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Analysis of Circulating Fatty Acid Profiles in Free-Ranging and Managed Care Marine Toads (*Rhinella marina*) with a Comparison of Whole-Blood Vial and Whole-Blood Dried Blood Spot Card Analyses

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Abstract: This study provides novel data on circulating concentrations of whole-blood fatty acids (FAs) in marine toads (*Rhinella marina)* via dried blood spot (DBS) card technology as a potential amphibian model species. Free-ranging (n = 10) animals were compared to managed populations fed two diet strategies for 60 days (n = 6 per diet). Thirty-six individual FAs were analyzed, with 28 found in significant reportable quantities. Eight FA groupings were represented. Traditional whole-blood vial (WBV) FA percentages were also collected and compared to DBS after managed care for 60 days. Results showed eleven individual FAs and four FA groups were higher in free-ranging toads (n = 10; $p \leq 0.05$), while three FAs and three groups were higher in managed care (n = 12; $p \leq 0.05$). FA concentrations compared between DBS cards and WBV at day 60 generally agreed, although two individual FAs and one grouping were higher in DBS ($p \leq 0.05$). When free-ranging FAs were analyzed by sex, four individual FAs and two groupings were higher in females, while four individual FAs and one grouping were higher in males. Understanding normal FA circulating levels and how husbandry changes them may impact amphibian health. Additionally, DBS cards may provide a convenient sampling tool for fieldwork.

Keywords: amphibians; anurans; nutrition; toads

1. Introduction

Amphibian populations are rapidly declining worldwide due to habitat loss, pollution, and disease. According to the International Union for Conservation of Nature (IUCN), 40% of amphibian species are threatened or endangered [1]. In response to this increasing rate of extinction, the Association of Zoos and Aquariums (AZA) has developed multiple species survival plans (SSPs) to sustain threatened species [2]. An integral part of amphibian SSPs is ex situ breeding followed by reintroduction to the wild. AZA welfare standards for animals maintained under human care mandate that they should be housed and fed in a manner that optimizes their health and breeding performance. There is a relative lack of knowledge of many aspects of amphibian nutrition compared to that of other vertebrate groups. Many anurans begin their lives as herbivorous larvae, then evolve into carnivorous

adults, posing an additional challenge to optimizing amphibian nutrition in managed care (MC).

There are numerous reports of whole-body fatty acid profiles in various frog, salamander and toad species [3–5], yet studies on circulating concentrations of fatty acids within any amphibian species could not be found [6]. Whole body analyses require euthanizing and destruction of the entire animal for results which negates the goals of conservation reproductive programs, especially when studying endangered amphibians. Because of the lack of information available on the normal fatty acid profiles of wild amphibians, there is little understanding of how diets in MC impact circulating fatty acid profiles and the overall health of amphibians. Animals in MC have been shown to alter their circulating fatty acid profiles when compared to their wild counterparts in a variety of non-amphibious species [7–10]. These changes in fatty acid profiles may have impacts on animal health and reproductive capability [7,8,10–12]. For example, it has been shown that Eiffinger's tree frog (*Kurixalus eiffingeri)* tadpoles fed chicken egg yolk grew slower than tadpoles fed eggs of their conspecifics [12]. The *K. eiffingeri* eggs contained more n-3 fatty acids (18:3n-3 (α-Linolenic acid) and 20:5n-3 (Eicosapentaenoic acid)) than chicken egg yolk, and tadpoles fed conspecific eggs contained more of these fatty acids [12,13]. Thus, potentially theorizing that incorrect dietary fatty acid ratios (amongst other nutritional factors) may alter amphibian growth and development.

In general, amphibian field studies present significant challenges, especially for blood-based analyses. Collecting samples of whole blood, serum, or plasma requires equipment for collection and processing as well as proficiency in blood draws and a large sample size [14]. Additionally, many assays have specific time and temperature constraints between collection and analysis of samples [14]. Fortunately, dried blood spot (DBS) cards have fewer processing, transport, and storage requirements, as well as requiring smaller quantities of whole blood than serum or plasma analysis [14,15]. Blood spot cards have been utilized for most measures as traditional blood liquids, including nutrient evaluations (cardiac and cholesterol markers, fat soluble vitamins, fatty acids, insulin, thyroid hormones, and select minerals (Cd, Cu, Hg, Mg, Se, Zn), etc.) [9,14–17]. Comparisons between DBS and whole-blood samples are imperative for validation and interpretation of DBS use in amphibians [14–16].

Marine toads (*Rhinella marina*) have been classified in the top 100 most destructive invasive species due to their toxicity, successful reproduction, and outcompeting native species [18–22]. Population control programs have been used to manage the impact of this invasive species and toads in this trial were collected as part of a population control program to be humanely euthanized. Thus, biological samples from these animals were collected to gather data as a potential amphibian model similar to prior publications [23–28]. The objectives of the current study were to: (1) report novel values of known circulating fatty acids in the marine toad as a potential model for anuran species; (2) compare marine toad fatty acid profiles of free-ranging (FR) animals to those in MC for 60 days; and (3) compare the analysis of DBS fatty acid samples to traditionally collected whole-blood samples in marine toads.

2. Materials and Methods

2.1. Animals

This trial was conducted under the approval of the NC State University Institutional Animal Care and Use Committee (IACUC) (IACUC #20-207), Zoo Miami Animal Care and Use Committee (#2020-6, 1 July 2020), and the North Carolina Zoo (NC Zoo) research review board.

