GHENT UNIVERSITY
FACULTY OF VETERINARY MEDICINE
Academic year 2016 - 2017

NUTRITION OF THE TITICACA WATER FROG (*TELMATOBIUS CULEUS*)

by

Stéphane KNOLL

Promoters: Muñoz-Saravia Arturo
Prof. dr. Janssens Geert P. J.

Research Report as part of the
Master's Dissertation

Disclaimer

GHENT UNIVERSITY
FACULTY OF VETERINARY MEDICINE
Academic year 2016 - 2017

NUTRITION OF THE TITICACA WATER FROG (*TELMATOBIUS CULEUS*)

by

Stéphane KNOLL

Promoters: Muñoz-Saravia Arturo
Prof. dr. Janssens Geert P. J.

Research Report as part of the
Master's Dissertation

Foreword:

This research has been conducted as a part of the master's degree at the Faculty of Veterinary Medicine of the Ghent University, Belgium. As third master student, graduating in the field of research, conducting a research project is an important part of the master dissertation.

I feel very lucky for being assigned such an interesting topic and had the chance to travel abroad. For this reason, I would like to thank the Ghent University for the amazing opportunity to conduct research abroad and get some international experience. I am very thankful I was able to travel to Bolivia for four months and execute this research project. It was an absolutely incredible experience and I would love to continue doing research in the field of animal nutrition.

I would like to express a great amount of gratitude to prof. dr. Geert P. J. Janssens and especially Arturo Muñoz-Saravia for making all this possible. Thankyou Arturo for organizing this research project and for all you hospitality and expertise here in Belgium and in Bolivia.

Furthermore, I would like to thank the directory team at the Museo de Historia Natural Alcide d'Orbigny in Cochabamba, Bolivia for all their help and support. A big thankyou to Ricardo Céspedes and Eliana Lizarraga. Fourthly, the team of BAI working in the captive breeding program in Cochabamba, as well as the BAI-research team in Bolivia also deserve a great amount of gratitude for all their help. In particular I would like to thank Gabriel Callapa for working with me in the field and helping me get around in Bolivia. Furthermore I would also like to thank Adriana Aguila Sainz, Alejandra Suarez Telles, Andrea Sandoval, Boris Bozo Herrera, Natalia Sanchez, Nazaria Lazzo, Ricardo Zurita Ugarte, Sophia M. Barron Lavayen, for the help I received catching and sorting potential prey items in the Titicaca Lake. Next, another thanks goes out to Arturo Muñoz-Saravia for helping me with the statistical analysis and processing of the data and a big thanks to Andrea Brenes Soto for helping with the nutrient intake calculations.

Last but not least a big thank you goes out to my wonderful parents for their emotional, mental and financial support during this journey. I couldn't have done it without them!

Finally I would like to express how blessed I feel to be able to conduct research that will potentially help out endangered species and will put amphibian nutrition is a brighter light. It has always been a passion of mine to work in the field of wildlife conservation and being able to contribute to the preservation of our world's wonderful biodiversity. Exotic animals, especially little critters like amphibians, fish, reptiles, and even insects have always had a special place in my heart. For as long as I can remember I have been catching and studying these animals in my free time. So being able to do the same on a semi-professional level and making a difference for these animals is a dream come true. I have to say that I have really found myself in the field of research and again, I would like to thank prof. dr. Geert P. J. Janssens and Arturo Muñoz-Saravia for this and, for making this project possible. I hope we will be working together again in the near future!

Contents:

Summary:

Nutrition is a considerable bottleneck in the *ex situ* maintenance of amphibians and is increasingly important in the context of captive breeding programs and worldwide amphibian declines. For these reasons, the dietary nutrient profile of the critically endangered Titicaca water frog (*Telmatobius culeus*) was determined and an attempt was made to deduce its nutritional requirements. During this research, a total of 12 types of potential prey items were attained and proximate analysis was carried out, together with mineral analysis, in order to determine their nutrient contents. Next, this data was combined with results of a previously performed gut content analysis to generate *T. culeus'* dietary intake. Frogs were found to get most nutrients from three prey items, i.e. *Hyalella* spp., *Heleobia* sp. and *Biomphalaria* sp. Furthermore, *Hyalella* spp. appeared to be the main source of all nutrients investigated, except phosphorus. The wild diet of *T. culeus* was determined to contain moderate crude protein (34 %), average fat (21 %) and extremely high ash contents (45 %), with a moderately low gross energy value (9908 kJ/kg). Furthermore, high ash contents translated into unrealistically high contents for most examined minerals. This being said, the extremely high Zn, Fe and Hg concentrations strongly suggest to be the result of environmental contamination in the Titicaca Lake. Finally, the extremely high ash contents made it practically impossible to determine the nutritional requirements of *T. culeus*. Regardless, by redistributing excess ash to the remaining macronutrients respectively, an estimation of these frog's nutrient recommendations was made. In conclusion, Titicaca water frogs are believed to have higher protein, fat and energy requirements compared to what the original dietary intake figures would suggest.

Keywords:

Amphibian, Nutrition, Nutrient intake, Nutritional requirements, Diet, Prey selection, Titicaca water frog, *Telmatobius culeus*

Samenvatting:

