

## Article

# Isotopic Niche Segregation among Darwin's Finches on Santa Cruz Island, Galápagos

Mariana Villegas <sup>1,2,\*</sup>, Catherine Soos <sup>1,2</sup> , Gustavo Jiménez-Uzcátegui <sup>3</sup>, Shukri Matan <sup>4</sup> and Keith A. Hobson <sup>2,4</sup>

<sup>1</sup> Department of Veterinary Pathology, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada; catherine.soos@canada.ca

<sup>2</sup> Environment and Climate Change Canada, Science & Technology Branch, Saskatoon, SK S7N 0X4, Canada; khobson6@uwo.ca

<sup>3</sup> Charles Darwin Research Station, Charles Darwin Foundation, Puerto Ayora, Galápagos 200350, Ecuador; gustavo.jimenez@fcdarwin.org.ec

<sup>4</sup> Department of Biology, University of Western Ontario, London, ON N6A 5B7, Canada; smatan2@uwo.ca

\* Correspondence: mariana.villegas@gmail.com; Tel.: +1-591-71294780

† Current address: Instituto de Ecología, Universidad Mayor de San Andrés, Calle 27 Campus Universitario de Cota Cota, 0001 La Paz, Bolivia.

**Abstract:** Darwin's finches are a classic example of adaptive radiation involving differential use of dietary resources among sympatric species. Here, we apply stable isotope ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^2\text{H}$ ) analyses of feathers to examine ecological segregation among eight Darwin's finch species in Santa Cruz Island, Galápagos collected from live birds and museum specimens (1962–2019). We found that  $\delta^{13}\text{C}$  values were higher for the granivorous and herbivorous foraging guilds, and lower for the insectivorous finches. Values of  $\delta^{15}\text{N}$  were similar among foraging guilds but values of  $\delta^2\text{H}$  were higher for insectivores, followed by granivores, and lowest for herbivores. The herbivorous guild generally occupied the largest isotopic standard ellipse areas for all isotopic combinations and the insectivorous guild the smallest. Values of  $\delta^2\text{H}$  provided better trophic discrimination than those of  $\delta^{15}\text{N}$  possibly due to confounding influences of agricultural inputs of nitrogen. Segregation among guilds was enhanced by portraying guilds in three-dimensional isotope ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^2\text{H}$ ) space. Values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were higher for feathers of museum specimens than for live birds. We provide evidence that Darwin's finches on Santa Cruz Island tend to be generalists with overlapping isotopic niches and suggest that dietary overlap may also be more considerable than previously thought.

**Keywords:** Darwin's finches; foraging guild; Galápagos; isotopic niche; carbon-13; nitrogen-15; deuterium; trophic position



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## 1. Introduction

Darwin's finches of the Galápagos Islands are a classic example of adaptive radiation and ecological segregation [1,2]. Currently, 17 species that derived from a common ancestor are recognized and they occupy a variety of individual ecological niches. It has been suggested that behavioral flexibility and innovation were the main drivers in the radiation of Darwin's finches [3–5]. Today, ground finches (*Geospiza* spp.) feed primarily on seeds, while tree finches (*Camarhynchus* spp.) feed on fruits and arthropods in trees, the single vegetarian finch (*Platyspiza crassirostris*) on leaves and fruits, and warbler finches (*Certhidea* spp.) on arthropods [1,6]. These differences in diet are also famously associated with differences in beak size and shape. *Geospiza* finches show temporal and spatial variation in their diets and there is dietary overlap among species [6,7], but in general, tree and ground finches show opportunistic resource use across seasons and years [6,8,9]. Ground finches represent an adaptive radiation, where they may be characterized as being 'imperfect

generalists' that have evolved to use a variety of overlapping resources, and are mostly opportunistic feeders [6]. However, like many avian groups, information on precise diets is lacking. The frequent environmental changes and climatic variation likely drive the use of versatile feeding strategies and dietary overlap among Darwin's finches [6,10].

Measurements of naturally occurring stable isotope ratios in consumer tissues have become a useful tool to reconstruct aspects of dietary, ecological, and environmental histories in terrestrial and marine ecosystems [11–15]. Such measurements can be used to trace movements of nutrients, compounds, particles and organisms across landscapes, and provide information on the source of nutrients to foodwebs and trophic positions of consumers [12], thus augmenting more conventional approaches to studying diets.

Isotope studies of diet and habitat use are based on the principle that isotope values of consumer tissues reflect those of diet and drinking water [16]. In particular, stable-carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotope ratios have been used to evaluate source of feeding and trophic level, respectively. Stable carbon isotope ratios are particularly useful in evaluating relative contributions to foodwebs from plants with different photosynthetic pathways (i.e., C3, C4 or CAM) [17,18]. In addition to providing such direct information on diets, these two isotopes are also linked to climate and anthropogenic landuse practices [19–22]. Stable hydrogen ( $\delta^2\text{H}$ ) isotope ratios in consumer tissues can reflect those of diet and drinking water [16,23]. Stable hydrogen isotopes in precipitation also show well-known and predictable continental patterns, and so have been used to track animal movements [21,24,25], but tissue  $\delta^2\text{H}$  values can also be used to investigate diet and habitat use at more local scales [26].

Birds in the Galápagos Islands have access to both terrestrial and marine nutrients. Marine nutrients can be derived directly through foraging in intertidal or beach zones or indirectly through cascading effects of nutrient transfer from marine sources to terrestrial foodwebs. Consumers that rely on marine resources typically have higher  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values compared to those consuming terrestrial foods [27]. Within terrestrial systems, changes in altitude can result in different vegetation zones due to associated changes in precipitation and temperature and these influence local foodweb stable isotope values [28–31]. Despite the potential isotopic complexity of foodwebs of birds on the Galápagos Islands, the use of a multi-isotope approach to examining avian diets in this system can be informative (e.g., [32–34]). Moreover, the development of multidimensional isotopic niches in ecology has allowed the examination of interspecific niche overlap and segregation thus allowing insight into how animal communities are structured [13]. While isotopic niches are not necessarily true ecological niches [35,36], they nonetheless provide a "first look" into potential evolutionary patterns resulting in coexisting species communities [37,38].

