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No longer a leap in the dark: the importance of protein as an energy source in amphibians

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Amphibian nutrition has been highlighted as one of the disciplines requiring more investigation to support ex situ conservation programs. Specifically, anuran metabolism related to dietary nutrients is not yet investigated in detail. Thirty (n = 30) free-range frogs from four families (Telmatobiidae, Hylidae, Leptodactylidae, Bufonidae) were collected in Bolivia, and opportunistic blood samples were drawn to determine acylcarnitines and amino acid profiles in order to evaluate metabolic activity. The overall profiles showed Telmatobiidae with higher numerical values of amino acids, while comparison with Hylidae displayed differences ($p < 0.05$) in metabolites related to amino acid catabolism, suggesting specific ketogenic pathways in Telmatobiidae as an adaptation to its extreme environmental (hypoxic) conditions. Multivariate analysis demonstrated both lipids and amino acids as the main forces in frog energy metabolism, confirming the carnivorous nature of anurans. Pathways detecting free carnitine and long chain acylcarnitines driving fat metabolism, as well as protein-derived utilisation of amino acid catabolites documented glucose sparing and energy production through both proteinogenic and ketogenic routes. Moreover, malonyl carnitine is suggested to play a role as a modulator of food intake and feeding status of frogs.

Keywords: acylcarnitines, amino acids, frogs, metabolism, nutrition

Global amphibian decline and extinction has become one of the greatest conservation challenges of the century, and solutions for their survival are of major concern (Baillie et al. 2004, Bishop et al. 2012). There is no single cause for this crisis, rather interactions among several factors contribute to the death of local populations (Hayes et al. 2010); global climate change and climate-derived epidemics are considered as immediate threats (Pounds et al. 2006), while environment pollutants, increased diseases and altered patterns of predation are also factors influencing the removal of individuals from populations (Hayes et al. 2010). Likewise, decreased nutritional status from scarcity of food items as well as nutritional diseases – resulting in, or as a consequence of – other health issues, are outlined as factors affecting this situation (Hayes et al. 2010, Olea-Popelka et al. 2014). Thus, amphibian nutrition must receive crucial focus, given its significance in the overall development, physiology, reproductive success and disease resistance of these creatures (Venesky et al. 2012, Dugas et al. 2013, Ferrie et al. 2014, Brenes-Soto et al. 2017).

Captive breeding programs arose as one of the alternatives to support amphibian conservation efforts, with the accompanying multidisciplinary challenge to meet rather unknown husbandry guidelines to assure the success of ex situ populations. Nutrition remains one of the scientific disciplines requiring more information and targeted effort to define needs and optimize care for this group of animals (Lee et al. 2006, Zippel et al. 2006). Although amphibians have been managed in captivity for many purposes, the basics of their nutritional metabolism have not been studied in detail, and many gaps still remain in nutrition research (Olea-Popelka et al. 2014). Common captive diets for amphibians are empirical, based on other species' requirement models, and limited by commercial availability of foods, without knowledge of suitability for meeting amphibian metabolic needs. Current diets and practices may thus be insufficient to ameliorate the incidence of health problems related to nutrient deficiencies or toxicities (Densmore and Green 2007, Ferrie et al. 2014).

In this regard, data from the wild is needed to serve as a basis for ex situ conservation efforts. Likewise, ecological

studies that investigate the impact of different threats on amphibians must understand the effects of a changing food web and the concomitant supply of at least the essential macronutrients such as protein, lipids and carbohydrates in their diets, considering the limited capacity of amphibians to change both gut morphology and digestive physiology when facing changes in food quality (Naya et al. 2005, Courtney-Jones et al. 2015). Although most amphibians are classified as carnivores (insectivores) in their adult stage (Duellman and Trueb 1994), there is yet no solid evidence of specific dietary macronutrient utilisation as energy sources. Several studies on nutrient use in amphibians often claim roles for carbohydrates and lipids as main energy supplies, based on changes in enzyme activities, glycogen and lactate mobilisation as well as lipid reserves (Hanke and Neumann 1972, Grafe 1996, Bevier 1997, Wells 2001, 2007). These authors conclude a metabolic strategy with only limited proof of nutrient availability.

From the nutritional point of view, wild arthropods, though very diverse due to the large number and variability of species, comprise on average ~50% protein (dry matter basis) and up to 50% fat, while carbohydrate content remains very low (1–8%) (Kinyuru et al. 2013, Finke and Oonincx 2014, Kouřimská and Adámková 2016, Muñoz-Saravia et al. 2017); therefore, carbohydrate (less chitin) is essentially of low significance in diets of wild amphibians. Clearly, protein and fat would become the major sources of energy in animals eating these items. From experimental work with *Xenopus laevis*, protein and lipids have been identified as preferred energy sources, and their use in metabolism is associated with voluntary food intake (Brenes-Soto et al. 2018), denoting anurans as 'true carnivores' with a unique energy and glucose metabolism, determined through both amino and fatty acid anabolic and catabolic routes (Case et al. 2000). Yet, no substantial data are available on nutrient metabolism of amphibians in the wild. Since *Xenopus laevis* has been held captive for many decades (Green 2010), they may not be the most suitable physiologic model of nutrient metabolism for free-living anuran species.

Acylcarnitine profile analysis has been used for the biochemical screening of disorders of fatty acid oxidation and organic acid metabolism in humans (Rinaldo et al. 2008), but recently has been also applied in animal studies to identify metabolites related to mitochondrial acetyl coenzyme A (acetyl-CoA) and potential available substrates for the citric acid cycle. Multiple studies have evaluated blood and plasma acylcarnitine concentrations in relation to mineral digestibility in zebu cattle (Dermauw et al. 2013), modulation of glucose metabolism in cats (Verbrugghe et al. 2009), as well as evaluating metabolic responses to changes in water temperature in carp and tilapia (Geda et al. 2017), hence demonstrating promise as a useful tool to assess metabolic processes across species.