In de laatste decennia zijn amfibieën getroffen door een wereldwijde uitstervingscrisis met wezenlijke gevolgen voor een groot aantal soorten. Vooralsnog is hiervoor nog geen oplossing gevonden en is deze crisis dus nog steeds gaande. Vooral species die "enigmatische" dalingen ondergaan, voornamelijk afkomstig uit het neotropisch gebied, worden ernstig bedreigd. Tot op heden zijn *ex situ* fokprogrammas de enige beschikbare optie voor het behoud van dergelijke getroffen soorten. Hoe dan ook, het houden van rendabele amfibieënpopulaties in gevangenschap is geen eenvoudige zaak. Een aanzienlijk probleem waarmee zulke programmas te kampen hebben vloeit voort uit het feit dat de voeding van amfibieën (dieet, nutriënteninname, voedingsbehoefte,...) nog steeds een grote onderzoekskloof vormt. Om deze reden werd in dit onderzoek de nutriënteninname van de kritisch bedreigde neotropische Titicacakikker (*Telmatobius culeus*) onderzocht. Daarenboven werd een poging gedaan om de voedingsbehoeften van deze kikkers af te leiden. Het algemene doel van dit onderzoek is dus om inzicht te verkrijgen in de voedingsgewoontes van deze kikkersoort en zo de kweekinspanningen voor deze soort te gerieven. Gedurende dit onderzoek werden potentiële prooidieren van *T. culeus* aan de oevers van het Titicacameer verzameld en vervolgens werd de voedingswaarde van deze dieren geanalyseerd. In totaal werden 12 soorten potentiële prooidieren bekomen. Deze prooidieren werden onderworpen aan een proximale analyse en aan mineralenanalyse, om zo hun voedingswaarde te bepalen. Deze gegevens werden vervolgens gecombineerd met de resultaten van eerder uitgevoerde darminhoudsanalyse om zo de nutriënteninname van de kikkers te berekenen. Kikkers bleken het merendeel van hun voedingsstoffen te verkrijgen van slechts drie prooidiersoorten, namelijk; *Hyalella* spp., *Heleobia* sp. en *Biomphalaria* sp.. Verder bleek *Hyalella* spp. de belangrijkste bron te zijn voor alle onderzochte voedingsstoffen, met uitzondering van fosfor. In het bijzonder bleek *Hyalella* spp. een uitstekende bron van calcium te zijn, hoewel het niet duidelijk is hoeveel van dit mineraal uiteindelijk beschikbaar is voor de kikkers. Daarnaast werd ook aangetoond dat de occasionele inname of grotere prooidieren, zoals *Orestias ispi*, substantiële voedingsvoordelen voor de kikkers betekent. In het algemeen bleek het dieet van *T. culeus* in het wild matige hoeveelheden ruw eiwit (34 %) en gemiddelde hoeveelheden vet (21 %) te bevatten. De bruto energiewaarde van het dieet van deze kikkers was matig laag (9908 kJ / kg). Ten slotte bleek het as gehalte van dit dieet extreem hoog te zijn (46 %), wat zich ook vertaalde in onrealistisch hoge gehaltes voor de meeste mineralen bepaald gedurende dit onderzoek. Er werd vastgesteld dat een groot deel van de overmatige as afkomstig was van de onverteerbare slakkenhuizen van *Heleobia* sp. en *Biomphalaria* sp., waardoor de proportionele waarden van de hierboven genoemde macronutriënten aanzienlijk verlaagd werd. Gezien het feit dat *Telmatobius culeus* deze slakken actief lijkt te selecteren, werd gespeculeerd dat deze slakkenhuizen een alternatieve functie zouden hebben in het spijsverteringsstelsel van de kikkers. Vervolgens maakte dit extreem hoge asgehalte, samen met het feit dat de verteerbaarheid van de prooidelen, noch de beschikbaarheid van de voedingsstoffen in deze prooidieren bekend is, de bepaling van de voedingsvereisten van de Titicacakikker praktisch onmogelijk. Hoe dan ook, door de overmaat aan as proportioneel te herverdelen naar de overige macronutriënten, werd een schatting bekomen van de voedingsaanbevelingen voor deze kikkers. Deze theoretische aanbevelingen werden vervolgens beoordeeld door deze te vergelijken met bestaande voedingsaanbevelingen voor amfibieën. Ter conclusie werd aangenomen dat Titicacakikkers hogere eiwit-, vet- en energievereisten hebben dan de oorspronkelijk vastgestelde nutriënteninnamecijfers suggereerden. Ten slotte is het noemenswaardig dat de gehalten aan Zn, Fe en Hg in het dieet van de kikkers zo onrealistisch hoog waren, dat men kon concluderen dat die het gevolg waren van milieuvervuiling in het Titicacameer.

Trefwoorden:

Amfibie, Voeding, Nutriënteninname, Voedingsbehoefte, Dieet, Prooidierselectie, Titicacakikker, *Telmatobius culeus*

RESEARCH

1. INTRODUCTION

Amphibians are a complex and diverse group of animals inhabiting every continent on the planet, except for Antarctica *(*Bishop et al., 2012). The class *Amphibia* is classified into 3 major orders: *Anura* (frogs and toads), *Caudata* (salamanders and newts), and *Gymnophiona* (caecilians) (Latney et Clayton., 2014). Currently, the total number of amphibian species is 7,874 (May 26, 2018), counting 6951 *Anura*, 715 *Caudata and* 208 *Gymnophiona* (AmphibiaWeb, 2018).

For the last few decades, amphibians have been undergoing a global extinction crisis (Stuart et al., 2004; Bishop et al., 2012; Alroy, 2015; Muths and fisher, 2015; Tapley et al., 2015). This immense rate of extinction has been labeled as one of the six mass extinctions in history (Whiles et al., 2013). Consequently, in 2001, the International Union for Conservation of Nature (IUCN) launched a global assessment of all known amphibian species: “The World Conservation Union Global Amphibian Assessment” (GAA) (Bishop et al., 2012; Harding et al., 2016). This assessment revealed that at least 43 % of all amphibian species were undergoing some form of population decline and that 32.5 % were globally threatened. Furthermore, 427 amphibian species were labeled as critically endangered (Stuart et al., 2004; Harding et al., 2016). Nowadays this number has increased to 552 amphibian species worldwide (IUCN, 2017). In addition, these figures are assumed to be underestimated as many amphibian species have not been sufficiently studied to allow any conclusions (Stuart et al., 2004; Bishop et al., 2012; Tapley et al., 2015).

According to Stuart et al. (2004), amphibian species can be divided into three groups based on the cause of their population decline. Firstly, amphibian declines are caused by “overexploitation”. This form of population decline is predominantly seen in Eastern and Southeast Asia, where amphibians are extracted for human consumption. In the second group, amphibian declines are caused by “reduced-habitat”, which occurs more widely around the globe. The remaining declining species fall under so called “enigmatic decline”. This form of population decline is called “enigmatic” because no clear underlying factor has been identified for this population effect. This being said, diseases (for example chytridiomycosis) and climate change are suspected to be culpable. Enigmatic declines are typically seen in the Neotropical realm and Australia (Stuart et al., 2004).

This last type of population effect poses an especially big threat for the survival of many amphibian species (Stuart et al., 2004). First of all, the Neotropical realm, consisting of the Caribbean and the entire Southern Americas, hosts nearly half of all amphibian species on earth (Bishop et al., 2012) and moreover, amphibian species living in this area seem to be undergoing the largest proportional population decline (Stuart et al., 2004; Becker and Zamudio, 2011). Second of all, conservationists have not yet been able to figure out a way to ensure the survival of enigmatic declining species in the wild. The only available conservation option for such species are captive breeding programs (CBPs) (Stuart et al., 2004).