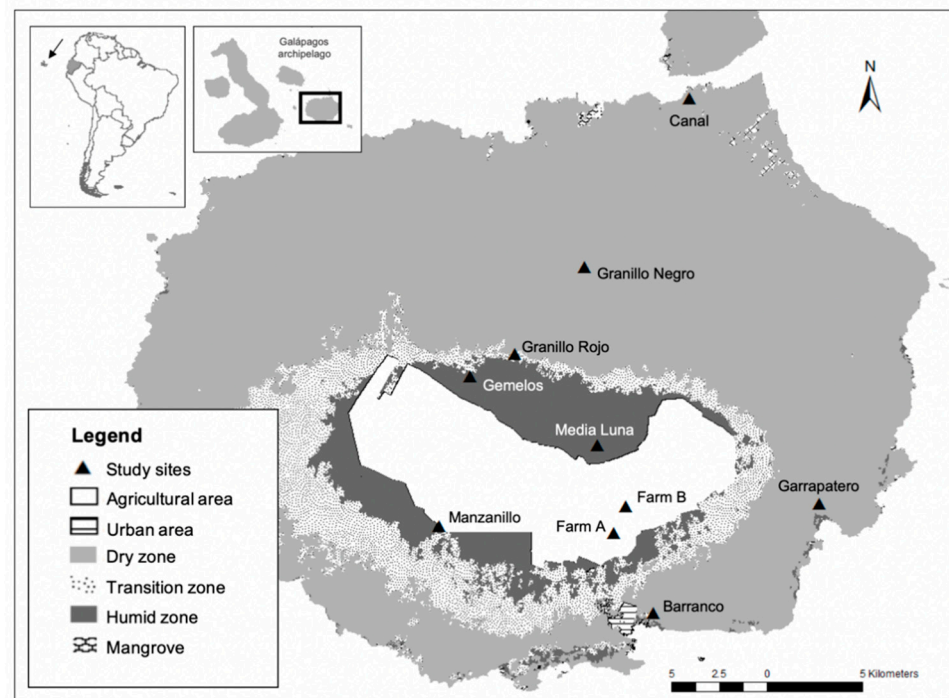
Here, we build upon the extensive foundation of studies on Darwin's finches, by introducing the use of stable isotope analyses of feathers to examine mechanisms of ecological segregation among these iconic sympatric species. We hypothesized that variation in environmental conditions and nutritional sources throughout different habitats on Santa Cruz Island, will be generally reflected in the isotopic signals of feathers but that individual species' isotopic niches would reveal strong segregation at each community level. We used a three-isotope ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^2\text{H}$ ) approach to examine feathers collected from both contemporary field campaigns and long-term museum collections, which also allowed us to examine niche characteristics through time [16,39].

## 2. Materials and Methods

### 2.1. Field Collections

Santa Cruz Island is the second largest island in the Galápagos archipelago (985 km<sup>2</sup>, maximum altitude 864 m; Figure 1) [29,30]. In June 2019, we collected feather samples at ten study sites (Figure 1), in the following habitats [29–31,40]: (1) Dry zone (up to ~120 m above sea level), comprised of deciduous forest, shrubland and grassland; (2) Transition zone (~120–300 masl), comprised of evergreen (seasonal) forest and shrubland; (3) Humid

zone, (>300 masl), comprised of three distinctive vegetation areas: *Scalesia* forest, *Miconia* shrubland and fern area (>650 masl); and (4) agricultural lands, located within the transition and humid zones (Figure 1). We also coordinated with the road-kill mortality project PC-09-18 conducted by the Fundación Charles Darwin–Dirección del Parque Nacional Galápagos (FCD-DPNG), in which wing flight feather samples were collected by G.J.U. at roadside margins in 2018.



**Figure 1.** Location of study sites in Santa Cruz Island, Galápagos, Ecuador. Dry zone (4 sites): Barranco, Garrapatero, Granillo Negro and Canal; Transition zone (2 sites): Granillo Rojo and Manzanillo; Humid zone (2 sites): Gemelos and Media Luna; and Agricultural lands (2 sites): Farm A and Farm B (map modified from shapefiles kindly provided by Rivas-Torres et al. [31]).

At each site, birds were captured using an array of six mistnets (6 m and 12 m) operated for two days (0600 h–1130 h). Nets were checked continuously. Birds were removed from mist nets and placed in individual cloth bags in a shaded area until processed. Birds were weighed (nearest 0.1 g) using a digital or string Pesola scale and morphometric measurements (head-bill length, exposed culmen, tarsus) were taken with a Vernier dial caliper with a precision of 0.02 mm. A measurement of the unflattened, right wing-chord was taken with a wing ruler (1 mm). Individuals were banded with a uniquely coded aluminum band before release. From each individual, we sampled 2–3 secondary wing coverts; all feathers from one individual were stored in a single paper coin envelope. Envelopes were stored in a plastic box containing silica gel, and kept at room temperature.

Feathers were sampled from 231 live birds, belonging to eight species (Table 1 and Supplementary Figure S1): Small Ground Finch (SGF, *Geospiza fuliginosa*); Medium Ground Finch (MGF, *Geospiza fortis*); Large Ground Finch (LGF, *Geospiza magnirostris*); Common Cactus Finch (CF, *Geospiza scandens*); Small Tree Finch (STF, *Camarhynchus parvulus*); Woodpecker Finch (WF, *Camarhynchus pallidus*); Green Warbler Finch (GWF, *Certhidea olivacea*); Vegetarian Finch (VF, *Platyspiza crassirostris*). We assigned all species to three foraging guilds (following [6,41]). Guilds were: (1) granivorous ground finches (i.e., opportunist feeders primarily on seeds) which included two species, SGF and MGF; (2) herbivorous finches (i.e., feeders on large seeds, leaves, fruits, and nectar), which included LGF, CF and VF; and (3) insectivorous finches (i.e., feeding primarily on small invertebrates), which included STF, GWF and WF (Table 1, Tables S1 and S2).