Sixty-six marine toads with a minimum weight of 35 g were collected from the grounds of Zoo Miami in Miami, Florida, on the night of 19 August 2020, during a routine invasive species population control program. The first 10 toads collected (average weight of 128.7 g) were anesthetized by a veterinarian by placing them individually in a bath of MS-222 (10 g/L, buffered with sodium bicarbonate) (Tricaine methanesulfonate, Syndel,

Ferndale, Washington, DC, USA) until they reached a surgical plane of anesthesia. Then, the veterinarian collected whole blood via cardiocentesis and transferred 80 µL directly to 1 spot on a Perkin-Elmer Spot Saver card (PerkinElmer, Waltham, MA, USA). The toads were then placed back into the MS-222 bath until euthanized. After drying, the dried blood spot (DBS) cards were closed and stored overnight at room temperature until shipped via overnight priority the next day to Lipid Technologies, LLC for fatty acid analyses.

2.2. Housing and Diet

The remaining 56 toads were deemed healthy upon veterinary visual inspection and were individually identified with a subcutaneous passive integrated transponder (Biomark Inc., Boise, ID, USA). The animals were then transported to the North Carolina (NC) Zoo in Asheboro, North Carolina, as part of a nutrition study. However, only 12 of these 56 animals were used for this fatty acid research. Upon arrival at the NC Zoo, the toads were again deemed healthy upon veterinary inspection. All 56 toads were blocked by weight, randomly allotted by Microsoft Excel's shuffle function (Microsoft Office 2013, Redmond, WA, USA) to 1 of 2 diet treatments, and then evenly sorted among 6 housing tubs. The majority of the toads were humanely euthanized throughout the separate short-term nutritional studies, but the 12 MC toads used for this research were in the final group that remained for 60 days.

Four days after arrival at NC Zoo, all toads were weighed, dewormed with Ivermectin at 0.2 mg/kg orally, and received another physical by NC Zoo veterinarians. Toads were group housed in 6 Waterland (CA) tubs (178.8 × 81.28 × 35.56 cm) with access to hide boxes and a shallow pool of reconstituted reverse osmosis (RO) water. The tubs were cleaned daily due to waste and leftover food and minimally disinfected (chlorhexidine gluconate 2% solution diluted 1 oz per gallon of water) weekly. Animals were kept at ambient temperature, ranging from 20.5 to 27.7 °C, without supplemental heating or cooling. The light cycle was maintained according to the working hours of the animal husbandry staff (0800–1700 h). Each tub had a mesh screen covering affixed to the top of the tubs to prevent escape and to partially block the artificial lighting. Temperature and humidity readings of the room were recorded daily using an AcuRite® indoor temperature and humidity monitor.

The toads were fed adult brown house crickets (*Acheta domestica*) (Catawba Cricket Hatchery Inc. (Charlotte, NC, USA)) that were gut-loaded using Mazuri® Cricket Diet 5M38 (St. Louis, MO, USA) plus small amounts of free choice sweet potato and carrot. As part of a simultaneous nutrition study, half the toads were fed the control diet (Diet 1), which consisted of the gut-loaded crickets alone (Table 1). The other half of the toads were fed a treatment diet that was identical to the control diet, with the exception that the crickets were dusted with the supplement Repashy® Superfoods Vitamin A Plus (La Jolla, CA, USA) immediately before being provided to the toads (Diet 2; Table 1). Toads were offered approximately 12 crickets per toad at 4 pm daily, seven days a week for the first 4 weeks. Based on the number of crickets left uneaten, the feeding frequency was decreased to 9 crickets per toad 5 days a week beginning on week 5. Any uneaten crickets were removed at 11 am each following day and disposed of. All toads were individually visually assessed for health daily.

Table 1. Nutrient analyses (dry matter basis) of cricket diet [1], nutrient-dusting supplement [2], and adult brown house cricket (*Acheta domestica*) [3] after consuming the cricket diet for three days (72 h) with and without supplement dusting.

	Unit	Cricket Diet	Dusting Supplement	Gut-Loaded Crickets (Diet 1)	Gut-Loaded and Dusted Crickets (Diet 2)
Moisture	%	7.7	2.2	70.5	69.0
Dry Matter	%	92.3	97.8	29.5	31.0
Crude Protein	%	20.2	20.5	67.3	63.3
Nitrogen Free Extract	%	39.5	ND	-	-
Acid Detergent Fiber	%	12.4	ND	35.2	44.2
Neutral Detergent Fiber	%	13.8	ND	-	-
Water Soluble Carbohydrates	%	3.9	ND	-	-
Starch	%	20.7	ND	-	-
Crude Fat (ether extract)	%	3.8	ND	-	-
Crude Fat (acid hydrolysis)	%	-	-	16.4	17.0
Crude Fiber	%	10.4	ND	-	-
Ash	%	23.8	ND	6.2	9.6
Ca	%	8.1	22.7	0.8	2.3
P	%	0.6	0.3	1.0	0.9
Ca:P ratio	1 to	13.8	78.2	0.8	2.5
Mg	%	0.3	0.2	0.1	0.1
K	%	0.9	0.5	1.2	1.1
Na	%	0.2	0.4	0.4	0.4
Cl	%	ND	0.3	-	-
S	%	0.3	0.5	0.6	0.6
Fe	ppm	297	247	61	73
Zn	ppm	74	10	184	168
Cu	ppm	16	ND	21	20
Mn	ppm	99	55	39	39
Mo	ppm	1.7	1.7	1.7	1.3
Co	ppm	ND	0.5	-	-

[1] Mazuri ®Cricket Diet 5M38, Mazuri Exotic Animal Nutrition (St. Louis, MO, USA). [2] Repashy®Vitamin A Plus, Repashy Specialty Pet Products (La Jolla, CA, USA). [3] Catawba Cricket Hatchery Inc. (Charlotte, NC, USA). ND = Not detected. - = Not tested.