This study investigated opportunistically collected bloodspot samples from a highly diverse group of anuran species from Bolivia, to obtain a real image of nutrient use in anuran metabolism in the wild. We hypothesised that protein is utilised as a primary energy source in free-living anurans, with utilisation directly linked to fatty acid metabolic activity. Further, we proposed that variability among individuals and species is linked with extrinsic factors such as habitat and season.

Methods

Thirty adult frogs were collected from the wild in eight sites in Bolivia, over a period of two years (Table 1). Animal collections and all procedures performed were approved by the General Biodiversity Directorate of Bolivia, as a project of the Bolivian Amphibian Initiative, permit UMABCC#0919/11. The animals used in this study were collected for other purposes, and opportunistic blood samples were taken to perform the analysis.

Animals were collected randomly or during VES (visual encounter survey) transects (Heyer et al. 2014), by hand, wearing nitrile gloves, and individually maintained in plastic bags. Euthanasia was performed by submerging the animals in 5% clove essence oil over 10 min (Davis et al. 2015), and blood samples were drawn immediately from the heart using a 1 ml syringe and a 27 G × 1 cm needle. Whole blood samples were then spotted onto Protein Saver cards (Whatman 903), and stored frozen at −20°C until analysis. Acylcarnitines and amino acid profiles of dried blood spots were determined using tandem mass spectrometry (Zytkovicz et al. 2001). All procedures were carried out following guidelines to minimise suffering of the animals.

Statistical analysis

Descriptive statistics were used to show the typical metabolic profile of the frogs, expressed as means and standard deviations (SD) of the relative values (percentage of metabolite per total carnitines). Univariate ANOVA analysis was performed between individuals from Telmatobiidae and Hylidae to evaluate the effect of family together with habitat (aquatic versus terrestrial, respectively) on amino acid and acylcarnitine profiles, with statistical significance accepted at $p < 0.05$. In addition, a multivariate analysis was carried out with all the combined frog data to determine relationships among metabolites, using principal components analysis (PCA), deemed relevant with a value above 0.5 and below −0.5 in each component, as well as determining Pearson correlations coefficients, declaring significance at $p < 0.05$ and high significance at $p < 0.01$. The analysis was conducted using SPSS ver. 24.

Results

Metabolite values from the different frog families are presented in Table 2. Individuals from Telmatobiidae displayed a numerically higher content of all amino acids, except citrulline and ornithine, while acylcarnitines varied among families. However, it is interesting to note that Leptodactylidae exhibited the highest citrulline, ornithine, free carnitine and isovaleryl carnitine concentrations, whereas Bufonidae had the higher values of both long chain (LCA) and the 3-hydroxy long chain (3OH-LCA) acylcarnitines.

When ANOVA was performed between Telmatobiidae and Hylidae, significant differences ($p < 0.05$) were only

Table 1. Frog species and field collection sites of the Bolivian study.

Family/species	n	Site	Latitude*	Longitude*
Telmatobiidae (n = 8)				
Telmatobius culeus	1	Sahuiña	−16.192731	−69.127511
	1	Sicuani	−16.084601	−69.120439
	3	Titicaca Lake	−16.08961	−69.12108
	3	Isla 2	−16.26414	−68.83331
Hylidae (n = 13)				
Dendropsophus joannae	1	Valle del Sacta	−17.15543	−64.75408
Dendropsophus leucophyllatus	1	Valle del Sacta	−17.15543	−64.75408
Boana fasciata	2	Valle del Sacta	−17.15543	−64.75408
Boana geographica	1	Valle del Sacta	−17.15543	−64.75408
Boana punctata	1	Valle del Sacta	−17.15543	−64.75408
Boana riojana	1	Apote	−17.308065	−66.24714
Phyllomedusa vaillanti	2	Valle del Sacta	−17.15543	−64.75408
Scinax cf. *ruber*	2	Valle del Sacta	−17.15543	−64.75408
Sphaenorhynchus lacteus	1	Valle del Sacta	−17.15543	−64.75408
Trachycephalus typhonius	1	Valle del Sacta	−17.15543	−64.75408
Leptodactylidae (n = 5)				
Leptodactylus cf. *leptodactyloides*	4	Valle del Sacta	−17.15543	−64.75408
Pleurodema cinereum	1	Sorata	−15.773333	−68.644806
Bufonidae (n = 4)				
Rhinella margaritifera	3	Valle del Sacta	−17.15543	−64.75408
Rhinella marina	1	Valle del Sacta	−17.15543	−64.75408

* Coordinates taken using Garmin GPSMAP60CSX Global Positioning System.

found in the ratio of citrulline:ornithine, free carnitine, acetyl carnitine, isovaleryl- and 3-hydroxyisovaleryl carnitine, with Telmatobiidae presenting higher values in the carnitines related to amino acid catabolism, whilst Hylidae showed the highest values of metabolites related to fatty acid mobilisation and oxidation (Fig. 1).

The PCA plot shows the association between the metabolites of the assembled group of frogs, explaining 51.2% of the variance (Fig. 2). From component 1, a positive association can be seen between the branched-chain amino acids, methionine, the ratio of long chain acylcarnitines as well as acetyl-CoA and the catabolites isovaleryl-, 3-hydroxyisovaleryl- and 3-hydroxybutyrylcarnitine (group B); likewise, this group is inversely related to free carnitine. Moreover, from component 2, and independent of the metabolites already mentioned, ornithine, 3-hydroxy long chain acylcarnitines and malonyl-carnitine remain grouped in a positive association (group A).

Table 2. Selected blood amino acid and acylcarnitine profiles in free ranging frogs from Bolivia (average + SD[1], % of total carnitines).