**Table 1.** Isotopic values (means and standard deviations in ‰) in feathers of live birds and museum specimens (see Table S2 for details on museum specimens). Differences per species, foraging guilds and habitats were examined with ANOVAs with Tukey's post-hoc tests (different superscripts represent statistical differences at  $p < 0.05$ ).

		n	$\delta^{13}\text{C}$ Mean $\pm$ SD	$\delta^{15}\text{N}$ Mean $\pm$ SD	$\delta^2\text{H}$ Mean $\pm$ SD
<b>LIVE BIRDS (N = 231)</b>					
<b>Per species:</b>					
<b>Species</b>	<b>Common name</b>				
<i>Geospiza fuliginosa</i>	Small Ground Finch (SGF)	83	$-20.18 \pm 3.53^a$	$9.67 \pm 1.53^a$	$-54.33 \pm 9.95^b$
<i>Geospiza fortis</i>	Medium Ground Finch (MGF)	51	$-22.07 \pm 2.17^{ab}$	$9.93 \pm 1.32^a$	$-56.74 \pm 9.53^{bc}$
<i>Geospiza magnirostris</i>	Large Ground Finch (LGF)	27	$-20.96 \pm 2.01^{ab}$	$9.83 \pm 2.02^a$	$-64.17 \pm 8.22^c$
<i>Geospiza scandens</i>	Common Cactus Finch (CF)	9	$-19.27 \pm 3.39^a$	$10.84 \pm 2.13^a$	$-50.77 \pm 13.93^{ab}$
<i>Camarhynchus parvulus</i>	Small Tree Finch (STF)	32	$-21.78 \pm 2.88^{ab}$	$10.33 \pm 2.20^a$	$-47.88 \pm 8.56^{ab}$
<i>Camarhynchus pallidus</i>	Woodpecker Finch (WF)	6	$-22.82 \pm 1.16^{ab}$	$11.12 \pm 1.80^a$	$-40.91 \pm 7.52^a$
<i>Certhidea olivacea</i>	Green Warbler Finch (GWF)	10	$-23.85 \pm 0.70^b$	$10.76 \pm 1.01^a$	$-47.86 \pm 6.27^{ab}$
<i>Platypiza crassirostris</i>	Vegetarian Finch (VF)	13	$-21.45 \pm 1.46^{ab}$	$10.13 \pm 1.26^a$	$-54.59 \pm 9.78^{bc}$
<b>Per guilds:</b>					
	Granivorous (SGF, MGF)	134	$-20.89 \pm 3.21^a$	$9.77 \pm 1.45^b$	$-55.25 \pm 9.52^b$
	Herbivorous (LGF, CF, VF)	65	$-20.15 \pm 3.13^a$	$10.41 \pm 2.04^{ab}$	$-58.99 \pm 11.05^c$
	Insectivorous (STF, GWF, WF)	51	$-22.23 \pm 2.52^b$	$10.61 \pm 1.97^a$	$-46.62 \pm 8.53^a$
<b>Per habitats:</b>					
	Dry Zone	85	$-20.77 \pm 2.72^{ab}$	$10.17 \pm 1.37^a$	$-53.36 \pm 10.80^a$
	Transition zone	49	$-22.19 \pm 2.62^c$	$10.05 \pm 1.97^a$	$-54.85 \pm 10.97^a$
	Humid zone	53	$-21.91 \pm 2.62^{bc}$	$9.96 \pm 1.92^a$	$-55.95 \pm 10.99^a$
	Agricultural areas (farms)	44	$-19.91 \pm 3.52^a$	$9.64 \pm 1.55^a$	$-53.87 \pm 6.51^a$
<b>MUSEUM SPECIMENS (N = 19)</b>					
<b>Species</b>	<b>Common Name</b>				
<i>Geospiza magnirostris</i>	Large Ground Finch (LGF)	9	$-20.15 \pm 1.47$	$9.83 \pm 1.21$	$-65.81 \pm 4.36$
<i>Geospiza scandens</i>	Common Cactus Finch (CF)	7	$-15.77 \pm 5.88$	$13.35 \pm 1.67$	$-48.97 \pm 9.13$
<i>Camarhynchus parvulus</i>	Small Tree Finch (STF)	3	$-20.54 \pm 1.42$	$12.08 \pm 2.08$	$-40.53 \pm 13.37$

## 2.2. Museum Collections

We collected wing secondary coverts from 19 museum specimens stored at the Vertebrate Collection of the Charles Darwin Research Station (VCCDRS) in Puerto Ayora, Santa Cruz (specimens ranged from 1962 to 2011; Table S2). From these specimens, we pulled 2-3 secondary wing coverts feathers per specimen. All feathers from one specimen were placed in a single coin envelope, and stored as described above.

## 2.3. Laboratory Analyses

All feather samples were cleaned of surface contaminants using a 2:1 chloroform: methanol rinse and air dried overnight in a fume hood. Feather  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  assays were processed at the stable isotope laboratory of Environment and Climate Change Canada in Saskatoon, Canada. Subsamples ( $0.5 \pm 0.02$  mg) of vane material were weighed into tin capsules and combusted at  $1030^\circ\text{C}$  in a Eurovector 3000 elemental analyser (Milan, Italy). The resulting  $\text{N}_2$  and  $\text{CO}_2$  were separated chromatographically and introduced to an Elementar Isoprime (Manchester, UK) or a Nu Instruments Horizon (Wrexham, UK) isotope ratio mass spectrometer. We used two internal reference materials to calibrate results to Vienna Pee Dee Belemnite (VPDB:  $\delta^{13}\text{C}$ ) and AIR ( $\delta^{15}\text{N}$ ), BWBIII (baleen) keratin ( $\delta^{13}\text{C} = -20.18$ ,  $\delta^{15}\text{N} = +14.31$ ‰) and the commercial gelatin PRCgel ( $\delta^{13}\text{C} = -13.64$ ,  $\delta^{15}\text{N} = +5.07$ ‰). Within-run ( $n = 5$ ) precisions as determined from both reference and sample duplicate analyses were  $\pm 0.1$ ‰ for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

For the  $\delta^2\text{H}$  measurements of feathers ( $\delta^2\text{H}_w$ ),  $0.35 \pm 0.02$  mg of feather vane was weighed into silver  $3.5 \times 5$  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 (Bremen, Germany) isotope-ratio mass spectrometer 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. The autosampler was then held under positive helium pressure for the duration of the analytical run. Two USGS keratin standards, EC-01 (formerly CBS: Caribou Hoof Standard) and EC-02 (formerly 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  $\delta^2\text{H}$  of non-exchangeable hydrogen were derived using the comparative equilibration approach of Wassenaar and Hobson [42] and calibrated to VSMOW using EC-01 ( $\pm 1.9\%$ , 1 SD,  $n = 18$ , accepted  $\delta^2\text{H} = -197.0\%$ ) and EC-02 ( $\pm 1.6\%$ ,  $n = 17$ , accepted  $\delta^2\text{H} = -54.1\%$ ). Overall measurement error for EC-01 and EC-02  $\delta^2\text{H}$  was  $\sim 2\%$ .