After 60 days in MC, 12 toads (6 from both Diet 1 and Diet 2) were anesthetized with MS-222 (10 g/L, buffered with sodium bicarbonate), and 1.0 mL of blood was collected via cardiocentesis using a 22-gauge needle on a 3 mL syringe prior to the toad being euthanized via pithing. Approximately 80 µL of blood was placed on a spot on a DBS card, and the remaining blood was placed in a lithium heparin tube. DBS cards were allowed to dry and then stored at room temperature. Whole-blood vials (WBV) were frozen and stored at −80 °C before being shipped to the Lipid Technologies, LLC (Austin, MN, USA) lab for fatty acid profile testing.

2.3. Lab Testing Methods

Lipid Technologies, L.L.C. analyzed the Mazuri ® Cricket Diet 5M38, the Repashy® Vitamin A Plus supplement, ground gut-loaded (72 h) crickets (Diet 1) (Table 2), DBS cards, and WBV samples for this study. Using established methods, samples were transmethylated using acidified methanol. Fatty acid methyl esters were analyzed using traditional gas chromatography and quantified by area percent [16,17]. The gas chromatography protocol specifics are based on the American Oil Chemists Society Official Method Ce 1-62. Percentages of 36 individual fatty acids representing 8 class groups were analyzed using this method. Values are presented on a percent of total fatty acids present.

Table 2. Total lipid fatty acid profile (quantified by area % and values are provided on a percent of total fatty acids present) in the commercial diet [1], dusting supplement [2], and the crickets [3] (*Acheta domestica*) gut-loaded for 72 h and fed as Diet 1 to marine toads (*Rhinella marina*).

Number	Common Name	Diet Item Analyzed		
		Cricket-Dusting Supplement	Cricket Diet	Gut-Loaded Crickets (Diet 1)
Individual Fatty Acids				
12:0	Lauric acid	0.47	0.00	0.00
14:0	Myristic acid	0.69	0.65	0.58
14:1	Myristoleic acid	0.24	0.00	0.00
15:0	Pentadecylic acid	0.92	0.20	0.07
15:1	Pentadecenoic acid	ND	ND	ND
16:0	Palmitic acid	44.60	19.52	27.17
16:1n5	Myristoleic acid	ND	ND	ND
16:1n7	Palmitoleic acid	10.12	1.24	1.07
17:0	Margaric acid	ND	ND	ND
17:1	Heptadecenoic acid	ND	ND	ND
18:0	Stearic acid	0.74	7.08	8.78
18:1n5	13-Octadecenoic acid	ND	ND	ND
18:1n7	Vaccenic acid	ND	ND	ND
18:1n9	Oleic acid	1.89	29.85	21.00
18:2n6	Linoleic acid	19.59	34.24	36.26
18:3n6	γ-Linoleic acid	12.69	0.08	0.00
18:3n3	α-Linolenic acid	0.12	2.50	0.99
18:4n3	Stearidonic acid	0.21	0.04	0.05
20:0	Arachidic acid	0.09	0.47	0.50
20:1n7	Paullinic acid	ND	ND	ND
20:1n9	Gondoic acid	0.00	0.68	0.13
20:2n6	Eicosadienoic acid	0.23	0.24	0.04
20:3n9	Mead acid	0.00	0.00	0.22
20:3n6	Dihomo-γ-Linoleic acid	0.16	0.04	0.02
20:4n6	Arachidonic acid	0.09	0.06	0.25
20:3n3	Eicosatrienoic acid	0.08	0.00	0.00
20:4n3	Eicosatetraenoic acid	0.25	0.00	0.00

Table 2. *Cont.*

Number	Common Name	Diet Item Analyzed		
		Cricket-Dusting Supplement	Cricket Diet	Gut-Loaded Crickets (Diet 1)
20:5n3	Eicosapentaenoic acid	0.32	0.18	0.39
22:0	Behenic acid	0.15	0.20	0.04
22:1n9	Erucic acid	0.12	0.02	0.02
22:4n6	Adrenic acid	0.03	0.13	0.18
22:5n6	n6 Docosapentaenoic acid	0.04	0.07	0.19
22:5n3	n3 Docosapentaenoic acid	0.06	0.11	0.08
22:6n3	Docosahexanoic acid	0.05	0.08	0.10
24:0	Lignoceric acid	0.11	0.20	0.05
24:1	Nervonic acid	0.00	0.01	0.13
Other		5.90	2.09	1.66
Fatty Acid Groupings				
Monoenes		2.27	30.56	21.28
Saturates		47.77	28.33	37.19
Highly Unsaturated Fatty Acids		14.12	3.32	2.50
Poly Unsaturated Fatty Acids		33.93	37.79	38.80
n-3 Fatty Acids		1.09	2.92	1.63
n-6 Fatty Acids		32.83	34.84	36.95
n-9 Fatty Acids		2.24	29.88	21.15
n6: n3 Fatty Acid Ratio		30.15	11.92	22.72

[1] Mazuri ® Cricket Diet 5M38, Mazuri Exotic Animal Nutrition (St. Louis, MO, USA). [2] Repashy® Vitamin A Plus, Repashy Specialty Pet Products (La Jolla, CA, USA). [3] Catawba Cricket Hatchery Inc. (Charlotte, NC, USA).