Metabolite (%)	Telmatobiidae n = 8	Hylidae n = 13	Leptodactylidae n = 5	Bufonidae n = 4
Amino acids				
Leucine	6.4±5.8	4.1±2.0	3.4±0.8	5.3±0.9
Phenylalanine	2.5±3.2	1.5±0.6	1.4±0.4	2.0±0.5
Valine	7.1±4.3	5.1±1.4	4.8±0.6	6.1±1.2
Methionine (Met)	1.4±1.2	1.3±1.5	0.8±0.2	1.4±0.9
Citrulline (Cit)	0.3±0.1	0.2±0.1	0.4±0.4	0.3±0.2
Ornithine (Orn)	0.8±0.4	0.9±0.4	1.0±0.6	0.9±0.5
Carnitine esters				
Free carnitine (CO)	0.6±0.1	0.7±0.1	0.8±0.1	0.6±0.1
Acetyl (C2)	0.3±0.1	0.2±0.1	0.4±0.04	0.3±0.1
Malonyl (C3-DC)	0.001±0.0005	0.0009±0.0005	0.0008±0.007	0.003±0.002
Butyryl (C4)	0.02±0.005	0.01±0.01	0.008±0.0005	0.008±0.001
Isovaleryl (C5)	0.01±0.003	0.003±0.002	0.02±0.001	0.004±0.001
3-Hydroxy-Butyryl (3OH-C4)	0.003±0.002	0.002±0.001	0.001±0.0006	0.001±0.0006
3-Hydroxy-Isovaleryl (3OH-C5)	0.006±0.002	0.002±0.001	0.002±0.001	0.003±0.001
Total LCA	0.009±0.003	0.01±0.008	0.01±0.005	0.02±0.01
Total 3OH-LCA	0.004±0.002	0.005±0.003	0.003±0.001	0.006±0.003
Ratios				
Met:CO	2.7±2.6	1.8±2.2	1.0±0.3	2.3±1.5
Cit:Orn	0.4±0.2	0.2±0.1	0.4±0.2	0.3±0.1
C2:CO	0.5±0.3	0.3±0.1	0.2±0.1	0.6±0.1
Tot 3OH-LCA:Tot LCA	0.4±0.2	0.3±0.2	0.3±0.2	0.3±0.1

[1] Standard deviation.
C: number of carbons, 3OH: 3-hydroxy, DC: dicarboxylic acid in the acyl group, LCA: long chain acylcarnitines, 3OH-LCA: 3-hydroxy long chain carnitines.

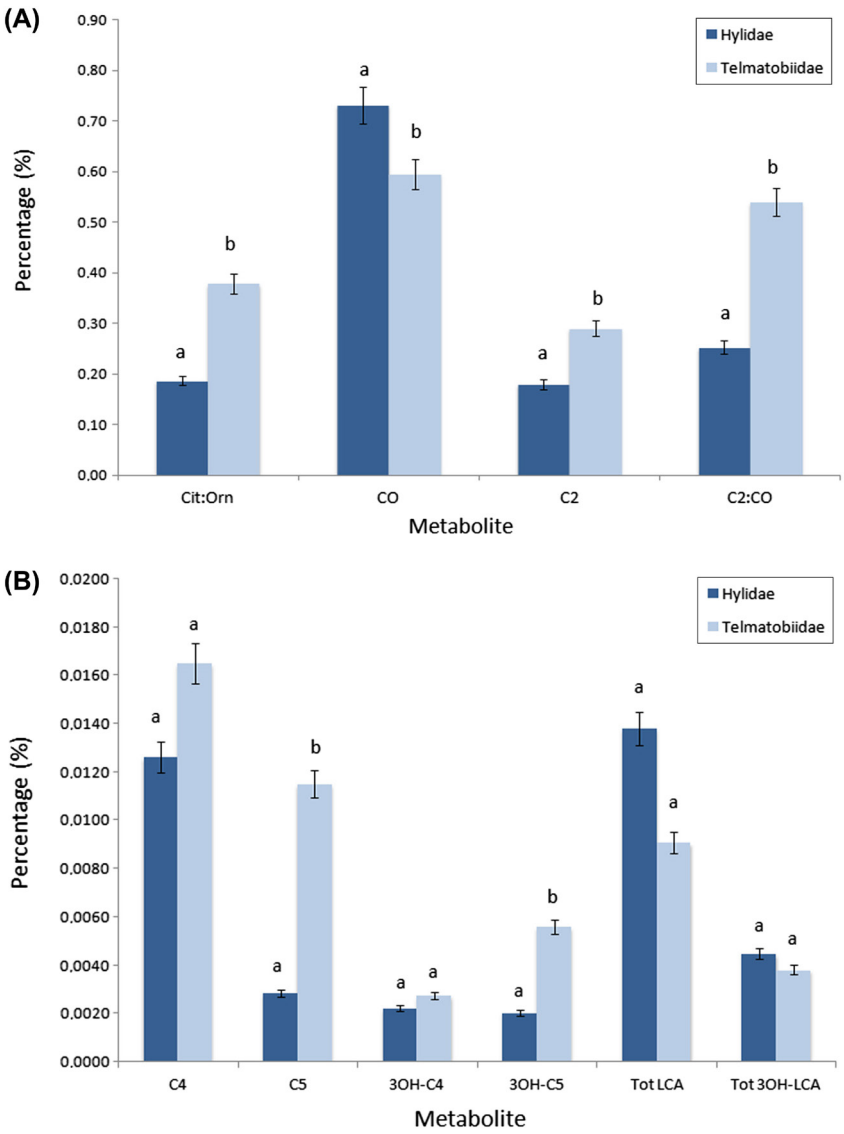

Figure 1. Metabolite values (means) of free-range Telmatobiidae and Hylidae frogs from Bolivia (bars with different letters differ statistically, $p < 0.05$). (A) Cit: citrulline, Orn: ornithine, CO: free carnitine, C2: acetyl carnitine. (B) C4: butyryl carnitine, C5: isovaleryl carnitine, 3OH-C4: 3-hydroxybutyryl carnitine, 3OH-C5: 3-hydroxyisovaleryl carnitine, LCFA: long chain fatty acid acylcarnitine, 3OH-LCFA: 3 hydroxy long chain fatty acid acylcarnitine.