Subsequently, following the publication of the GAA in 2004, “the Amphibian Conservation Action Plan” (ACAP) was set up, wherein the implementation of CBPs was recommended as a means of buying time and ensuring the survival of threatened amphibian species. This initiative led to the foundation of “the Amphibian Ark” (AArk), an organization which focusses on the *ex situ* conservation of amphibians (Bishop et al., 2012; Tapley et al., 2015; Harding et al., 2016). After conducting “Amphibian Conservation Need Assessments”, AArk recommended that at least 360 amphibian species needed to be incorporated in CBPs, with other estimates going even higher. Later, the Amphibian Survival Alliance (ASA) was set up which further emphasized the need for CBPs as one of their three main objectives in the effort against amphibian declines (Bishop et al., 2012).

This being said, maintaining viable amphibian populations in such CPBs is often easier said than done. Amphibians are a diverse class of animals and many species have very specific husbandry requirements (Densmore and Green, 2007; Tapley et al., 2015). Various species require specific microhabitats taking into consideration: moisture, temperature, pH, lighting and refuges (McWilliams, 2008; Bishop et al., 2012; Tapley et al., 2015). For example, many of the Neotropical amphibian species are therefore hard to maintain under *ex situ* conditions (Stuart et al., 2004).

Even though considerable progress has been made in the last few decades, amphibian husbandry is still a vital research topic in the efforts to combat amphibian declines worldwide. Apart from the various factors named above, amphibian nutrition is also a considerable bottleneck in maintaining these animals under *ex situ* conditions. Many of the species in this class of animals have specific nutritional requirements that regularly vary over different life stages (Densmore and Green, 2007; Tapley et al., 2015). Additionally, to date, not much is known about amphibian nutrition. So far, little evidence-based research has been conducted on the nutritional value of wild amphibian diets, resulting in inadequate knowledge of the requirements of many amphibian species (Densmore and Green, 2007; McWilliams, 2008; Ferrie et al., 2014; Latney and Clayton, 2014).

Commonly, the diet of both aquatic and terrestrial amphibians mainly consists of invertebrates. For this reason, research on insect nutritional composition is vital to amphibian husbandry (Latney and Clayton, 2014). Luckily, the nutritional composition (minerals, vitamins, protein, fat, carbohydrate) of many commercially available “feeder insects” has already been analyzed in the past (Ferrie et al., 2014; Latney and Clayton, 2014). Table 1 consists of a list of previously analyzed invertebrates, as found in Latney and Clayton (2014). However, little comparative research has yet been conducted on wild and commercially available invertebrates (Ferrie et al., 2014).

Even though these comparative national analyses are limited, research has shown that commercially available invertebrates usually have poor nutritive value (Latney and Clayton, 2014). For example, the calcium and phosphorus ratio, as well as the fat and protein concentration of many of these insects are inadequate or imbalanced (Browne, 2009). In general, feeder insects are low in calcium, vitamins A, D, E, thiamin and omega-3 fatty acids (Finke, 2015). Additionally, the composition of insect diets given to captive amphibians is habitually limited by the commercial availability of these food sources. Therefore, *ex situ* amphibians are often fed a small variety of insect species (Densmore and Green, 2007; Ferrie et al., 2014; Tapley et al., 2015) whereas, wild diets of many amphibians consist of a large assortment of invertebrates, with variable nutrient composition (Ferrie et al., 2014).

Poor nutritive value of many feeder insects and limited commercial availability of various insect species result in high occurrence of nutritional deficiencies in captive amphibians. Consequently, diseases such as hypovitaminosis A and metabolic bone disease are a common sight among these animals (Densmore and Green, 2007; McWilliams, 2008; Ferrie et al., 2014).

Because of the important role that CBPs play in the conservation of amphibians, and the lack of data regarding management and nutritional requirements of this class of vertebrates, this research determined the nutrient intake of the endangered Titicaca water frog (*Telmatobius culeus*), with the goal to optimize captive breeding efforts for this species. Potential prey items of the Titicaca water frog were captured on the shores of Lake Titicaca and analyzed for their nutrient content. Next, this data was combined with results from gut content analysis of *T. culeus*, carried out by Muñoz-Saravia et al. (2018a, unpublished data) in order to create a nutritional profile, portraying the nutrient intake of the Titicaca water frog in the wild. Finally, an attempt was made to deduce the nutritional requirements of these frogs based on the data generated during this research. The present study is the first attempt to determine the nutrient composition of an amphibian’s wild diet, rendering crucial information for successful breeding under human care.

Tabel 1. List of insects that have been evaluated for their nutrient composition, as found in Latney and Clayton (2014).

Common name	**Latin name**	**references**
Domestic cricket	Acheta *domesticus*	Jones et al., 1972; Allen et Oftedal, 1989; Anderson, 2000; Finke, 2002; Finke et el., 2005
Earthworm	*Lumbricus terrestris*	Barker et al., 1998; Finke, 2002
Silkworm	*Bombyx mori*	Finke, 2002; Finke, 2007
Mealworm larvae and beetles	*Tenebrio molitor*	Fraenkel, 1950; Jones et al., 1972; Martin et al., 1976; Zwart et Rulkens, 1979; Barker et al., 1998; Klasing et al., 2000; Hunt et al., 2001; Finke, 2002; Ooninctx et Dierenfeld, 2012
Fruit flies	*Drosophila melanogaster*	Van Den Sande et Van Den Bergh, 1976; Barker et al., 1998; Ooninctx et Dierenfeld, 2012; Finke, 2013
Waxworm	*Galleria mellonella*	Strzelewicz et al., 1985; Barker et al., 1998; Finke, 2002
Superworm larvae and beetle	*Zoophobas morio*	Barker et al., 1998; Finke, 2002; Oonincx et Dierenfeld, 2012
Termites	*Nasutitermes* spp.	Oyarzun et al., 1996
Black soldier fly larvae or phoenix worm	Hermetia illucens	St-Hilaire et al., 2007; Dierenfeld et King, 2008
Madagascar hissing cockroaches	*Gromphadorhina portentosa*	Oonincx et Dierenfeld, 2012
Butterworm worms	*Chilecomadia moorei*	Finke, 2013
Turkistan or red rusty cockroaches	*Blatta Lateralis*	Oonincx et Dierenfeld, 2012; Finke, 2013
Adult house flies	*Musca domestic*	Finke, 2013
Wood louse	*Porcellio scaber*	Oonincx et Dierenfeld, 2012
False katydid	*Microcentrum rhombifolium*	Oonincx et Dierenfeld, 2012
Migratory locust	Locusta migratoria	Van Den Sande et Van Den Bergh, 1976; Oonincx et van der Poel, 2011
Six-spotted cockroach	*Eublaberus distani*	Oonincx et Dierenfeld, 2012
German cockroach	*Blatella germanica*	Pennino et al., 1991
Honey bee	*Apis mellifera*	Pennino et al., 1991
Gypsy moth	*Porthetria dispar*	Pennino et al., 1991
Slugs	*Arion subflavus*	Pennino et al., 1991
Dung beetle	*unknown species*	Pennino et al., 1991
Dragonfly nymphs	*unknown species*	Pennino et al., 1991
Krill	*Euphasia pacifica*	Pennino et al., 1991
Squid	*Loligo pealei*	Pennino et al., 1991
Crayfish	*Procambarus blandingi*	Pennino et al., 1991