2.4. Cricket Feed and Cricket Analyses

Frozen samples (−80 °C) of the Mazuri® Cricket Diet 5M38 (St. Louis, MO, USA) and Repashy® Vitamin A Plus (La Jolla, CA, USA) were sent frozen overnight to Zooquarius® Laboratory Services (Ithaca, NY, USA) for their Feed 1 profile (#190) (Table 1). Crickets indicative of each diet treatment were euthanized via isoflurane and immediately frozen (−80 °C) prior to being sent frozen overnight to Zooquarius® for their Insect 1 (#191) profile after consuming the 5M38 diet for 3 days with and without the Repashy® Vitamin A Plus supplement. Methodology for the Insect 1 (#191) profile and euthanasia technique was previously explained in Cerreta et al., 2021 [29], and diet analyses were identical other than fat being analyzed as ether extract (Association of Official Agricultural Chemists, May 2003).

2.5. Statistical Analyses

Statistical analyses were performed using various Proc GLM (general linear models) procedure model statements in SAS 9.4 (Cary, NC, USA). The main effects were considered significant at $p \leq 0.05$. The following fatty acid percentage comparisons were conducted: (1) differences among FR toad DBS cards, MC toad DBS cards and MC toad WBV; (2) differences between fatty acids by sex of the FR toads; and (3) differences among the marine

toad diet strategies: FR, MC consuming gut-loaded crickets (Diet 1), and MC crickets consuming gut-loaded and supplement-dusted crickets (Diet 2).

3. Results

All marine toads remained visually healthy throughout the duration of the research study. The average temperature and humidity for the study were: 21–28 °C and 42–88%, respectively.

Toad sex was determined upon necropsy and confirmed on histology. The FR animals consisted of five females and five males, while the 12 toads euthanized on day 60 of MC consisted of two females and ten males. The FR toads (128.7 ± 15.81) tended to be lighter than the toads on Diet 1 and Diet 2 (171 ± 25.71 and 186.3 ± 15.59, respectively) (p = 0.085) though non-significant.

Of the 36 individual fatty acids analyzed, 28 were present in sufficient concentrations to be quantified in both FR and MC marine toad samples (Table 3). Additionally, pentadecylic acid (15:0) is not quantifiable in DBS cards and was only reported for WBV. Lauric acid (12:0), myristoleic acid (16:1n5), 13-octadecenoic acid (18:1n5), vaccenic acid (18:1n7), gondoic acid (20:1n9), eicosatrienoic acid (20:3n3), mead acid (20:3n9), and lignoceric acid (24:0) did not have high enough values in any sample to make comparisons. All eight fatty acid groups were analyzed and quantified in both FR and MC samples (Table 3).

Table 3. Whole-blood fatty acid profile percentages (LSmean ± SEM) in marine toads (*Rhinella marina*) compared among free-ranging animals (FR) at day 0 and those housed in managed care (MC) for 60 days that were tested using either dried blood spot cards (DBS) or whole-blood vials (WBV). Results are quantified by area %, and values are provided on a percent of total fatty acids present [1].

Fatty Acid	Common Name	Sample Location and Testing Method		
		Free-Ranging DBS (n = 10)	Managed Care DBS (n = 12)	Managed Care WBV (n = 12)
Individual Fatty Acids				
12:0	Lauric acid 12:0	ND	ND	ND
14:0	Myristic acid	0.9 ± 0.15	0.9 ± 0.11	0.9 ± 0.11
14:1	Myristoleic acid	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.02
15:0	Pentadecylic acid	NQ	NQ	3.6 ± 0.24
15:1	Pentadecenoic acid	0.2± 0.02	0.1 ± 0.02	0.1 ± 0.02
16:0	Palmitic acid	15.3 ± 0.64	16.9 ± 0.59	16.7 ± 0.59
16:1n5	Myristoleic acid	ND	ND	ND
16:1n7	Palmitoleic acid	2.5 ± 0.53 [a]	4.9 ± 0.48 [b]	5.3 ± 0.48 [b]
17:0	Margaric acid	1.0 ± 0.13 [a]	0.3 ± 0.12 [b]	0.5 ± 0.12 [b]
17:1	Heptadecenoic acid	0.6 ± 0.09 [a]	0.3 ± 0.08 [b]	0.2 ± 0.08 [b]
18:0	Stearic acid	6.3 ± 0.45	5.4 ± 0.41	5.7 ± 0.41
18:1n5	13-Octadecenoic acid	ND	ND	ND
18:1n7	Vaccenic acid	ND	ND	ND
18:1n9	Oleic acid	27.6 ± 0.93 [a]	19.1 ± 0.85 [b]	19.4 ± 0.85 [b]
18:2n6	Linoleic acid	28.2 ± 1.35 [a]	46.1 ± 1.23 [b]	42.3 ± 1.23 [c]
18:3n6	γ-Linoleic acid	0.3 ± 0.02 [a]	0.05 ± 0.02 [b]	0.06 ± 0.02 [b]
18:3n3	α-Linolenic acid	2.6 ± 0.39 [a]	0.9 ± 0.35 [b]	0.8 ± 0.35 [b]
18:4n3	Stearidonic acid	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.03