#### 2.4. Statistical Analyses

Our response variables were feather  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values and the explanatory variables used were Species, Habitat, Site, Altitude, Foraging guild and Mass. We conducted separate ANOVAs and Tukey's post-hoc tests to examine differences in feather isotopic values among categories of interest such as species, foraging guilds and habitats. To evaluate the importance of these explanatory variables on feather isotope values, we used generalized linear models, with a Gaussian distribution and the "identity" link function. Given that  $\delta^{15}\text{N}$  values had a non-normal distribution, skewed to the lower values, we applied a "log-link" to  $\delta^{15}\text{N}$  values rather than the "identity" link function. Best models were selected and compared using Akaike Information Criterion scores corrected for small sample sizes (AICc). The model with the lowest AICc was considered to have the best fit. Model sets were conducted separately for: (1) live birds, which were the majority (eight species); and (2) species sampled from museum specimens and live birds (only CF, LGF and STF) for which we used Origin (i.e., museum or live) as an explanatory variable instead of Mass. We also examined individual relationships among feather  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values and with each of the explanatory variables considered using simple general linear models. In addition, with general linear models we tested whether feather isotope values differed among guilds in each of the habitats.

Isotopic niche was evaluated among foraging guilds using standard ellipse areas (SEA) with the correction for small sample sizes ( $\text{SEA}_c$ ), estimated using a Bayesian approach in the SIBER package [43]. We also used this Bayesian approach in SIBER to estimate niche overlap between ellipses of sampling categories. In order to graph the isotopic niche in a three-dimensional space, we followed the model and code proposed by Rossman et al. [33], which represents an extension of Jackson et al. [43] and measures standard ellipse volumes instead of areas. These approaches allowed us to make a comparative analysis of isotopic niches among species and category levels of the different explanatory variables, and allowed a characterization of niche overlap and segregation among Darwin's finch species. All statistical analyses were conducted in R (Version 4.0.3, [44]).

### 3. Results

Feather  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values are summarized in Table 1. Values of  $\delta^{13}\text{C}$  were lowest for GWF, highest for CF and SGF, and intermediate for MGF, LGF, STF and WF (Table 1). Foraging guilds differed for all isotopes, whereby the insectivorous guild had the lowest  $\delta^{13}\text{C}$  values and highest  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values (Table 1). Habitat groupings differed only in  $\delta^{13}\text{C}$  values (Table 1).

For live birds, the model with the lowest AICc score and strongest support both for  $\delta^{13}\text{C}$  and  $\delta^2\text{H}$  values included Mass, Species and Site as explanatory variables (Table 2 and Supplementary Table S3). Values of  $\delta^{15}\text{N}$  did not differ among species (Table 1). For  $\delta^{15}\text{N}$

values, the model with lowest AICc score and strongest support included Mass, Foraging guild and Habitat (Table 2 and Supplementary Table S3). Values of  $\delta^2\text{H}$  were highest for WF, CF, STF and GWF and lowest for LGF (Table 1) and lowest for sites San José farm, Gemelos and Granillo Rojo (Supplementary Table S3).

**Table 2.** Summary of results from the best generalized linear models (GLM) explaining the response variables (i.e.,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values) in each dataset analyzed: (A) Live birds (N = 231), and (B) Species with live and museum data (N = 85).

Model	AICc	Delta AICc	Weight
<b>(A) Live birds (N = 231)</b>			
$\delta^{13}\text{C}\sim\text{Mass} + \text{Species} + \text{Site}$	1121.12	0.00	0.674
$\delta^{13}\text{C}\sim\text{Mass} + \text{Guild} + \text{Habitat}$	1123.87	2.75	0.170
$\delta^{13}\text{C}\sim\text{Species} + \text{Altitude} + \text{Habitat}$	1125.09	3.97	0.092
$\delta^{13}\text{C}\sim\text{Species} + \text{Habitat}$	1127.84	6.72	0.023
$\delta^{13}\text{C}\sim\text{Habitat} + \text{Site} + \text{Species}$	1128.39	7.27	0.018
$\delta^{13}\text{C}\sim 1$	1159.05	37.93	<0.001
$\delta^{15}\text{N}\sim\text{Mass} + \text{Guild} + \text{Habitat}$	892.46	0.00	0.452
$\delta^{15}\text{N}\sim\text{Mass}$	895.02	2.56	0.125
$\delta^{15}\text{N}\sim\text{Guild} + \text{Altitude}$	895.43	2.96	0.102
$\delta^{15}\text{N}\sim\text{Guild}$	895.69	3.23	0.089
$\delta^{15}\text{N}\sim 1$	899.54	6.42	0.018
$\delta^2\text{H}\sim\text{Mass} + \text{Species} + \text{Site}$	1658.24	0.00	0.951
$\delta^2\text{H}\sim\text{Species} + \text{Altitude} + \text{Habitat}$	1664.43	6.18	0.043
$\delta^2\text{H}\sim\text{Habitat} + \text{Site} + \text{Species}$	1670.11	11.87	0.002
$\delta^2\text{H}\sim\text{Species} + \text{Site}$	1670.11	11.87	0.002
$\delta^2\text{H}\sim 1$	1749.64	91.40	<0.001
<b>(B) Species with live and museum data (N = 85)</b>			
$\delta^{13}\text{C}\sim\text{Species} + \text{Habitat}$	426.77	0.00	0.194
$\delta^{13}\text{C}\sim\text{Habitat} + \text{Site}$	427.00	0.23	0.173
$\delta^{13}\text{C}\sim\text{Site}$	427.00	0.23	0.173
$\delta^{13}\text{C}\sim\text{Guild} + \text{Site}$	427.16	0.38	0.159
$\delta^{13}\text{C}\sim 1$	447.02	20.24	<0.001
$\delta^{15}\text{N}\sim\text{Origin} + \text{Species}$	370.48	0.00	0.305
$\delta^{15}\text{N}\sim\text{Species} + \text{Habitat}$	371.63	1.15	0.172
$\delta^{15}\text{N}\sim\text{Species} + \text{Altitude}$	372.88	2.39	0.092
$\delta^{15}\text{N}\sim\text{Species} + \text{Altitude} + \text{Guild}$	372.88	2.39	0.092
$\delta^{15}\text{N}\sim 1$	378.99	8.51	0.004
$\delta^2\text{H}\sim\text{Species} + \text{Habitat}$	612.09	0.00	0.636
$\delta^2\text{H}\sim\text{Species} + \text{Altitude} + \text{Habitat}$	614.49	2.40	0.191
$\delta^2\text{H}\sim\text{Guild} + \text{Site}$	616.59	4.50	0.066
$\delta^2\text{H}\sim\text{Habitat} + \text{Site} + \text{Species}$	617.32	5.22	0.046
$\delta^2\text{H}\sim 1$	698.95	58.75	<0.001