2. THE TITICACA WATER FROG (*TELMATOBIUS CULEUS*)

2.1. OCCURRENCE AND HABITAT

Telmatobius culeus (Garman, 1875), or the Titicaca water frog, is a fully aquatic species of frog (IUCN, 2017), part of the *Telmatobiidae* family. This family consists of 64 frog species (Frost, 2017), inhabiting high elevation aquatic habitats in the South American Andes, ranging from Ecuador (02° 05' S) to Argentina (29° 39' S) (Benavides et al., 2002; De La Riva, 2005; Frost, 2017). Generally, this genus of frogs occupies lakes and streams at altitudes between 1000-4500 m, with the species living in the highlands being fully aquatic (De La Riva, 2005). The Titicaca water frog predominantly inhabits Lake Titicaca, located at 3,810 m above sea level (De La Riva, 2005; IUCN, 2017). The Titicaca Lake is situated between Peru (to the west) and Bolivia (to the east), and covers about 8,300 square km (De La Riva and Reichle, 2014; UNESCO, 2017), making it the largest freshwater lake in South America. Besides that, this lake is known as the highest of the world's large lakes (UNESCO, 2017). At this elevation, low temperature ranges can be expected, as well as large nocturnal and diurnal temperature variations. Regardless, due to the stable nature of the Titicaca water frog's lacustrine microhabitat, this species rarely experiences temperatures lower than 10 °C (Navas, 1997). In addition to the Titicaca Lake, *T. culeus* has been found in neighboring lakes and rivers, like Lago Saracocha, Laguna Arapa and Río Ilave (De La Riva, 2005).

The Titicaca water frog is a bottom-dwelling frog, mostly seen on the bottom of the Titicaca Lake. These frogs can be found ranging from the shorelines, nearing the surface, to depths of over 20 m (De La Riva, 2005; Hutchison et al., 1976). Prints left behind by these frogs were even recorded at a depth of 120 m in an underwater expedition with two Cousteau Sea Fleas (Diving Almanac & Book of Records, 2018). Juvenile frogs seem to prefer shallow habitats, whereas adults frogs are usually found in deeper waters (Batko, 2014; Genova, 2011). Preliminary research conducted by Genova (2011), North-West of the Copacabana peninsula in the Bolivian part of Lake Titicaca revealed that adult frogs would prefer muddy and sandy areas with some rocks present. Juveniles would strongly favor areas with a high prevalence of rocks. Habitat choice of juvenile frogs was vastly correlated to the presence of invertebrates like crustaceans, gastropods and leaches (Genova, 2011). Another preliminary study on the habitat preference of *Telmatobius culeus*, conducted by Schimmel (2015) on Isla de la Luna in Bolivia revealed additional information. Per this study, adult frogs would prefer areas with large amounts of vegetation, as well as a rich diversity in plants. High presence of rocks seems to have a negative impact on the abundance of adult frogs. Furthermore, this study showed that juvenile frogs tend to favor a more mixed bottom structure (Schimmel, 2015). Nonetheless, these results should be interpreted with care as this study only examined frogs in a limited area of the Titicaca Lake and for a short period of time. In general, Schimmel (2015) concluded that the highest number of frogs could be found in "mixed" areas with a rich vegetation fauna together with the presence of some rocks. The author speculated that plants function as refuges for the frogs, as well as for the frog's food-items (invertebrates). Furthermore, the abundance of plants would lead to well oxygenated water, which would be beneficial for the frogs.

2.2. MORPHOLOGY AND PHYSIOLOGY

Telmatobius culeus is the largest frog species found in the Titicaca Basin and is morphologically and physiologically well adapted for a bottom dwelling life. Males measure around 120 mm and females around 138 mm from snout to cloaca (snout-vent length) (De La Riva, 2005) and can weigh over 250 g (Hutchison et al., 1976). Titicaca water frogs can be distinguished by their large body size, bulky flattened head and a round snout, which has a pointed aspect in lateral view (Benavides et al., 2002; De La Riva, 2005). These frogs also have a thick dorsal disc (Benavides et al., 2002) and a variable amount of webbing, ranging from 1/2 to 2/3 of the length of the toes (De La Riva, 2005). However, *T. culeus'* most prominent characteristics are its supratympanic and tarsal dermal folds, as well as its extreme "bagginess" around the tights and flanks. These folds are most pronounced in larger individuals (Benavides et al., 2002; De La Riva, 2005). Because of these excessive dermal folds, the Titicaca water frog is also commonly referred to as "the scrotum frog" (Pappas, 2016). Generally, the skin of these frogs is smooth with a highly vascularized surface (Benavides et al., 2002). Overall, the coloration of the Titicaca water frog is quite variable. These frogs range from a brown to a greenish-brown color and can have diverse lightly colored dorsal patterns. Ventral colorations usually consist of white or gray tones and can also have varying colored patterns (De La Riva, 2005). Finally, it is noteworthy to mention that specimen within this species can differ substantially in morphology and body size, making phylogenetic identification difficult (Benavides, 2005; De La Riva, 2005). Some examples of *T. culeus* are given in Figure 1.

Figure 1. An illustration of the diversity in coloration and the prominent dermal folds of *Telmatobius culeus*. (Pictures taken at Isla de la Luna, Titicaca, Bolivia, 2016-2017 by Knoll Stéphane).