These findings are corroborated with the correlation coefficients, where very strong positive associations ($p < 0.01$) were determined between the amino acids and their catabolites, as well as the ratio of long chain acylcarnitines and some amino acids. Conversely, strong inverse associations were also confirmed between free carnitine (CO) with acetyl-carnitine and the amino acids' catabolites (Table 2).

Discussion

The current information on amino acids and acylcarnitines shows a broad descriptive picture of the typical metabolic profile of free-ranging frogs. In general, whole blood amino acid values were lower in frogs compared to other species such as the laboratory mouse (Rivera et al. 1987), fish *Cyprinus carpio* and *Carassius auratus* (Van der Boon et al. 1991) and Yorkshire pigs (Keith et al. 1977), excepting Telmatobiidae, which were within the lower range of leucine and phenylalanine values reported for pigs. In relation to acylcarnitine profiles, at the moment very little information has been reported in animals, and many of the studies have analysed plasma metabolites (Alhomida et al. 1995, Verbrugghe et al. 2009, Dermauw et al. 2013). However, the few studies conducted on whole blood samples report higher values of free carnitine, acetyl and butyryl carnitine, as well as long chain acylcarnitines in Arabian sand gazelles, *Gazella subgutturosa marica* (Al-Eissa and Alhomida 1997) and laboratory mice (Spiekerkoetter et al. 2004), compared to frogs from this study.

Lower values found in these frogs could be related to their low metabolic rate in comparison to endotherms, which is partly coupled with differences in the size of internal organs but also to differences in cell metabolism (McNab 1988, Hulbert and Else 2004). Nonetheless, although the environment determines an ectotherm's body temperature and

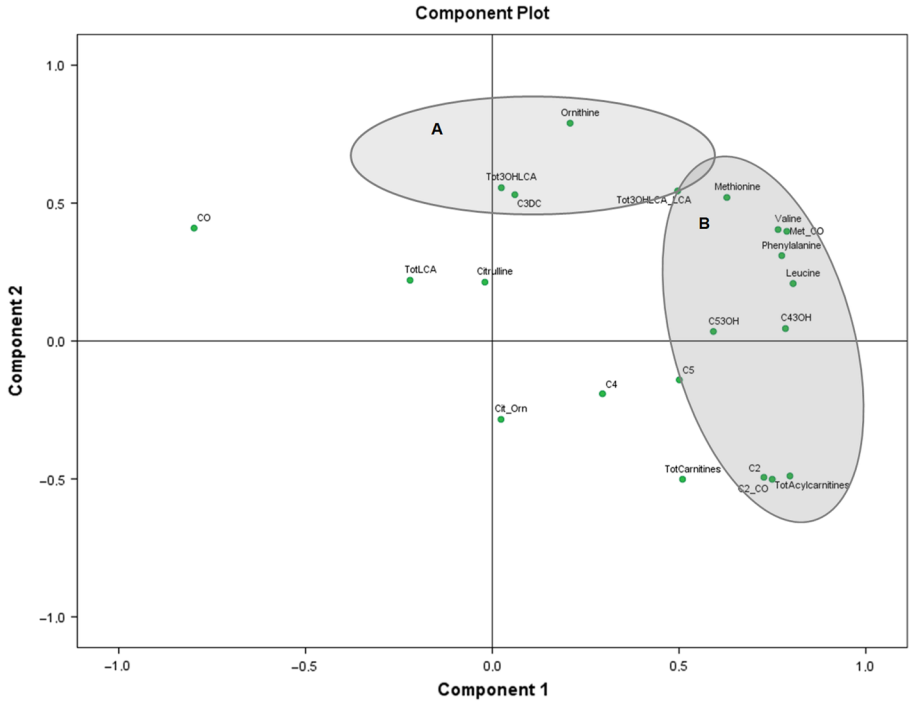

Figure 2. Principal component analysis (PCA) plot of blood amino acids and acylcarnitines of the combined frog data from Bolivia (expressed as percentages of total carnitines). CO: free carnitine. Group A strong relationships: C3-DC: malonyl carnitine, Tot 3OH-LCA: total 3 hydroxy long chain carnitine, Tot 3OH-LCA:Tot LCA: ratio of the total 3 hydroxy and the long chain acylcarnitines. Group B strong relationships: Met:CO: ratio of methionine with free carnitine, C2: acetyl carnitine, C5: isovaleryl carnitine, 3OH-C4: 3-hydroxybutyryl carnitine, 3OH-C5: 3-hydroxyisovaleryl carnitine.

therefore the chemical reactions and occurrence of physiological processes (Van de Pol et al. 2017), it was demonstrated that ectotherms' ratios of respiratory enzymes in mitochondria as well as citric acid cycle intermediaries are very similar to endotherms (Gumbmann and Tappel 1962). Furthermore, the ratio between resting and maximum metabolic rates are likely the same in ectotherms and endotherms (Bennett and Ruben 1979). Since ectotherms do not need to invest in maintenance of a relatively high and constant body temperature, energy demand per unit body mass is much lower than an endothermic animal (Van de Pol et al. 2017). In this case, the low metabolite values in frogs would not imply fewer metabolic processes, but rather a more efficient and economical use of the energy source.