Table 3. *Cont.*

Fatty Acid	Common Name	Sample Location and Testing Method		
		Free-Ranging DBS (n = 10)	**Managed Care DBS (n = 12)**	**Managed Care WBV (n = 12)**
20:0	Arachidic acid	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.02
20:1n7	Paullinic acid	0.3 ± 0.04 [a]	0.2 ± 0.03 [b]	0.2 ± 0.03 [b]
20:1n9	Gondoic acid	ND	ND	ND
20:2n6	Eicosadienoic acid	0.4 ± 0.02	0.3 ± 0.02	0.3 ± 0.02
20:3n3	Eicosatrienoic acid	ND	ND	ND
20:3n6	Dihomo-γ-Linoleic acid	0.6 ± 0.04 [a]	0.2 ± 0.03 [b]	0.2 ± 0.03 [b]
20:3n9	Mead acid	ND	ND	ND
20:4n6	Arachidonic acid	7.1 ± 0.40 [a]	1.6 ± 0.37 [b]	1.3 ± 0.37 [b]
20:4n3	Eicosatetraenoic acid	0.0 ± 0.03 [a]	0.1 ± 0.03 [a]	0.2 ± 0.03 [b]
20:5n3	Eicosapentaenoic acid	1.0 ± 0.24	0.7 ± 0.22	0.5 ± 0.22
22:0	Behenic acid	0.2 ± 0.01 [a]	0.1 ± 0.01 [b]	0.04 ± 0.01 [c]
22:1n9	Erucic acid	0.2 ± 0.07 [a]	0.1 ± 0.06 [a]	0.5 ± 0.06 [b]
22:4n6	Adrenic acid	0.6 ± 0.07 [a]	0.2 ± 0.06 [b]	0.2 ± 0.06 [b]
22:5n6	n6 Docosapentaenoic acid	0.1 ± 0.02 [a]	0.0 ± 0.02 [b]	0.06± 0.02 [b]
22:5n3	n3 Docosapentaenoic acid	1.1 ± 0.10 [a]	0.5 ± 0.10 [b]	0.4 ± 0.10 [b]
22:6n3	Docosahexanoic acid	0.6 ± 0.14	0.3 ± 0.13	0.2 ± 0.13
24:0	Lignoceric acid	ND	ND	ND
24:1	Nervonic acid	0.2 ± 0.03 [a]	0.1 ± 0.03 [b]	0.1 ± 0.03 [b]
Fatty Acid Groupings				
Monoenes		28.6 ± 0.94 [a]	19.6 ± 0.86 [b]	20.3 ± 0.86 [b]
Saturates		24.4 ± 0.84 [a]	23.9 ± 0.77 [a]	27.2 ± 0.77 [b]
Highly Unsaturated Fatty Acids		11.2 ± 0.73 [a]	3.6 ± 0.67 [b]	2.9 ± 0.67 [b]
Poly Unsaturated Fatty Acids		42.8 ± 1.56 [a]	51.0 ± 1.42 [b]	46.4 ± 1.42 [a]
n-3 Fatty Acids		5.4 ± 0.69 [a]	2.6 ± 0.63 [b]	2.1 ± 0.63 [b]
n-6 Fatty Acids		37.4 ± 1.39 [a]	48.4 ± 1.26 [b]	44.3 ± 1.26 [c]
n-9 Fatty Acids		28.1 ± 0.92 [a]	19.3 ± 0.84 [b]	20.0 ± 0.84 [b]
n-6:n-3 Fatty Acid Ratio		8.6 ± 1.63 [a]	23.1 ± 1.48 [b]	22.0 ± 1.48 [b]

[1] Differing superscripts (a,b,c) indicate statistical differences ($p \leq 0.05$). ND = not detected; NQ = not quantifiable.

Comparisons between FR and MC samples using the DBS technique showed differences between 14 of the individual fatty acids. Eleven fatty acids had higher concentrations in the FR samples: margaric acid (17:0), heptadecenoic acid (17:1), oleic acid (18:1n9), α-linolenic acid (18:3n3), paullinic acid (20:1n7), dihomo-γ-Linoleic acid (20:3n6), arachidonic acid (20:4n6), behenic acid (22:0), n-3 docosapentaenoic acid (22:5n3), n-6 docosapentaenoic acid (22:5n6), and nervonic acid (24:1). Three fatty acids had higher concentrations in the MC samples: palmitoleic acid (16:1n7), linoleic acid (18:2n6), and γ-linolenic acid (18:3n6). Seven of the fatty acid groups demonstrated differences between FR and MC. Monoenes, omega-3 fatty acids, omega-9 fatty acids and HUFA had higher concentrations in FR DBS while omega-6 fatty acids, the omega-6: omega-3 ratio, and PUFA had higher concentrations in MC samples.

Comparisons between the DBS and WBV fatty acid analytical technique conducted on day 60 showed two individual fatty acids and one fatty acid group had significant differences. Linoleic acid (18:2n6), behenic acid (22:0), and the omega-6 fatty acid grouping were present in higher concentrations in the DBS samples.

There were only two statistical differences between MC toads fed gut-loaded crickets alone and those fed crickets dusted with the Repashy® supplement DBS cards (Table 4). Erucic acid (22:1n9) and nervonic acid (24:1) were higher in Diet 2, although both fatty acids were present in very small quantities. Based on these results, the MC diets were combined in Tables 3 and 5.

Table 4. Whole-blood fatty acid dried blood spot cards percentages (LSmean ± SEM) in managed care (MC) marine toads (*Rhinella marina*) compared between those consuming gut-loaded [1] crickets (Diet 1) or gut-loaded and supplement[2]-dusted crickets (Diet 2) for 60 days in managed care. [2,3] Results are quantified by area %, and values are provided on a percent of total fatty acids present.