In order to survive in the oxygen poor, and low temperature environment of the South American Andes mountains, *T. culeus* had to develop some interesting adaptations. Accordingly, the Titicaca water frog has a high hematocrit and high hemoglobin concentrations, as well as the lowest metabolic rate reported in any frog (Hutchison et al., 1976; Navas, 1997). Furthermore, these frogs also have extremely small erythrocytes. All these adaptations help these animals to maximize their oxygen uptake and usage in this extreme habitat (Hutchison et al., 1976). Additionally, the frog's abundant dermal folds increase their skin surface area substantially, allowing for augmented oxygen resorption from the surrounding water (Hutchison et al., 1976; Feder and Burggren, 1985). Rich subepidermal plexi with capillaries that reach through to the outer layer of the stratum corneum facilitate this process. Moreover, by exhibiting behavior described as 'bobbing', the Titicaca water frog can increase oxygen resorption from the surrounding water (if needed). For this purpose, frogs slightly propel themselves of the bottom by pushing up with their hind legs. As they come back down, the frog's dermal folds undergo a 'wave-action', ventilating the skin surface, increasing contact with oxygenated water. In addition, the Titicaca water frog also possesses a pair of lungs. Although greatly reduced, flattened and poorly vascularized, these allow breathing above water. Nevertheless, these frogs have never been observed surfacing for air. As the water of Lake Titicaca is relatively well oxygenated (100 mm Hg dissolved oxygen at saturation), due to strong winds and constant wave action, *T. culeus* relies on cutaneous gas exchange as its primary breathing method. Experimental reduction of the oxygen concentration in the water has shown that these frogs can tolerate P_{O2} (dissolved oxygen) nearing zero, proving this cutaneous breathing mechanism to be very effective (Hutchison et al., 1976).

Amphibians living at high altitudes usually have a short breeding season (Beebee and Griffith, 2005). For *T. culeus*, breeding is believed to occur year round, leading to the perpetual presence of a limited number of nests (Schimmel, 2015). Furthermore, the clutch size of this frog species is small compared to other frogs and consists of 80 to 500 eggs (Barrionuevo and Mangione, 2006). These eggs are typically deposited in shallow waters near the coastline (Stuart et al., 2008; IUCN, 2017). Interestingly males tend to guard the eggs until hatching (personal comment Arturo Muñoz-Saravia). Furthermore, the cool temperatures at these altitudes cause slow larval development, meaning a long tadpole phase (which can be several months) and large size at metamorphosis (Beebe and Griffith, 2005; Genova, 2011). The actual length of the tadpole phase of the Titicaca water frog in the wild is not known. In captivity, this phase can last anywhere from two months up to a year (personal comment Arturo Muñoz-Saravia). In addition, a long time is needed before sexual maturity is reached. All these factors combined result in a slow reproductive rate (Beebe and Griffith, 2005; Genova, 2011). The generation length of *T. culeus* is presumed to be five years (IUCN, 2017).

2.3. DIET

Information regarding the diet of *Telmatobius culeus* is limited and typically based on short-term research (Muñoz-Saravia et al., 2018a, unpublished data). In any case, *Telmatobius culeus* is assumed to mostly consume macro-invertebrates. Inspection of these frog's stomach contents, conducted by Allen (1922), revealed; amphipods (small crustaceans), snails, aquatic insects, tadpoles, and fish. No terrestrial insects were found to be ingested (Allen, 1922).

Recently, a more thorough study of *T. culeus*' feeding ecology was conducted by Muñoz-Saravia et al. (2018a, unpublished data), generating additional insights on diet composition and prey selection of the Titicaca water frog. In this study, gut contents of 44 *T. culeus* frogs were analyzed and eight prey species were identified. Overall, seven macro-invertebrate and one fish species were found. An overview of these prey items can be found in Table 2, together with the total number, mass and index of relative importance based on mass (IRI m) for each ingested prey item, as found in Muñoz-Saravia et al. (2018a, unpublished data) (note: a distinction is made between larval and adult *Austrelmis s.l.*). These authors believe some prey item consumption may be underreported by applying this gut content analysis method, as soft-bodied prey (like *Helobdella s.l.* and *Girardia* sp.) could have been digested prior to sampling (Muñoz-Saravia et al., 2018a, unpublished data). Additionally, Muñoz-Saravia et al. (2018a, unpublished data) report the visual observation of the consumption of *Orestias cf. luteos, Orestias cf. olivaceus* and smaller individuals of *Telmatobius culeus* (cannibalism) by the Titicaca water frog.

Tabel 2. Frequency, total number (Total No.), mass (expressed in grams of dry matter) and index of relative importance (based on mass: IRI m) of prey items ingested by *T. culeus* as reported by Muñoz-Saravia et al. (2018a, unpublished data), together with the proportional IRI m, expressed in %.

Prey	**Frequency**	**Total No.**	**Mass (g)**	**IRI m**	**IRI m (%)**
Austrelmis s.l.	3 (9.38)	3 (0.55)	0.003	6.13	0.062
Austrelmis s.l. larvae	4 (12.50)	10 (1.84)	0.007	25.78	0.259
Anisanculus sp.	2 (6.25)	3 (0.55)	0.014	6.29	0.063
Chironomidae	2 (6.25)	2 (0.37)	0.000	2.31	0.023
Hyalella spp.	23 (71.88)	308 (56.62)	0.974	6353.90	63.955
Heleobia sp.	15 (46.88)	179 (32.90)	0.732	2662.19	26.796
Orestias ispi	2 (6.25)	2 (0.37)	0.884	182.03	1.832
Biomphalaria sp.	11 (34.38)	31 (5.70)	0.438	686.14	6.906
Trichoptera	2 (6.25)	5 (0.92)	0.022	10.17	0.102

Per Muñoz-Saravia et al. (2018a, unpublished data), main prey items consumed by the Titicaca water frog are amphipods (*Hyalella* spp.) and snails (*Heleobia* sp. and *Biomphalaria* sp.), representing 89.7 % of all prey consumed (based on count). Amphipods, the most abundant prey species available for *T. culeus* was also the most frequently consumed. Yet, frogs seem to actively select *Heleobia* sp. out of all available food items, as other relatively abundant prey items were comparatively less consumed.

This finding suggests *T. culeus* might not to be an opportunistic feeder, as is currently believed to be the case.