Differences between Hylidae and Telmatobiidae

The results obtained demonstrated how the carnitine profiles and particular conditions (in this case habitat selection) can be associated with specific metabolic features. Although it is acknowledged that the dataset from Hylidae comprises several frog species while Telmatobiidae only includes the Titicaca frog, *T. culeus*, both groups of animals present very distinct physical and physiological characteristics. Hylids are extremely variable in size and are classified as terrestrial/arboreal in the adult stage, having an impervious skin that protects them from desiccation (Duellman and Trueb 1994); the individuals sampled for this study were all collected at low elevations. Likewise, *T. culeus* demonstrates unique adaptations for a fully aquatic life at high altitude and low temperatures in the Titicaca Lake, including a highly vascularised greater skin surface and the lowest metabolic rate measured among anurans (Hutchison et al. 1976, Navas and Chauí-Berlinck 2007).

The significantly higher values of acetyl carnitine, isovaleryl carnitine and 3-hydroxyisovaleryl carnitine shown in *T. culeus*, together with the highest value of leucine, could be indicators of increased use of amino acid catabolites as a major source of energy in the form of acetyl-CoA via acetoacetyl-CoA (Lehninger 1973) compared to terrestrial anurans. Moreover, the potential activation of leucine's exclusive ketogenic pathway (McGilvery 1970) may reflect the capacity of these frogs to use ketone bodies as a source of energy. Studies in rats have demonstrated that animals at high altitudes and with low metabolic rates can mobilise fatty acids (McClelland et al. 2001), but also can enhance the generation of ketone bodies as an adaptive strategy in case of limited oxygen supply (Ni et al. 2014). Likewise, the use of ketone bodies may also serve as a mechanism to spare glucose to be utilised in other functions, such as the protection of erythrocytes and the increase of tissue solute concentrations in order to cope with low temperatures, mechanisms already reported in several frog species living under those conditions (Costanzo et al. 1993).

Associations between metabolites of the combined frog data from Bolivia

The PCA plot (Fig. 2) emulates very well the metabolic activity of the free-ranging frogs, showing, from the component 1, the relationships among the free carnitine (CO), as an indicator of fatty acid oxidation, contrasting with group B,

Table 3. Pearson's correlation coefficients of blood metabolites of the combined data from Bolivian frogs (n = 30).

	Val	Leu	Met	Phe	Orn	CO	C2	C5	3OH-C4	3OH-C5	C3-DC	Ratio LCA
Val	1	0.926**	0.546**	0.923**	0.546**	−0.374*	NS	NS	0.521**	0.474**	NS	0.458*
Leu	0.926**	1	0.496**	0.960**	0.422*	−0.435*	0.387*	NS	0.580**	NS	NS	0.409*
Met	0.546**	0.496**	1	0.508**	0.378*	NS	NS	NS	0.617**	NS	0.404*	0.500**
Phe	0.923**	0.960**	0.508**	1	0.509**	NS	NS	NS	0.552**	NS	NS	0.485**
Orn	0.546**	0.422*	0.378*	0.509**	1	NS	NS	NS	NS	NS	NS	0.494**
CO	−0.374*	−0.435*	NS	NS	NS	1	−0.967**	−0.617**	−0.490**	−0.532**	NS	NS
C2	NS	0.387*	NS	NS	NS	−0.967**	1	0.534**	0.383*	0.448*	NS	NS
C5	NS	NS	NS	NS	NS	−0.617**	0.534**	1	0.432*	0.626**	NS	NS
3OH-C4	0.521**	0.580**	0.617**	0.552**	NS	−0.490**	0.383*	0.432*	1	0.387*	NS	0.450*
3OH-C5	0.474**	NS	NS	NS	NS	−0.532**	0.448*	0.626**	0.387*	1	NS	0.385*
C3-DC	NS	NS	0.404*	NS	NS	NS	NS	NS	NS	NS	1	NS
Ratio LCA	0.458*	0.409*	0.500**	0.485**	0.494**	NS	NS	NS	0.450*	0.385*	NS	1

* Correlation significant at $p < 0.05$ level.
** Correlation significant at $p < 0.01$ level.
Amino acids: Val: valine, Leu: leucine, Met: methionine, Phe: phenylalanine, Cit: citrulline, Orn: ornithine.
Carnitines: CO: free carnitine, C2: acetyl carnitine, C5: isovaleryl carnitine, 3OH-C4: 3-hydroxybutyryl carnitine, 3OH-C5: 3-hydroxyisovaleryl carnitine, C3-DC: malonyl carnitine, Ratio LCA: ratio of the total 3-hydroxyl- and the long chain acylcarnitines.

which illustrates protein breakdown and catabolism, whilst component 2 displays an intriguing association between metabolites related to fatty acid activity (group A). Although the bloodspot analysis in this study contained no direct markers of carbohydrate utilisation, herein can be seen that most of the metabolism is guided by amino acid and lipid activity. Free carnitine, having an inverse relationship with amino acids and amino acid-derived acylcarnitines (Table 3), provides evidence of lipids as one of the forces involved in frog metabolism.

The lipogenic route in this case does include carnitine, which transports activated fatty acids (in a shuttle system with coenzyme A) through the mitochondrial membrane for degradation via the β-oxidation pathway, leading to acetyl-CoA and hence to energy in form of ATP (McGilvery 1970, Bremer 1983). The rate of entry of these acyl groups into the mitochondria is regulated by the action of the hormones insulin and glucagon, but also is modulated by the levels of both carnitine O-palmitoyltransferase activity and malonyl-CoA (Michal and Schomburg 2012). Additionally, carnitine acts as a buffer for the mitochondrial acetyl-CoA, permitting a shift of the 'acetyl surplus' from the mitochondria to the cytosol, due to the fact that carnitine is usually present in higher concentration than acetyl-CoA (Bremer 1983); this explains the high inverse association shown by free carnitine and acetyl carnitine in the frogs. The concentration gradients between the pools of extracellular carnitine and intracellular acylcarnitine (in the form of esters of short-, medium- or long-chain organic and fatty acids) is also a result of the feeding status, due to food intake control through nutrient concentrations, metabolites and/or hormones that signal the need to start or stop feeding (Rebouche and Seim 1998, McDonald et al. 2011).