When analyzing the species dataset including both live birds and museum specimens, models with lowest AICc scores and highest support were (1) for  $\delta^{13}\text{C}$  values, the best model included Species and Habitat as explanatory variables; (2) for  $\delta^{15}\text{N}$  values the best model included Origin and Species; and (3) for  $\delta^2\text{H}$  values, the best model included Species and Habitat (Table 2 and Supplementary Table S3). Museum specimens had higher  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values than live individuals (Table 3). Values of  $\delta^{15}\text{N}$  were highest in the humid zone and for museum specimens, and were lowest for LGF (Supplementary Table S3). Values of  $\delta^2\text{H}$  were lowest for LGF and at the farm habitat and were highest at the transition zone (Supplementary Table S3).

When examining feather isotope values among guilds within each habitat separately, we found significant differences in all isotope values, especially for the insectivorous foraging guild in the humid habitat (Supplementary Table S4).

**Table 3.** Simple linear relationships between feather  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values and each of the explanatory variables, for each dataset analyzed: (A) Live birds (N = 231), and (B) Species with live and museum data (N = 85). Significant models are in bold.

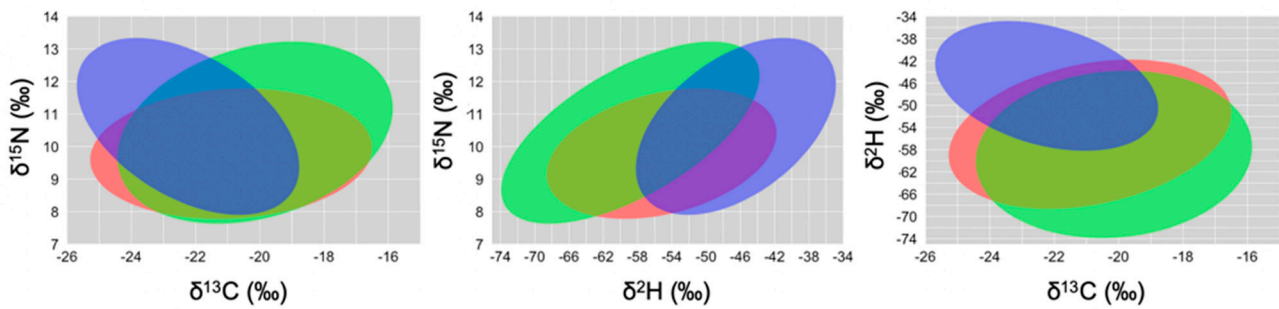
Model	F Value	Residual SE	DF	p-Value
<b>(A) Live birds (N = 231)</b>				
$\delta^{13}\text{C}\sim\text{Species}$	4.685	2.801	223	<b>&lt;0.0001</b>
$\delta^{13}\text{C}\sim\text{Guild}$	4.928	2.905	228	<b>0.008</b>
$\delta^{13}\text{C}\sim\text{Habitat}$	6.706	2.85	227	<b>0.0002</b>
$\delta^{13}\text{C}\sim\text{Site}$	3.283	2.831	221	<b>0.0008</b>
$\delta^{13}\text{C}\sim\text{Altitude}$	12.03	2.886	229	<b>0.0006</b>
$\delta^{13}\text{C}\sim\text{Mass}$	0.263	2.954	227	0.608
$\delta^{15}\text{N}\sim\text{Species}$	1.716	1.664	223	0.1063
$\delta^{15}\text{N}\sim\text{Guild}$	3.665	1.663	228	<b>0.027</b>
$\delta^{15}\text{N}\sim\text{Habitat}$	0.985	1.682	227	0.4007
$\delta^{15}\text{N}\sim\text{Site}$	1.554	1.664	221	0.131
$\delta^{15}\text{N}\sim\text{Altitude}$	1.372	1.681	229	0.243
$\delta^{15}\text{N}\sim\text{Mass}$	0.0123	1.693	227	0.912
$\delta^2\text{H}\sim\text{Species}$	9.326	9.745	223	<b>&lt;0.0001</b>
$\delta^2\text{H}\sim\text{Guild}$	19.85	9.832	228	<b>&lt;0.0001</b>
$\delta^2\text{H}\sim\text{Habitat}$	0.707	10.63	227	0.5483
$\delta^2\text{H}\sim\text{Site}$	5.265	9.82	221	<b>&lt;0.0001</b>
$\delta^2\text{H}\sim\text{Altitude}$	17.51	10.25	229	<b>&lt;0.0001</b>
$\delta^2\text{H}\sim\text{Mass}$	11.82	10.38	227	<b>0.0006</b>
<b>(B) Species with live and museum data (N = 85)</b>				
$\delta^{13}\text{C}\sim\text{Species}$	9.73	2.973	84	<b>0.0001</b>
$\delta^{13}\text{C}\sim\text{Guild}$	7.16	3.15	85	<b>0.0008</b>
$\delta^{13}\text{C}\sim\text{Altitude}$	2.42	3.266	83	0.1236
$\delta^{13}\text{C}\sim\text{Origin}$	9.83	3.105	85	<b>0.0023</b>
$\delta^{15}\text{N}\sim\text{Species}$	5.689	2.081	84	<b>0.0048</b>
$\delta^{15}\text{N}\sim\text{Guild}$	0.00001	2.204	85	0.9972
$\delta^{15}\text{N}\sim\text{Altitude}$	0.3327	2.217	83	0.5656
$\delta^{15}\text{N}\sim\text{Origin}$	5.344	2.138	85	<b>0.0232</b>
$\delta^2\text{H}\sim\text{Species}$	36.05	8.978	84	<b>&lt;0.0001</b>
$\delta^2\text{H}\sim\text{Guild}$	32.11	10.37	85	<b>&lt;0.0001</b>
$\delta^2\text{H}\sim\text{Altitude}$	0.1189	12.23	83	0.7311
$\delta^2\text{H}\sim\text{Origin}$	0.077	12.16	85	0.7812