Further findings of this study indicate a shift in prey consumption according to the body size (and age) of the frogs. Smaller (post-metamorphic and juvenile) frogs would predominantly feed on amphipods, while gradually increasing consumption diversity with increasing body size. Hence, larger frogs would also consume larger quantities of snails and even add fish to their diet, as was stated above (Muñoz et al., 2018a, unpublished data).

2.4. CONSERVATION STATUS

Since the beginning of the 21st century, severe population declines have been reported for several *Telmatobius* species, including *Telmatobius culeus* (Angulo, 2008). It is estimated that populations of this frog species have shrunk more than 80 % over the last three generations (IUCN, 2017). Consequently, in 2004, the Titicaca water frog was added to the IUCN Red List and currently is labeled as critically endangered (IUCN Red List version 3.1) (De La Riva and Reichle, 2014; IUCN, 2017). Nowadays, *Telmatobius culeus* is also included in the Red Book of Bolivian Vertebrates, a book portraying the endangered vertebrates of the region (De La Riva, 2005).

Threats of major concern to the survival of *Telmatobius culeus* can be divided into six categories:

1) Overharvesting of adult frogs for human consumption and use in medical products (De La Riva and Reichle, 2014; IUCN, 2017); Although frog legs are hardly served anymore, frog meat used to be a common dish in restaurants around the Titicaca Lake (Reichle, 2006). These days, the Titicaca water frog is more frequently used in traditional medicinal practices. For example, these frogs are used to make a drink called ‘extracto de rana’, a concoction that is believed to have curing effects and improve general health (Genova, 2011). Titicaca water frogs are also blended into “frog juice” or “rana y maca”, a drink that is supposed to have aphrodisiac properties. Many other traditional medicinal uses exist for *Telmatobius culeus* (Amphibianark, 2010).

2) Habitat destruction; Water extraction from the Titicaca Lake and human activities close to shore cause loss of reproductive habitat for these frogs (De La Riva, 2005; Reichle, 2006; De La Riva and Reichle, 2014; IUCN, 2017).

3) Domestic and agricultural water pollution (De La Riva, 2005; De La Riva and Reichle, 2014; IUCN, 2017); As these frogs are fully aquatic, alterations in water quality is of great concern for the wellbeing of the *T. culeus* population (Schimmel, 2015).

4) Climatic change; For example: increasing water temperatures (De La Riva and Reichle, 2014; IUCN, 2017).

5) Presumed predation of larvae by nonindigenous fish (De La Riva and Reichle, 2014; IUCN, 2017); The presence of rainbow trout (*Oncorhynchus mykiss*), introduced in the Titicaca Lake in 1939, is believed to be one of the main reasons for the frog’s declines (Genova, 2011).

6) Disease; Chytridiomycosis, a deadly fungal infection in amphibians, may additionally pose a substantial threat to the survival of *T. culeus* (IUCN, 2017). Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd), is believed to be one of the major causes of the world wide amphibian extinction crisis (Stuart et al., 2004). Although unclear if this disease played any role in the decline of the Titicaca water frog, Barrionuevo and Mangione (2006) argue that, for several reasons, the genus *Telmatobius* would be particularly vulnerable for Bd infections in the future. Recent research has also indicated that Bd is widely present in various Andean habitats, affecting a wide range of other frog species (De La Riva and Reichle, 2014). Additionally, a recent survey, sampling the feet of various water bird carcasses in museum collections, confirmed the presence of this fungus in the Titicaca Lake

(Burrowes and De La Rive, 2017). On top of that, the first case of chytridiomycosis in the Titicaca Lake has already been reported (BAI, 2015a).

Summary:

1) Overharvesting of adult frogs for human consumption and use in medical products.
2) Habitat destruction.
3) Domestic and agricultural water pollution.
4) Climatic change.
5) Presumed predation of larvae by nonindigenous.
6) Disease.

Although small die-offs of *T. culeus* have occurred in prior years in the Titicaca Lake, recently, several episodes of mass deaths have been recorded. In April 2015, thousands of dead frogs were discovered on the shores of Lago Menor in Bolivia (BAI, 2015a; Stiftung-Artenschutz, 2015) and more recently, in 2016, approximately 10,000 dead *T. culeus* were found on the Peruvian side of Lake Titicaca (Collyns, 2016). In both cases, fluctuations in water quality as a result of pollution was suspected to be the culprit (BAI, 2015a; Collyns, 2016).

So far, several conservation efforts regarding the Titicaca water frog have been initiated, including *in situ* and *ex situ* conservation measures (BAI, 2015a; Stiftung-Artenschutz, 2015). Two captive breeding programs have been set up, counting one in Peru (Huachipa Zoo, Lima) and one in Bolivia (Museo de Historia Natural Alcide d'Orbigny, Cochabamba) (De La Riva, 2005; AmphibiaWeb, 2018), where, at the last, in 2012, the first captive individuals were bred (AmphibiaWeb, 2018). These programs aim to establish a steady captive population that ensures species survival and, if necessary, could reintroduce individuals into the wild (BAI, 2015a). Additionally, at the end of 2015, some individuals were sent to the Denver Zoo as part of a new conservation project (Pappas, 2016). Other conservation efforts include; investigation of recent die-offs, research towards pollution, water quality and disease in the Titicaca Lake, monitoring of frog populations in different part of the lake, improving and expanding existing CBP's and educational awareness programs regarding the conservation of amphibians (BAI, 2015a).

Sidenote:
This particular research has been conducted in cooperation with the Bolivian Amphibian Initiative (BAI) and its captive breathing efforts in the Museo de Historia Natural Alcide d'Orbigny in Cochabamba, with the aim to optimize husbandry and health for the frogs in this CBP as well as frogs held in similar programs.

3. THE DIGESTIVE TRACT OF AMPHIBIANS

The digestive tract or gastro-intestinal tract (GIT) of amphibians follows the general anatomical composition as seen in mammals, with a few exceptions (Latney and Clayton, 2014). Overall, the GIT of amphibians can be described as "simple", as it is short and lacking a cecum (Hadfield et al., 2006; McWilliams, 2008). Just like in mammals, this organ system can be divided into two major components; a digestive tube, where food is digested and nutrients are absorbed, and digestive glands, that produce various secretions. Subsequently, the digestive tube can be divided into several specialized areas namely; the oral cavity, the pharynx, the esophagus, the stomach, the small and the large intestine and finally the cloaca. The digestive glands are composed of uni- and multicellular structures in the wall of the digestive tube, together with two major secretory organs; the liver and the pancreas. Morphologically, the digestive tract of most amphibians is quite similar, although some structural differences can be observed (for example: the digestive tract of frogs tends to be shorter) (Vitt and

Caldwell, 2013).