The proteinogenic/ketogenic route (Fig. 1B) is suggested as the other main force in frog metabolism. The use of the oxidative degradation of amino acids to yield energy through different pathways (McDonald et al. 2011) is something already known in obligate carnivores such as the cat, the fox and the mink, where the carbon skeletons of amino acids are converted into acetyl-CoA and other citric acid cycle intermediaries to form ATP and glucose via gluconeogenesis, as well as in other metabolic pathways (Lehninger 1973, Case et al. 2000, Zoran 2002). Cats for instance, continue using proteins even in cases of low dietary availability, demonstrating the high need of this macronutrient (Verbrugghe and Bakovic 2013). The very high correlation coefficients between amino acids from this study are a clear reflection of their common use in frog metabolism. The elevated requirement of protein in carnivores is also a response to high endogenous glucose demand (Verbrugghe and Bakovic 2013), which may work in the same manner in the frogs from this study. In these pathways, valine and 3-hydroxy butyryl-CoA give rise to the formation of succinyl-CoA via methylmalonyl-CoA, while phenylalanine is further metabolised with the ultimate formation of fumarate, both continuing downstream to oxaloacetate, reaching the synthesis of glucose via phosphoenolpyruvate (McGilvery 1970, Michal and Schomburg 2012). In anurans, these routes have not been investigated in detail, although studies in bullfrog *Rana* (*Lithobates*) *catesbeiana* tadpoles suggest an

uptake of endogenous leucine and phenylalanine during metamorphosis (Kistler et al. 1980).

These neosynthetic routes are particularly important in the case of anurans, where glucose has been demonstrated to be a very critical compound in several functions of ecological significance, involving both anaerobic and/or anaerobic pathways in muscles as a compensatory mechanism during temperature acclimation throughout the year (Kiss et al. 2009), locomotion and calling performance (Wells 2007), or special adaptations to extreme freezing environmental conditions (Costanzo et al. 1993). In all cases glucose needs to be mobilised to serve as a primary fuel; however, as strict carnivores with low expected carbohydrate intake (Case et al. 2000), animals depend on protein when glucose is required, as well as for structural and synthetic purposes (Zoran 2002). The ketogenic amino acids leucine and phenylalanine may also have a substantial function, being catabolised to acetoacetate as an alternative source of energy when acetyl-CoA is insufficient, necessary to spare glucose for other purposes (Holum 1969).

Circling back to the lipogenic 'force', component 2 (Fig. 2A), shows in the first instance the lipid dynamics in the form of activated long chain acylcarnitines as well as the ratio of activated/non activated long chain acylcarnitines, as an independent source of energy. Additionally, the ratio of long chain acylcarnitines overlapping both groups of nutrients (Fig. 2A–B) does indicate its contribution with both contrasts in metabolism, whereas ornithine in that group acts as a marker of the active use of proteins. In anurans, fatty acids are stored in the fat bodies as well as in small deposits in the carcass, and are the major energy reserve of anurans, utilised to fill energy demands in specific ecological and/or physiological situations (Fitzpatrick 1976). Several studies have demonstrated the role of lipids in frogs maintaining a favourable energy balance in determined seasons (Blem et al. 1986), for the regulation of reproductive events (Fitzpatrick 1976), for survival of long periods of dormancy (Lillywhite et al. 1973) and foraging behaviour (Duellman and Trueb 1994). The presence of fats as multiple droplets may be related to a rapid mobilisation requirement to meet the demands of the body and/or gonads (Zancanaro et al. 1996).

Alongside the metabolic relationships already examined, malonyl carnitine shows an intricate positive association with the activated form of long chain acylcarnitines. Malonyl-CoA is an intermediary to synthesize fatty acids under circumstances of fat scarcity, but in this case it may be acting as a modulator of food ingestion, rather than other functions. This compound has been identified as a biological regulator of food intake in non-ruminant mammals, linked with the responsive fatty acid synthesis response in the hypothalamus, as a mediator of energy expenditure and feeding behaviour, and acting in consequence to changes in the orexigenic and anorexigenic neuropeptides responsible for the monitoring and homeorhesis in energy balance (Hu et al. 2003, Dunshea et al. 2018). In the case of the current study, malonyl-CoA may mediate the signals for suppression of food intake, meaning that animals could have been in a positive energy balance at the moment of the sampling. From the metabolic perspective, the circulating levels of the metabolite might reflect its hypothalamic concentrations, namely, when energy status in the animal is low, low concentrations of malonyl-CoA stimulate orexigenic peptides, increase lipolysis and the energy expenditure is reduced, whereas when the energy status in the animals is high, the increase of malonyl-CoA enhances the anorexigenic peptides, stimulating lipogenesis and energy expenditure (Lane et al. 2005, Dunshea et al. 2018). In this case, it is assumed that free-range anurans could share these metabolic regulations, though the state of knowledge in this regard is very limited at this moment, and biological variation within populations should also be taken into account.

In conclusion, the metabolic profiles of free-range frogs from Bolivia showed striking unique mechanisms from such a singular group of animals. The overall profile displayed lower values of some amino acids and carnitines compared to other groups of animals, suggesting more efficient pathways for energy regulation as ectotherms with low metabolic rates. Telmatobiidae confirmed its peculiar physiology and metabolism, having a potential affinity to use the ketogenic routes to obtain energy when glucose is necessary to be utilised for other functions related to adaptations at high altitudes and low temperatures. Moreover, the metabolites' associations confirmed the carnivorous essence of these animals, using both lipogenic and proteinogenic/ketogenic pathways as the main forces in their metabolism, demonstrated development of glucogenic routes, and defined how food intake may play a noteworthy role in the regulation of overall nutrient use.