Fatty Acid	Common Name	Diet Type	
		Gut-loaded Crickets (Diet 1) (n = 6)	Gut-loaded and Dusted Crickets (Diet 2) (n = 6)
Individual Fatty Acids			
12:0	Lauric acid	ND	ND
14:0	Myristic acid	1.0 ± 0.14	0.8 ± 0.14
14:1	Myristoleic acid	0.1 ± 0.04	0.1 ± 0.04
15:0	Pentadecylic acid	NQ	NQ
15:1	Pentadecenoic acid	0.1 ± 0.02	0.1 ± 0.02
16:0	Palmitic acid	16.9 ± 0.56	17.0 ± 0.56
16:1n5	Myristoleic acid	ND	ND
16:1n7	Palmitoleic acid	5.0 ± 0.73	4.9 ± 0.73
17:0	Margaric acid	0.2 ± 0.13	0.4 ± 0.13
17:1	Heptadecenoic acid	0.2 ± 0.09	0.3 ± 0.09
18:0	Stearic acid	5.2 ± 0.25	5.7 ± 0.25
18:1n5	13-octadecenoic acid	ND	ND
18:1n7	Vaccenic acid	ND	ND
18:1n9	Oleic acid	18.3 ± 0.68	19.8 ± 0.68
18:2n6	Linoleic acid	46.7 ± 1.25	45.4 ± 1.25
18:3n6	γ-Linoleic acid	0.04 ± 0.02	0.06 ± 0.02
18:3n3	α-Linolenic acid	0.9 ± 0.04	0.9 ± 0.04
18:4n3	Stearidonic acid	0.2 ± 0.08	0.1 ± 0.08
20:0	Arachidic acid	0.2 ± 0.03	0.2 ± 0.03
20:1n7	Paullinic acid	0.2 ± 0.04	0.2 ± 0.04
20:1n9	Gondoic acid	ND	ND
20:2n6	Eicosadienoic acid	0.3 ± 0.03	0.3 ± 0.03

Table 4. *Cont.*

Fatty Acid	Common Name	Diet Type	
		Gut-loaded Crickets (Diet 1) (n = 6)	**Gut-loaded and Dusted Crickets (Diet 2) (n = 6)**
20:3n3	Eicosatrienoic acid	ND	ND
20:3n6	Dihomo-γ-Linoleic acid	0.2 ± 0.04	0.2 ± 0.04
20:3n9	Mead acid	ND	ND
20:4n6	Arachidonic acid	1.5 ± 0.53	1.7 ± 0.53
20:4n3	Eicosatetraenoic acid	0.06 ± 0.02	0.04 ± 0.02
20:5n3	Eicosapentaenoic acid	1.1 ± 0.42	0.4 ± 0.42
22:0	Behenic acid	0.1 ± 0.01	0.1 ± 0.01
22:1n9	Erucic acid	0.02 ± 0.02 [a]	0.09 ± 0.02 [b]
22:4n6	Adrenic acid	0.1 ± 1.33	0.2 ± 1.33
22:5n6	n6 Docosapentaenoic acid	0.04 ± 0.02	0.02 ± 0.02
22:5n3	n3 Docosapentaenoic acid	0.5 ± 0.10	0.5 ± 0.10
22:6n3	Docosahexanoic acid	0.5 ± 0.28	0.1 ± 0.28
24:0	Lignoceric acid	ND	ND
24:1	Nervonic acid	0.02 ± 0.02 [a]	0.08 ± 0.02 [b]
Fatty Acid Groupings			
Monoenes		18.8 ± 0.68	20.4 ± 0.68
Saturates		23.6 ± 0.53	24.2 ± 0.53
Highly Unsaturated Fatty Acids		4.0 ± 1.02	3.2 ± 1.02
Poly Unsaturated Fatty Acids		52.1 ± 1.32	49.9 ± 1.32
n-3 Fatty Acids		3.2 ± 0.85	2.0 ± 0.85
n-6 Fatty Acids		48.9 ± 1.30	47.9 ± 1.30
n-9 Fatty Acids		18.5 ± 0.67	20.1 ± 0.67
n-6: n-3 Fatty Acid Ratio		21.5 ± 2.60	24.7 ± 2.60

[1] Mazuri® Cricket Diet 5M38, Mazuri Exotic Animal Nutrition (St. Louis, MO, USA). [2] Repashy® Vitamin A Plus, Repashy Specialty Pet Products (La Jolla, CA, USA). [3] The superscripts (a,b) indicates a statistical difference ($p \leq 0.05$) between diets. ND = not detected; NQ = not quantifiable.

Table 5. Free-ranging marine toad (*Rhinella marina*) whole-blood fatty acid percentages via dried blood spot cards (LSmean ± SEM) compared by sex. [1,2] Results are quantified by area %, and values are provided on a percent of total fatty acids present.