The mouth of amphibians opens directly into a large and wide oral cavity (Clayton, 2005; Vitt and Caldwell, 2013; Latney and Clayton, 2014; Hedley, 2016). This facilitates prey capture (Latney and Clayton, 2014), as most prey is caught and swallowed whole (Wright, 2001; Hadfield et al., 2006; McWilliams, 2008). Amphibians possess poorly developed lips (Latney and Clayton, 2014) which are generally immobile (Vitt and Caldwell, 2013) and a choana that varies in structure among species (Clayton, 2005; Latney and Clayton, 2014). Homodont and polyphodont teeth are present in most amphibians and are generally used to hold on to prey instead of for chewing (Hadfield et al., 2006; McWilliams, 2008; Hedley, 2016). In frogs, teeth can be absent in the bottom jaw, and some species even lack teeth in the upper jaw. The primary palate forms the roof of the oral cavity and the tongue forms the floor (Vitt and Caldwell, 2013). Amphibian tongues show a large morphological variety (Vitt and Caldwell, 2013; Hedley, 2016) and are often quite specialized. A lot of amphibian species use this specialized muscular organ to actively hunt prey (Clayton, 2005; Latney and Clayton, 2014). This is especially true for terrestrial amphibians who commonly use their sticky, mucus-secreting tong, which can significantly be protracted, to capture prey items (Clayton, 2005; Hadfield et al., 2006; McWilliams, 2008; Latney and Clayton, 2014). Contrasting, aquatic amphibian species have less developed tongues and some species even lack this organ (for example: *Pipidae*: Clawed Frogs) (Clayton, 2005; McWilliams, 2008). Such species do not rely on their tongues for prey capture, instead, these animals create negative pressure by opening their mouth (‘buccal pump mechanism’), which sucks in water together with any nearby prey items (Clayton, 2005; Hadfield et al., 2006; McWilliams, 2008). Amphibians can additionally make use of their forelimbs to push food into their mouths. Lastly, the tongue of amphibians houses taste buds capable of tasting bitter, salty, sour and sweet (McWilliams, 2008).

Various uni- and multicellular glands are present in the lining of the oral cavity. These glands primarily produce a lubricating mucus that facilitates the passage of food. Terrestrial amphibians generally possess a greater number of these glands in comparison to aquatic species. Similar glands with similar functions reside in the wall of the rest of the digestive system. An intermaxillary gland, capable of producing a sticky substance, can additionally be present in the oral cavity (Vitt and Caldwell, 2013). Noteworthy; none of the oral secretions contribute to the digestion of food (Clayton, 2005; Latney and Clayton, 2014; Hedley, 2016).

When ingesta is swallowed, it passes the pharynx into a short and wide esophagus. The esophagus is delimited from the pharynx, cranially, and the stomach, caudally, by two muscular sphincters (Wright, 2001; Clayton, 2005; Vitt and Caldwell, 2013; Latney and Clayton, 2014). Peristaltic esophageal contraction, in coordination with ciliary movement and the presence of mucus, move the food bolus caudally towards the stomach (Clayton, 2005; Hadfield et al., 2006; Latney and Clayton, 2014). On top of the above mentioned mucus secreting glands, the esophagus of amphibians often possesses additional pepsinogen-secreting glands. In some species, esophageal pepsinogen production is even higher than that of the gastric mucosa. However, this pro-enzyme is not activated until the stomach is reached. Activation is prevented by the alkaline climate present in the esophagus due to the mucoid oral and esophageal secretions (Clayton, 2005; Latney and Clayton, 2014).

The stomach of amphibians is a muscular, thick-walled organ (Vitt and Caldwell, 2013) located on the left side of the body cavity. Histologically, the wall of the stomach can be divided into the *tunica muscularis*, the submucosa and the mucosa. The inner layer, the glandular gastric mucosa, produces hydrochloric acid, among other secretions (like pepsinogen) (Clayton, 2005; Latney and Clayton, 2014). This acid is secreted into the stomach lumen resulting in an acidic microclimate (low pH), allowing for the conversion of pepsinogen into pepsins. These pepsins are enzymes that play a key role in the breakdown of proteins into peptides and amino acids (McWilliams, 2008). Gastric contractions further help the breakdown of the ingesta as well as mix and move food along in the stomach. Stomach contractions can be divided into pyloric and fundic contractions respectively and occur in regular patterns (Clayton, 2005; Latney and Clayton, 2014).

After passing through the stomach, food enters the small intestine. Passage into this tubular organ is controlled by a muscular valve; the pyloric sphincter (Wright, 2001; Clayton, 2005; Vitt and Caldwell, 2013; Latney and Clayton, 2014). Overall, the small intestine of *amphibia* is structurally less complex than that of mammals. Consequently, the different anatomical regions (duodenum, jejunum, and ileum) are harder to distinguish (Clayton, 2005). The small intestine is the organ where the largest proportion of the digestion occurs. In the duodenum, the most cranial part of the small intestine, food is mixed with digestive juices produced in the liver and pancreas (Clayton, 2005; Vitt and Caldwell, 2013). The liver, the largest of these two major digestive glands produces bile (gall). After production, bile is stored in the gallbladder before being discharged into the duodenum through the bile duct (Vitt and Caldwell, 2013). The presence of an acidic mix in the duodenal lumen, originating from the stomach, stimulates this release (Clayton, 2005; Latney and Clayton, 2014). Bile, consisting of bile salts, helps the enzymatic breakdown of fats by organizing fat aggregations into smaller micelles (Clayton, 2005; McWilliams, 2008; Latney and Clayton, 2014). The pancreas, located between the duodenum and the stomach, also produces digestive juices (Wright, 2001; Clayton, 2005). Usually, one or two ducts drain from the pancreas into the small intestine (Clayton, 2005). Pancreatic juices contain a variety of enzymes which break down the ingesta into nutrients, for example; lipase breaks down fats and glycerides into fatty acids and glycerol, amylase cuts carbohydrates into simple sugars and trypsin furthers the breakdown of protein (McWilliams, 2008). Other enzymes, like chitinases (that break down chitin, a common component of invertebrates' exoskeletons) have been identified in the GIT of some amphibians (Oshima et al., 2002). The release of these enzymes from the pancreas is, just like bile, stimulated by the acidic content of the duodenal lumen. Next to digestion, active nutrient absorption additionally takes place in the small intestine (Clayton, 2005). This process is maximized by the presence of mucosal folds and villi that significantly increase the intestine's absorptive surface area (Vitt and Caldwell, 2013). Nevertheless, these folds and villi are less pronounced compared to mammalian intestine (Clayton, 2005).