#### Isotopic Niche Size, Overlap and Trophic Position

The herbivorous foraging guild had the largest SEA in all isotope biplots, followed by the granivorous guild, and the insectivorous guild had the smallest SEA (Figure 2, Table 4). Isotopic niche overlap between the insectivorous and each of the other two foraging guilds decreased when using  $\delta^2\text{H}$  values instead of  $\delta^{15}\text{N}$  (Table 4); segregation among foraging guilds (Figure 2 and Supplementary Figure S1) and among species (Supplementary Figure S2) was better when using  $\delta^2\text{H}$  values as well.

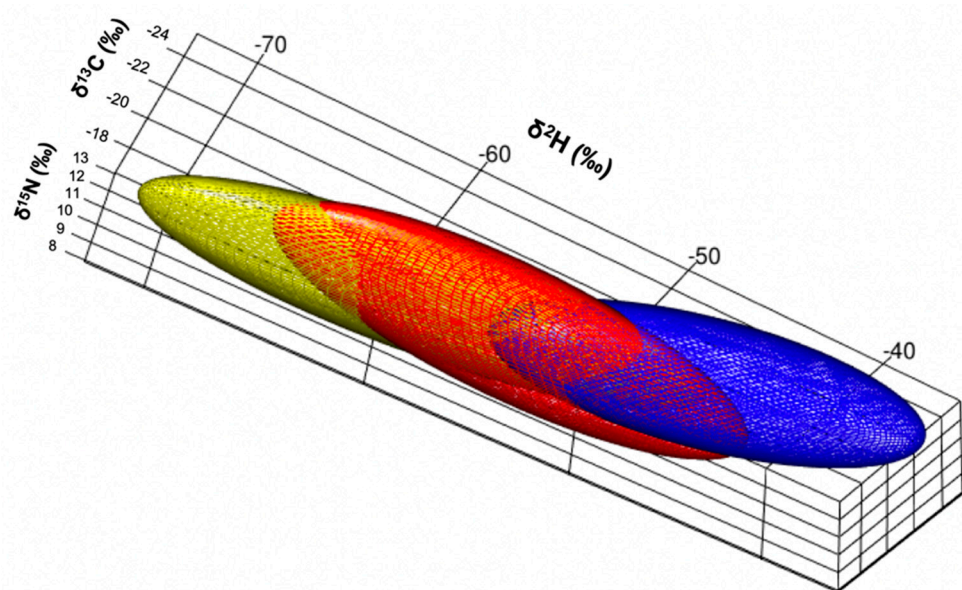
**Table 4.** Standard ellipse areas (SEA) and ellipse areas corrected for small sample size (SEA<sub>c</sub>), and pairwise isotopic niche overlap between these foraging guilds.

Foraging Guild	$\delta^{13}\text{C}\sim\delta^{15}\text{N}$		$\delta^{13}\text{C}\sim\delta^2\text{H}$	
	SEA (% <sup>2</sup> )	SEA <sub>c</sub> (% <sup>2</sup> )	SEA (% <sup>2</sup> )	SEA <sub>c</sub> (% <sup>2</sup> )
Granivorous	16.26	16.39	94.75	95.47
Herbivorous	13.25	13.53	79.24	80.92
Insectivorous	12.24	12.51	58.73	60.01
<b>Overlap between guilds (in %<sup>2</sup>)</b>				
Granivorous and herbivorous	74.30		405.72	
Granivorous and insectivorous	68.66		275.99	
Herbivorous and insectivorous	62.67		276.45	



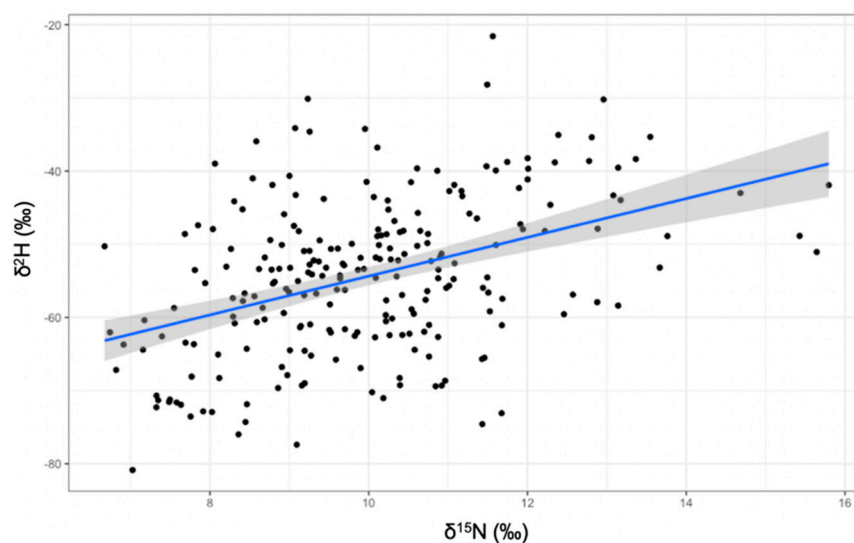
**Figure 2.** Two-dimensional graphs based on  $\delta^{13}\text{C}\sim\delta^{15}\text{N}$  (left),  $\delta^2\text{H}\sim\delta^{15}\text{N}$  (center) and  $\delta^{13}\text{C}\sim\delta^2\text{H}$  (right), showing the isotopic niches of foraging guilds: granivorous finches (red; SGF and MGF), herbivorous finches (green/yellow; LGF, CF and VF), and insectivorous finches (blue; STF, GWF and WF). We followed the code proposed by Rossman et al. [33].