Fatty Acid	Common Name	Female (n = 5)	Male (n = 5)
Individual Fatty Acids			
12:0	Lauric acid	ND	ND
14:0	Myristic acid	1.0 ± 0.22	0.8 ± 0.22
14:1	Myristoleic acid	0.1 ± 0.05	0.1 ± 0.05
15:0	Pentadecylic acid	NQ	NQ
15:1	Pentadecenoic acid	0.2 ± 0.21	0.1 ± 0.21
16:0	Palmitic acid	16.1 ± 0.49 [a]	14.4 ± 0.49 [b]
16:1n5	Myristoleic acid	ND	ND
16:1n7	Palmitoleic acid	2.6 ± 0.31	2.5 ± 0.31
17:0	Margaric acid	1.3 ± 0.20	0.7 ± 0.20
17:1	Heptadecenoic acid	0.9 ± 0.14 [a]	0.3 ± 0.14 [b]
18:0	Stearic acid	6.2 ± 0.47	6.3 ± 0.47
18:1n5	13-octadecenoic acid	ND	ND
18:1n7	Vaccenic acid	ND	ND
18:1n9	Oleic acid	27.4 ± 2.11	27.9 ± 2.11
18:2n6	Linoleic acid	28.8 ± 1.76	27.7 ± 1.76
18:3n6	γ-Linoleic acid	0.3 ± 0.06	0.3 ± 0.06
18:3n3	α-Linolenic acid	1.5 ± 0.92	3.7 ± 0.92
18:4n3	Stearidonic acid	0.1 ± 0.22	0.1 ± 0.22
20:0	Arachidic acid	0.2 ± 0.04	0.3 ± 0.04
20:1n7	Paullinic acid	0.35 ± 0.08	0.4 ± 0.08
20:1n9	Gondoic acid	ND	ND
20:2n6	Eicosadienoic acid	0.4 ± 0.02 [a]	0.3 ± 0.02 [b]
20:3n3	Eicosatrienoic acid	0.02 ± 0.01	0.04 ± 0.008
20:3n6	Dihomo-γ-Linoleic acid	0.5 ± 0.06 [a]	0.7 ± 0.06 [b]
20:3n9	Mead acid	ND	ND
20:4n6	Arachidonic acid	7.0 ± 0.85	7.2 ± 0.85
20:4n3	Eicosatetraenoic acid	0.01 ± 0.01	0.02 ± 0.01
20:5n3	Eicosapentaenoic acid	0.5 ± 0.27 [a]	1.5 ± 0.27 [b]
22:0	Behenic acid	0.2 ± 0.02	0.2 ± 0.02
22:1n9	Erucic acid	0.2 ± 0.05	0.1 ± 0.05
22:4n6	Adrenic acid	0.6 ± 0.11	0.6 ± 0.11
22:5n6	n6 Docosapentaenoic acid	0.1 ± 0.05	0.1 ± 0.05
22:5n3	n3 Docosapentaenoic acid	0.8 ± 0.18 [a]	1.4 ± 0.18 [b]

Table 5. *Cont.*

Fatty Acid	Common Name	Female (n = 5)	Male (n = 5)
22:6n3	Docosahexanoic acid	0.4 ±0.11 [a]	0.8 ± 0.11 [b]
24:0	Lignoceric acid	0.1 ± 0.02	0.08 ± 0.02
24:1	Nervonic acid	0.2 ± 0.06	0.3 ± 0.06
Fatty Acid Groupings			
Monoenes		28.4 ± 2.14	28.9 ± 2.14
Saturates		25.6 ± 0.62 [a]	23.1 ± 0.62 [b]
Highly Unsaturated Fatty Acids		10.1 ± 1.27	12.4 ± 1.27
Poly Unsaturated Fatty Acids		41.1 ± 2.61	44.5 ± 2.61
n-3 Fatty Acids		3.3 ± 1.17 [a]	7.5 ± 1.17 [b]
n-6 Fatty Acids		37.8 ± 1.92	37.0 ± 1.92
n-9 Fatty Acids		27.9 ± 2.10	28.4 ± 2.10
n-6: n-3 Fatty Acid Ratio		11.6 ± 0.625 [a]	5.5 ± 0.625 [b]

[1] The superscripts (a,b) indicate a statistical difference ($p \leq 0.05$) between female and male. [2] Average body weight (g) of females was 132.6 ± 22.88 and 124.8 ± 22.88 for males. ND = not detected; NQ = not quantifiable.

Due to the skewed sex population data for the MC animals (two females and 10 males), statistics on sex differences among the fatty acids were only conducted on the FR animals (n = 5 for both groups). The body weight for the FR animals did not differ by sex (132.6 ± 22.88 g and 124.8 ± 22.88 for the females and males, respectively). Of the 36 individual fatty acids that were analyzed, 30 were present in sufficient concentrations to be quantified in FR of both sexes. Eight differed by sex (Table 5), and all eight fatty acid groupings were present, with three differing by sex. Overall, females were higher in pentadecylic acid (15:0), palmitic acid (16:0), heptadecenoic acid (17:1), eicosadienoic acid (20:2n6), total omega 6: omega 3 ratio, and saturates, while males were higher in dihomo-γ-linoleic acid (20:3n6), eicosapentaenoic acid (20:5n3), n3 docosapentaenoic acid (22:5n3), docosahexanoic acid (22:6n3), and total omega 3 fatty acids.

4. Discussion

This study provides novel data that can suggest baseline parameters of fatty acid profiles for apparently healthy amphibians based on free-ranging and managed care marine toads. The differences in FR marine toads versus those in MC for 60 days demonstrate the potential impact of MC on circulating fatty acids in amphibians.

Being housed in MC (regardless of which diet was offered) for 60 days had a significant impact on 14 of the 27 (52%) detectable individual DBS fatty acids and seven of the eight (88%) fatty acid groups. In general, there was an overall increase in the omega-6 fatty acids along with a decrease in omega-3 fatty acids, resulting in an increase in the omega-6: omega-3 ratio for animals in MC. These data are consistent with findings of FR fatty acid profile in various other species compared to those in MC [7,9,10,30].

For most species with known data, there are two dietary essential fatty acids, linoleic acid (18:2n6) and α-linolenic acid (18:3n3), although all omega-3 and omega-6 fatty acids can be conditionally essential throughout their metabolic pathways due to the dietary, enzyme, and genetic limitations [31]. FR toads demonstrated higher concentrations of α-linolenic acid (18:3n3), dihomo-γ-Linoleic acid (20:3n6), arachidonic acid (20:4n6), omega-3 docosapentaenoic acid (22:5n3), and omega-6 docosapentaenoic acid (22:5n6). As these omega fatty acids are all at least conditionally essential in most species and have been linked to general health and disease prevention, particularly in humans, this may be a concern for MC diets [32]. The decreased growth and reproduction concerns noted in amphibians by Huang et al**.** (2003) with lower dietary α-linolenic acid (18:3n3) also support

these concerns [12]. Additionally, arachidonic acid is considered a third essential fatty acid in mammalian carnivores [33]. The current research presented here indicates arachidonic acid also may be essential in carnivorous toads since most other species can convert linoleic acid into arachidonic acid, and despite our MC diet being very high in linoleic acid, the arachidonic acid levels remain low.