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References

Al-Eissa, M. S. and Alhomida, A. S. 1997. A study of the distribution of total, free, short-chain acyl and long-chain acyl carnitine in whole blood and plasma of Arabian sand gazelles (*Gazella subgutturosa marica*). – Comp. Haematol. Int. 1: 65–69.

Alhomida, A. S. et al. 1995. Determination of L-carnitine and total carnitine levels in plasma and tissues of camel (*Camelus dromedaries*). – Comp. Biochem. Physiol. B 111: 441–445.

Baillie, J. E. M. et al. 2004. 2004 IUCN Red List of threatened species. A global species assessment. – IUCN.

Bennett, A. F. and Ruben, J. A. 1979. Endothermy and activity in vertebrates. – Science 206: 649–54.

Bevier, C. R. 1997. Utilization of energy substrates during calling activity in tropical frogs. – Behav. Ecol. Sociobiol. 41: 343–352.

Bishop, P. J. et al. 2012. The amphibian extinction crisis – what will it take to put the action into the amphibian conservation action plan? – Sapiens 5: 97–111.

Blem, C. R. et al. 1986. The thermal physiology of two sympatric treefrogs *Hyla cinerea* and *Hyla chrysoscelis* (Anura; Hylidae). – Comp. Biochem. Physiol. A 85: 563–570.
Bremer, J. 1983. Carnitine – metabolism and functions. – Physiol. Rev. 63: 1421–1480.
Brenes-Soto, A. et al. 2017. Colouration in amphibians as a reflection of nutritional status: the case of tree frogs in Costa Rica. – PLoS One 12: e0182020.
Brenes-Soto, A. et al. 2018. The interplay between voluntary food intake, dietary carbohydrate-lipid ratio and nutrient metabolism in an amphibian, (*Xenopus laevis*). – PLoS One 13(12): e0208445.
Case, C. et al. 2000. Canine and feline nutrition. – Mosby Inc, St. Louis, Missouri.
Costanzo, J. P. et al. 1993. Glucose concentration regulates freeze tolerance in the wood frog *Rana sylvatica*. – J. Exp. Biol. 181: 245–255.
Courtney-Jones, S. K. et al. 2015. Long-term changes in food availability mediate the effects of temperature on growth, development and survival in striped marsh frog larvae: implications for captive breeding programmes. – Conserv. Physiol. 3: 1–12.
Davis, D. J. et al. 2015. Effects of clove oil as a euthanasia agent on blood collection efficiency and serum cortisol levels in *Danio rerio*. – J. Am. Assoc. Lab. Anim. Sci. 54: 564–567.
Densmore, C. L. and Green, D. E. 2007. Diseases of amphibians. – ILAR J. 18: 235–254.
Dermauw, V. et al. 2013. Effects of trace element supplementation on apparent nutrient digestibility and utilization in grass-fed zebu (*Bos indicus*) cattle. – Livest. Sci. 155: 255–261.
Duellman, W. and Trueb, L. 1994. Biology of amphibians. – Johns Hopkins Univ. Press.
Dugas, M. et al. 2013. Carotenoid supplementation enhances reproductive success in captive strawberry poisson frogs (*Oophaga pumilio*). – Zoo Biol. 32: 655–658.
Dunshea, F. R. et al. 2018. Role of gut, melanocortin system and malonyl-CoA in control of feed intake in non-ruminant animals. – Anim. Prod. Sci. 58: 627–639.
Ferrie, G. M. et al. 2014. Nutrition and health in amphibian husbandry. – Zoo Biol. 33: 485–501.
Finke, M. D. and Oonincx, D. G. A. B. 2014. Insects as food for insectivores. – In: Morales-Ramos, J. A. et al. (eds), Mass production of beneficial organisms. Elsevier Inc., pp. 583–616.
Fitzpatrick, L. C. 1976. Life history patterns of storage and utilization of lipids for energy in amphibians. – Am. Zool. 16: 725–732.
Geda, F. et al. 2017. The response in fish to midly elevated water temperature relates to species-dependent muscular concentrations of imidazole compounds and free amino acids. – J. Therm. Biol. 65: 57–63.
Grafe, T. U. 1996. Energetics of vocalization in the African reed frog (*Hyperolius marmoratus*). – Comp. Biochem. Physiol. 11: 235–243.
Green, S. L. 2010. The laboratory *Xenopus* sp. – CRC Press, Florida, USA.
Gumbmann, M. and Tappel, A. L. 1962. The tricarboxylic acid cycle in fish. – Arch. Biochem. Biophys. 98: 262–270.
Hanke, W. and Neumann, U. 1972. Carbohydrate metabolism in Amphibia. – Gen. Comp. Endocrinol. 3: 198–208.
Hayes, T. B. et al. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. – J. Exp. Biol. 213: 921–933.
Heyer, R. et al. 2014. Measuring and monitoring biological diversity: standard methods for amphibians. – Smithsonian Institution.
Holum, J. R. 1969. Introduction to organic and biological chemistry. – Wiley.
Hu, Z. et al. 2003. Hypothalamic malonyl-CoA as a mediator of feeding behavior. – Proc. Natl Acad. Sci. USA 100: 12624–12629.
Hulbert, A. J. and Else, P. L. 2004. Basal metabolic rate: history, composition, regulation and usefulness. – Physiol. Biochem. Zool. 77: 869–876.
Hutchison, V. H. et al. 1976. Aquatic life at high altitude: respitaroty adaptations in the Lake Titicaca frog, *Telmatobius culeus*. – Respir. Physiol. 27: 115–129.
Keith, M. O. et al. 1977. Dietary effects on the concentrations of free amino acids in plasma and whole blood of pigs. – Can. J. Anim. Sci. 57: 295–303.
Kinyuru, J. N. et al. 2013. Nutrient composition of four species of winged termites consumed in western Kenya. – J. Food Compos. Anal. 30: 120–124.
Kiss, A. C. I. et al. 2009. Seasonal metabolic changes in a year-round reproductively active subtropical tree-frog (*Hypsiboas prasinus*). – Comp. Biochem. Physiol. A 152: 182–188.
Kistler, A. et al. 1980. Changes in amino acid metabolism and protein synthesis during spontaneous metamorphosis in the bullfrog tadpole liver. – Int. J. Biochem. 12: 395–400.
Kouřimská, L. and Adámková, A. 2016. Nutritional and sensory quality of edible insects. – NFS J. 4: 22–26.
Lane, M. D. et al. 2005. Role of malonyl-CoA in the hypothalamic control of food intake and energy expenditure. – Biochem. Soc. Trans. 33: 1063–1067.
Lee, S. et al. 2006. Captive-breeding programme for the Kihansi spray toad at the Wildlife Conservation Society, Bronx, New York. – Int. Zoo Yearb. 40: 241–253.
Lehninger, L. 1973. Short course in biochemistry. – Worth Publishers, Inc, New York.
Lillywhite, H. B. et al. 1973. The role of behavioral thermoregulation in the growth energetics of toad, *Bufo boreas*. – Ecology 54: 375–383.
McClelland, G. B. et al. 2001. High-altitude acclimation increases the triacylglycerol/fatty acid cycle at rest and during exercise. – Am. J. Physiol. Endocrinol. Metab. 281: E537–E544.
McDonald, P. et al. 2011. Animal nutrition, 7th edn. – Pearson Education Limited, Edinburgh Gate, Essex, England.
McGilvery, R. W. 1970. Biochemistry. A functional approach. – W.B. Saunders Company, London.
McNab, B. K. 1988. Complications inherent in scaling the basal rate of metabolism in mammals. – Q. Rev. Biol. 63: 25–54.
Michal, G. and Schomburg, D. 2012. Biochemical pathways: an atlas of biochemistry and molecular biology. – Wiley.
Muñoz-Saravia, A. et al. 2017. Habitat selection, temperature ranges and implications for nutrition and body condition in a high Andean amphibian, the Titicaca water frog (*Telmatobius culeus*). – In: Clauss, M. et al. (eds), EAZA Nutrition Group 9th European Zoo Nutrition Conference. Liberec, Czech Republic.
Navas, C. A. and Chauí-Berlinck J. G. 2007. Respiratory physiology of high-altitude anurans: 55 years of research on altitude and oxygen. - Respir. Physiol. Neurobiol. 158: 307–313.
Naya, D. E. et al. 2005. Digestive morphology and enzyme activity in the Andean toad *Bufo spinulosus*: hard-wired or flexible physiology? – Comp. Biochem. Physiol. 140: 165–170.
Ni, Q. et al. 2014. Impact of high altitude on the hepatic fatty acid oxidation and synthesis in rats. – Biochem. Biophys. Res. Commun. 446: 574–579.
Olea-Popelka, F. et al. 2014. Leaping forward in amphibian health and nutrition. – Zoo Biol. 33: 586–591.
Pounds, J. A. et al. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. – Nature 439: 161–167.
Rebouche, C. and Seim, H. 1998. Carnitine metabolism and its regulation in microorganisms and mammals. – Annu. Rev. Nutr. 18: 39–61.