When incorporating  $\delta^2\text{H}$  values and examining isotopic niches for each guild as volumes (Figure 3), the herbivorous guild had the largest standard ellipse volume at a 97.5 credible interval ( $216\text{‰}^3$ ) followed by the granivorous guild ( $158\text{‰}^3$ ) and lastly, the insectivorous guild ( $138\text{‰}^3$ ). Distance between centroids was largest between herbivorous and insectivorous ( $0.92\text{‰}$ ), then between insectivorous and granivorous ( $0.89\text{‰}$ ), and lastly between granivorous and herbivorous finches ( $0.62\text{‰}$ ). The only significant relationship between isotopes, was that between  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values (Figure 4).



**Figure 3.** Estimated isotopic niches for the three foraging guilds: granivorous (red; SGF and MGF), herbivorous (yellow; LGF, CF and VF) and insectivorous finches (blue; STF, GWF and WF) graphed in three dimensions (following the code proposed by Rossman et al. [33]).





**Figure 4.** Significant relationship between  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values ( $F_{1,229} = 49.14$ ,  $p < 0.001$ ).

#### 4. Discussion

Darwin's Finches are considered a classic example of adaptive radiation and niche segregation [2,4,5] and so provided a compelling test of the stable isotope approach as a means of confirming or elucidating the degree to which expected biological/ecological niche segregation is mirrored in isotopic niche segregation [13,45,46]. Isotopic similarity among species and guilds by no means implies true biological similarity as we are at the mercy of how environmental drivers may differ isotopically. Indeed, we expect much overlap among diets that are trophically similar ( $\delta^{15}\text{N}$ ,  $\delta^2\text{H}$ ) or based on similar photosynthetic pathway ( $\delta^{13}\text{C}$ ,  $\delta^2\text{H}$ ) or microhabitat ( $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ ,  $\delta^2\text{H}$ ). Nonetheless, stable isotopes are well suited to revealing biological segregation when they differ and so can illuminate factors resulting in true biological segregation that would otherwise remain cryptic [46]. Our multi-isotope approach suggested considerable isotopic overlap among species and guilds of Darwin's finches on Santa Cruz Island at least during the period of feather molt [47].

Ours is the first characterization, as far as we are aware, of isotopic niches of Darwin's finches. Michel et al. [48] examined isotopic composition of feathers of some species of finches; however, their study was focused on the gut microbiome of the Vampire Finch (*Geospiza septentrionalis*), for which a complementary stable isotope analysis revealed that its feathers had exceptionally high  $\delta^{15}\text{N}$  values, resembling top marine predators. Our investigation confirms that Darwin's finches tend to be "imperfect generalists", as suggested by De León et al. [6]. We provide evidence confirming that Darwin's finches are opportunistic feeders and hence demonstrate large isotopic niche overlap across a number of sites and habitats (Tables 1 and 3, Supplementary Figure S3). Darwin's finches on Santa Cruz Island are composed primarily of granivorous and herbivorous foraging guilds, both with large isotopic niches (Figures 2 and 3). Specifically, the granivorous foraging guild (formed by the small and medium ground finches, SGF and MGF), is the most abundant [30], and previous evidence shows that SGF consumes the highest proportion of plants and is the most important seed disperser on the island [40,49]. These ground finches (i.e., SGF and MGF) occur in several habitat types and so can consume resources that span multiple trophic levels [50] and can depend on a variety of food webs [37]. Similarly, a study examining the degree of niche overlap among three sympatric species of thrush (i.e., *Turdus albicollis*, *T. amaurochalinus*, *T. rufiventris*) in Brazil confirmed they were generalist species, partitioning some resources but coexisting at the same trophic positions [38]. Buelow et al. [51] also demonstrated that birds in a mangrove forest in Australia had more generalist and opportunistic foraging strategies than previously assumed, and they suggest that the environmental heterogeneity in resource availability results in such strategies. Environmental

heterogeneity [37], often linked to variation in precipitation on the Galápagos [2,8] may also be an important factor driving niche overlap of Darwin's finches on Santa Cruz Island. Feathers of live birds analyzed in this research were likely formed after the breeding season in 2018 and 2019, around April–June [47]. This period constitutes the transition from the wet to the dry season (Supplementary Figure S3 shows precipitation in Santa Cruz Island from Dec 2017 to June 2019 [7,52]), at which time food availability decreases and this in turn, tends to increase diet overlap in Darwin's finches [6,7]. Additionally, an El Niño event occurred from 2018–2019 [53], which presumably affected our feather isotope data. During El Niño years, the surface ocean around Galápagos warms, and this considerably increases rainfall in the archipelago [54]. This combination of factors, along with the variable precipitation patterns in Galápagos in general, together may have contributed to the strong isotopic niche overlap we found and further studies during different phases of ENSO (El Niño Southern Oscillation) are encouraged.