Free-ranging and MC toads had low concentrations of the omega 3 fatty acid docosahexaenoic acid (22:6n3) (DHA), which is known to be an integral component of the phospholipids that make up cell membranes throughout the human body, particularly in the brain and retina [32]. While higher concentrations of omega-3 fatty acids in FR toads were expected, this was only visually represented (0.6 ± 0.14 % vs. 0.3 ± 0.13 %). Further research may be needed into DHA and the potential need for MC diet supplementation despite the lack of significant differences noted (Table 2) [34]. Higher concentrations of the other fatty acids in FR toads could be attributed to the diversity of their diet when compared to the diets of the MC toads in this study. Additionally, the prey items of FR toads have a more diverse diet than gut-loaded crickets fed to the toads in this study.

Successful reproduction is a vital yet often challenging goal of all SSPs, including those for amphibians [2]. The shift in fatty acid concentrations in the toads under MC may affect breeding success by altering the phospholipid membranes of sperm and egg cells as noted in poultry [35]. Omega-3 fatty acids and the omega-6: omega-3 ratio have been demonstrated to increase sperm quality and overall fertility in zebrafish (*Danio rerio*) [36] and Japanese eel (*Anguilla japonica*) [37]. It is important to note that all the toads within this study remained visually healthy and that, in humans, elevated mead acid (20:3n9) is used as a marker of essential fatty acid deficiency, which was not present in either FR toads or the toads at 60 days of MC [38]. In addition, there have been reported differences in the whole-body fatty acid composition of aquatic amphibians versus terrestrial amphibians [12,13]. Aquatic amphibian eggs have higher fractions of non-polar lipids than terrestrial amphibian eggs [12]. Therefore, future species-specific amphibian data are needed to identify optimal fatty acid profiles.

The implementation of DBS cards for fatty acid analysis is important to make fatty acid analysis more accessible, especially for field work on free-ranging animals. Rather than collecting a vial of whole blood (WBV) and needing to store it on ice, biologists can place a few drops of blood onto the DBS card and wait for it to dry. Then, the cards can safely be closed and stored at room temperature until analyses can be performed within the next several weeks. Research regarding the best storage methods (time and temperature) for DBS cards among zoological species is ongoing, although validating the novel use of DBS cards for fatty acid analysis in amphibians is essential for effective interpretation of DBS results [15,39,40].

Data from this study showed minimal differences between the fatty acid profiles when DBS cards were compared against WBV from the same blood sample. While DBS cards demonstrated statistically higher values for two fatty acids, along with the omega 6 fatty acid grouping, these differences are likely not clinically significant. This is due to the fact that the numerical difference was small for these significances, and despite DBS or WBV, MC samples remained different from FR. DBS results appear to be an acceptable replacement for WBV for most individual fatty acids, and since there is no comparison for prior published fatty acids in amphibians, future work using DBS cards is recommended to advance the research data. Further studies with larger sample sizes are needed to determine the efficacy of both fatty acid analyses in amphibian species.

Sex differences were only reviewed for the FR data (5 males: 5 females) due to the MC animals being heavily skewed toward males (10 males: 2 females). Numerous differences were noted, with the major impact being that males had higher concentrations of four essential/conditionally essential omega-6 fatty acids. This led to the male overall omega-3 fatty acid level being higher and the females having a higher omega-6: omega-3 ratio. This is contradictory to research that has shown that human and rodent males normally have a hormone-associated lower conversion of the dietary essential α-Linolenic acid (18:3n3) into

other omega-3 fatty acids, resulting in lower docosahexaenoic acid (22:6n3) [31,41,42]. The sex differences in fatty acids within toads need further research to determine if amphibians may be different in their fatty acid metabolism and biological pathways. Additionally, our FR sex ratio was evenly split, but our MC sex ratio was heavily skewed toward males. Thus, some FR-versus-MC results in this study may be influenced by this skewed sex ratio of our study population. While age may also affect fatty acid usage and metabolism, we were unable to access the age of our wild-caught population of toads. Additionally, the MC toads did tend to be heavier than FR. Some of this difference could be due to continued growth over time, but the MC's easy access to constant food also likely influenced the increased weight. Obesity in humans has been linked to increased quantities of fatty acids, but the variations noted in our FR to MC comparisons do not follow weight trends [43]. While marine toads were used in this study, it is important to note that these toads were from a small, localized population in the Miami, FL, USA area. Additionally, this study only lasted for 60 days and did not have a robust sample size for either FR or MC populations. Therefore, further study is vital to determine the long-term effects that altered MC fatty acids have on other criteria such as histopathology and the overall health and reproductive capability of amphibians in MC.

5. Conclusions

The data presented within this study provides a starting point to begin assessing fatty acid concentrations among free-ranging anurans. In addition, marked differences were noted between the fatty acid profiles of FR toads and toads in MC, which indicated that there needs to be an investigation to ensure that MC animals are being optimally fed. Finally, the accessible field collection method using DBS cards for blood collection appears effective for quantifying a wide range of fatty acids within amphibian species.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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