Rinaldo, P. et al. 2008. Acylcarnitine profile analysis. – Genet. Med. 10: 151–156.
Rivera, S. et al. 1987. Blood amino acid compartmentation in mice bearing Lewis lung carcinoma. – Cancer Res. 47: 5644–5646.
Spiekerkoetter, U. et al. 2004. Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA deshydrogenase-deficient mice subjected to stress. – Eur. J. Clin. Invest. 34: 191–196.
Van de Pol, I. et al. 2017. Comparative physiology of energy metabolism: fishing for endocrine signals in the early vertebrate pool. – Front. Endocrinol. 8: 36.
Van der Boon, J. et al. 1991. Free amino acids in whole blood and plasma of two cyprinids, *Cyprinus carpio* and *Carassius auratus* (Cyprinidae: Teleostei). – Comp. Biochem. Physiol. A 99: 391–399.
Venesky, M. D. et al. 2012. Dietary protein restriction impairs growth, immunity and disease resistance in southern leopard frog tadpoles. – Oecologia 169: 23–31.
Verbrugghe, A. and Bakovic, M. 2013. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. – Nutrients 5: 2811–2835.
Verbrugghe, A. et al. 2009. Oligofructose and inulin modulate glucose and amino acid metabolism through propionate production in normal-weight and obese cats. – Br. J. Nutr. 102: 694–702.
Wells, K. D. 2001. The energetics of calling in frogs. – In: Ryan, M. J. (ed.), Anuran communication. Smithsonian Inst. Press, pp. 45–60.
Wells, K. D. 2007. The ecology and behavior of amphibians. – Univ. of Chicago Press.
Zippel, K. et al. 2006. CBSG/WAZA Amphibian ex situ conservation planning workshop final report. – IUCN/SSC Conservation Breeding Specialist Group.
Zoran, D. L. 2002. The carnivore connection to nutrition in cats. – J. Am. Vet. Med. Assoc. 221: 1559–1567.
Zytkovicz, T. H. et al. 2001. Tandem mass spectrometric analysis for amino, organic and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. – Clin. Chem. 47: 1945–1955.
Zancanaro, C. et al. 1996. Fat body of the frog *Rana esculenta*: an ultrastructural study. – J. Morphol. 227: 321–334.