While tissue  $\delta^2\text{H}$  values have been largely used for tracking migratory animals due to the close correspondence with hydrogen precipitation isoscapes [25,55], recent studies have shown that microhabitat differences (e.g., forest interior versus coffee plantation) were good predictors of avian  $\delta^2\text{H}$  values [56] or for identifying aquatic and terrestrial food webs [57]. Here we found that feather  $\delta^2\text{H}$  values differentiated among foraging guilds and trophic positions (Figures 2 and 3, Supplementary Figures S1 and S2 [58]). Similarly, previous studies in Central America with birds [56] and bats [59] have shown that secondary consumers had higher tissue  $\delta^2\text{H}$  values than primary consumers. The lack of an increase in  $\delta^{15}\text{N}$  values from granivorous (or herbivorous) to insectivorous finches was surprising but we note that this may be linked to the inclusion of agricultural sites in our study whereby nitrogen from a variety of inputs to the foodweb was possible (see below). Conversely, we did find a significant increase in  $\delta^2\text{H}$  values (58–46‰) across this trophic gradient (but see [60]). These results highlight the potential application of  $\delta^2\text{H}$  values for studying habitat use and dietary specialization in animal communities, especially in areas where there is high sympatry and niche overlap [38]. However, a challenging characteristic of using  $\delta^2\text{H}$  values in this way is that the isotopic composition of consumer tissues reflect a blend of dietary and body water isotopic compositions that can vary considerably due to abiotic factors affecting environmental waters [26,61]. Here, we confirmed, for example, that both feather  $\delta^{13}\text{C}$  and  $\delta^2\text{H}$  values significantly decreased with altitude [62]. Local hydrological patterns must be considered and accounted for, then, in studies that use  $\delta^2\text{H}$  values as a proxy of trophic position.

Of considerable interest in community ecology is understanding the degree of individual dietary variation within species. Isotopic groups with high dietary diversity is consistent with individual dietary specialization [51]. Individual dietary variation is driven by several factors (e.g., sex, species, location, etc.) and stable isotope data can be definitive or ambiguous. Individuals that have different tissue stable isotope values will differ in location or type of diet but those with similar isotope values may also differ in these factors [63]. Anthropogenic factors on Santa Cruz Island clearly affect diet of ground finches and this, in turn, has been implicated in decreasing niche segregation among species [64]. During environmentally benign periods, finches tend to consume foods that are easily accessible and under such conditions, high dietary overlap is expected [64]. In the case of the MGF, human impacts such as habitat alteration, introduction of non-native plants and provisioning of foods are changing finch diets and consequently the adaptive landscape for beak size [65].

We only found difference among habitats for feather  $\delta^{13}\text{C}$  values (Tables 1 and 3). However, farms had higher  $\delta^{15}\text{N}$  values than other sites, and the two farms differed in this isotope. Farms grew crops of coffee and citrus fruits. For some species of Darwin's finches, feather samples at Farm B had higher  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values than Farm A. The farm A is organic and does not use fertilizers or herbicides, whereas at Farm B, inorganic fertilizer is spread on crops, which is known to result in higher foodweb  $\delta^{15}\text{N}$  values [19,20,66,67]. As noted above, this could have influenced our results of  $\delta^{15}\text{N}$  values and their failure to

discriminate among foraging guilds. However, when excluding Farm B from the analysis, results on  $\delta^{15}\text{N}$  values among foraging guilds were the same (results not included). The strong relationship between  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values (Figure 4) further supports our use of  $\delta^2\text{H}$  values as an alternative and additional means of determining isotopic niches of foraging guilds. When examining difference in feather  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values among guilds within each habitat, we found that the insectivorous guild differed from the other guilds, especially for the humid zone (Supplementary Table S4). This supports our findings that insectivorous finches are more differentiated from the other guilds, and also that granivorous and herbivorous finches are mainly composed of generalist species which tend to dominate the avian community on Santa Cruz Island. It is also likely that the humid zone provides different resources for Darwin's finches that are not available in other habitats, given the presence of native vegetation patches and its higher altitude and precipitation [29,31].

For our retrospective study involving museum specimens, we did find that feathers of museum specimens had significantly higher  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values than feathers from live birds. This could suggest a possible trophic shift over time [39,67], however, due to our small sample size we cannot say if there has been climatic variation over time or some other human factor affecting isotope values, hence further sampling of museum specimens of multiple Darwin's finch species is recommended.

We conclude that Darwin's finches are mainly generalist and opportunistic species, a finding largely consistent with more recent dietary considerations of these species [6,64]. We clearly demonstrate that stable isotope analysis constitutes a useful tool for studying variation in diet and ecological segregation among sympatric bird species because it provides information on dimensions rarely explored with just observational purposes. Future studies in the Galápagos should compare feather samples from an island with high human disturbance, such as Santa Cruz, with feathers from an island with no or with very little human disturbance, such as Fernandina or Santiago. Such future studies should also examine isotopic values of local foodwebs as well as birds, such analyses will provide a more powerful analysis of ecological segregation among species.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/d13040147/s1>, Table S1: Total numbers of individuals of Darwin's finches sampled in four different habitats in Santa Cruz Island and from museum specimens, Table S2: Museum collections at the Vertebrate Collection of the Charles Darwin Research Station (VCCDRS) in Puerto Ayora, Santa Cruz Island, Table S3: Best generalized linear models (GLM) that explain variation in feather  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^2\text{H}$  values in each dataset analyzed: (A) Live birds (N = 231), and (B) Samples with live and museum data (N = 85). Significant parameters are in bold, Table S4: Differences among foraging guilds within each habitat. Significant guilds are in bold, Figure S1: Standard ellipse areas (SEA) based on  $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$  (A) and  $\delta^{13}\text{C}$ – $\delta^2\text{H}$  (B), Figure S2: Biplots that show the trophic position of each Darwin's finch species, with the Y axis given by  $\delta^{15}\text{N}$  values (upper graph) or  $\delta^2\text{H}$  values (lower graph). Figure S3: Monthly precipitation (mean  $\pm$  SD) from December 2017 to June 2019, based on data from the two climatological stations on Santa Cruz Island (Puerto Ayora at 2 masl, and Bella Vista at 223 masl; [52]). This time frame covers breeding seasons and molt periods of feathers of live birds analyzed in this research. Breeding season in Darwin's finches starts around December when precipitation is higher, and molt after the breeding season, around April to June [47].

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**Data Availability Statement:** The data presented in this study are stored in Dryad at <https://doi.org/10.5061/dryad.nzs7h44r2>, accessed on 1 March 2021.

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