variance in wing δ^2H and $\delta^{13}C$ values may seem relatively minor for the purposes of assignment to isoscapes and hence migration studies, systematic isotopic differences among laboratories of the order of those determined here can indeed be significant. The worst case scenario would derive from a lack of solvent rinsing and any systematic sampling differences of sections of the wing that favor one pigment (i.e. orange vs. black) over the other. To appreciate this, in Table 2 we present the expected mean wing δ^2H and $\delta^{13}C$ values characterizing the various regions (e.g. Midwest, Northcentral, Northeast, Southeast, Southwest) of origin presented in Flockhart et al. (2017). A systematic difference of 5 ‰ for δ^2H and 0.5 ‰ for $\delta^{13}C$ would result in differences in assignment especially to Southeast and Southwest regions and among Midwest, Northeast and Northwest regions. The only way this could be avoided would be for each laboratory to use an assignment algorithm that accounted for such differences in regional categories and/or propagated any additional variance in measurement error. Fortunately, as we demonstrate here, this situation can be avoided by using a solvent rinse but further reductions in variance can also be expected from systematic and consistent sampling of one region of the

Table 2. Mean and SD (‰) of expected monarch wing stable isotope values used by Flockhart et al. (2017) to assign individual monarchs to regions of natal origin. This illustrates the problem of any systematic differences among laboratories amounting to as much as a 5‰ difference in δ^2H and a 0.5‰ difference in $\delta^{13}C$ that can arise from lack of solvent rinsing and differential use of black vs orange pigmented regions.

Region	Mean δ^2H	SD	Mean $\delta^{13}C$	SD
Southwest	-97.5	6.6	-28.9	1.1
Southeast	-95.5	6.2	-29.0	0.5
Midwest	-110.2	7.9	-27.6	1.1
North Central	-117.3	7.3	-27.1	0.9
Northeast	-112.0	10.8	-27.7	1.6
Northwest	-135.8	10.7	-27.9	0.6

wing. At the very least, authors need to report precisely how butterfly wings from migratory species have been sampled for assignment to origins.

The fact that solvent rinsing greatly reduced within-wing and between pigment isotopic variance for all isotopes to within measurement error strongly suggests that researchers should routinely adopt this sample pretreatment approach. Moreover, our own research and that of others has indicated that wing wear, in turn related to the age of an individual and distance travelled, can be quite variable among individuals and represents differential loss of scales containing pigment (Flockhart et al. 2013, Stefanescu et al. 2016). Thus, the routine removal of surface pigments is recommended to reduce the potential for within-wing variance related to natal origin (i.e. distance travelled and wing wear) and diet. Presumably, the wing membrane material remaining after solvent extraction of pigments and any adhered oils is primarily, but not exclusively (since some color still remains after washing), chitin and the non-exchangeable H portion of this component will be most appropriate to use on migratory assignments using δ^2H measurements in particular. Currently, the source(s) of variation in pigment δ^2H is not clear. This could be a result of differential isotopic discrimination during pigment synthesis or could reflect differential adsorption of ambient moisture by each pigment prior to measurement. The measurement of δ^2H in complex organic materials is challenging since a significant proportion of weakly bonded hydrogen can exchange with hydrogen from ambient water vapor. This effect can be controlled for by using the comparative equilibration approach employed here or use of modified carousel systems Meier-Augenstein et al. 2013, Wassenaar et al. 2015). Orange coloration is caused by the differential deposition of color pigments such as pterins and ommochromes on to wing scales Janssen et al. 2001). Melanin is derived from either fatty acids/acetyl CoA or tyrosine, both which are depleted of ^{13}C relative to compounds similarly involved in orange pigment synthesis. Tyrosine is a semi-essential amino acid, which can be obtained from the diet or synthesized from the essential amino acid, phenylalanine (McGraw et al. 2006). Given that diet as well as origin can contribute to the pigments in butterfly wings, for studies of migration and provenance, unnecessary isotopic variance result from diet needs to be reduced whenever possible.

Since no butterfly wing chitin standards exist that might also control for pigment content, researchers need to be especially vigilant in their analytical approaches using δ^2H of butterfly wing material (Meier-Augenstein et al. 2013). We routinely use the comparative equilibration approach of Wassenaar and Hobson (2003) that involves within-run analysis of keratin standards EC-01 and EC-02 (now available from the U.S. Geological Survey) to calibrate our monarch measurements to non-exchangeable hydrogen on the VSMOW scale. Until homogenized and calibrated insect chitin standards are developed that also control for pigment content, we recommend this approach, coupled with the solvent rinse procedure described here and consistent sampling of wing quadrant among individuals. The cryogenic homogenization of whole wing samples is ideal (Yang et al. 2015) but the calibration algorithm linking monarch wings to amount-weighted mean growing season average precipitation δ^2H used by most migration assignment studies is currently based on whole wing clippings.

Previous studies have reported effects of pigment type on $\delta^{13}C$ in feathers (Michalik et al. 2010) with melanin being generally depleted of ^{13}C compared to white pigmented regions. We similarly found that black pigmented regions of monarch wings had lower $\delta^{13}C$ compared to orange regions. In contrast, black wing regions tended to be enriched in ^{15}N compared to orange areas. Currently, $\delta^{13}C$ is useful in combination with δ^2H for dual isotope assignments to native origin. While more complex and poorly studied, wing $\delta^{15}N$ can provide information on the source of nitrogen to milkweed plants and on regional agricultural or land-use practices. We anticipate that our recommendations pertaining primarily to sampling and pretreatment for provenance assignments using δ^2H and $\delta^{13}C$ in monarch wings will be applicable also to any future studies using $\delta^{15}N$.

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