Peristaltic contractions drive the intestinal content towards the large intestine (Clayton, 2005). The large intestine can usually be distinguished by its wide diameter in comparison to the small intestine and, in some frog species, these two structures are partitioned by the presence of a valve (Clayton, 2005; Vitt and Caldwell, 2013; Latney and Clayton, 2014). No significant digestion is believed to occur in the large intestine. However, this organ is responsible for most of the water and mineral absorption in the GIT (Clayton, 2005).

Finally, the intestinal content reaches the cloaca, which for amphibians, like in birds and reptiles, is the final part of the digestive tract. In this sac-like cavity, contents of the digestive tract, urinary tract, as well as the genital tract debouch before being excreted (Clayton, 2005; Vitt and Caldwell, 2013).

4. METABOLISM

Overall, knowledge regarding amphibian metabolism is still quite deficient (McWilliams, 2008). Energy requirements for this class of animals will commonly vary between species and life stage. *Anurans* tend to have higher metabolic rates than salamanders, and terrestrial species generally have higher metabolic rates as compared to aquatic species (Wells, 2010; Ferrie et al., 2014).

Amphibians, just like reptiles, are ectothermic, meaning that these animals rely on external heat sources in order to regulate body temperature, rather than on their own metabolism. Consequently, amphibians exhibit a broad range of body temperatures, depending on the environmental conditions (Donoghue, 1998). Furthermore, this leads to significantly lower energy requirements as compared to endotherms (Latney and Clayton, 2014). Overall, amphibians have the lowest resting metabolic rate and the lowest maintenance energy requirement of any terrestrial vertebrate (Wells, 2010). Commonly, the metabolic rate of amphibians is about 1:4 of that in mammals (Donoghue, 1998). Latney and Clayton, (2014) state the energy requirements of amphibians to be approximately 1:10 of those of endotherms. Additionally, external temperatures will directly affect amphibian’s activity, energy needs and their metabolic rate (Donoghue, 1998). Metabolic rates will, in most cases, increase correspondingly to the surrounding temperature (Browne, 2009; Ferrie et al., 2014), as will feeding and digestion (Browne, 2009).

Generally, standard equations are used to calculate metabolic rates, and thus estimating daily calorie requirements. Such a formula has been developed for amphibians: “$33*(\text{weight in kg})^{.75}$” (Donoghue, 1998; McWilliams, 2008). As for all other animals, metabolic rates are calculated based on metabolic body size (Donoghue, 1998; Latney and Clayton, (2014). This concept dictates that the smaller the animal, the higher the metabolic rate per unit of body weight will be (Figure 2). On the other hand, some authors believe that the formula stated above estimates metabolizable energy (ME) instead of the metabolic rate, in amphibians (McWilliams, 2008). Metabolizable energy is the amount of net energy remaining after the energy costs for digestion and absorption have been deducted from the total energy value of a meal, and is measured in kilocalories per kilogram (kcal/kg). Additionally, ME can be calculated based on values obtained through proximate nutrition analysis of a meal, using the following equation: *“[(4 x gram of crude protein) + (9 x gram of crude fat) + (4 x gram of nitrogen-free extract)]”* (Latney and Clayton, 2014).

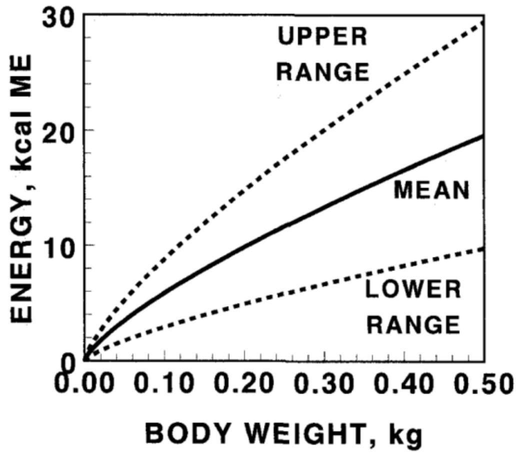


Figure 2. Approximate daily intakes of metabolizable energy (ME, kcal) of amphibians [standard metabolic rate = $33(\text{weight in kg})^{.75}$], as found in Donoghue (1998).

Standard energy intake rates for amphibians have been estimated based on clinical trials, although these rates are believed to be quite variable over time. Long term variations in energy intake, and thus energy requirements, are reflected in some annual cycles with periods of hyperphagia followed by periods of aphagia (Ferrie et al., 2014). For example, a lot of amphibian species hibernate or estivate, considerably lowering their metabolic rate during this period. Furthermore, daily cycles in energy requirement can also be observed, with nocturnal animals exhibiting higher metabolic rates at night and diurnal species having higher metabolic rates during the day. These short-term variations are primarily linked to variations in the amount of activity of the animal (Wells, 2010). Typically, amphibians will consume more food while in an active state and consume less or even stop eating when inactive (McWilliams, 2008). Metabolic rates of amphibians correspondingly tend to go up following the ingestion of food. This phenomenon is caused by the energy required for digestion and is called “specific dynamic action” (Wells, 2010).

5. NUTRITION

Overall, formulating general nutritional requirements for amphibians has proven to be quite challenging, predominantly due to the sheer diversity this class of animals holds (Ferrie et al., 2014). Tackling this task would require extensive field research on the nutritive values of wild amphibian’s diets (Latney and

Clayton, 2014), which is currently lacking for most species. To this day, this lack of data has led amphibian nutritional requirements to be mostly unknown, and thus represent a big research gap (Hadfield et al., 2006; Oonincx and Dierenfeld, 2012; Ferrie et al., 2014). However, relative macronutrient requirements are believed to be fairly consistent among amphibians, regardless of the specific diet of a particular species (Donoghue, 1998). In general, these animals will take in high amounts of protein and fat, and low levels of carbohydrates (Donoghue, 1998; Hadfield et al., 2006). Contrasting, as a result of possible physiological adaptations to specific environmental factors, amphibian micronutrient (minerals and vitamins) requirements are suggested to be quite variable among species. These adaptations may result in large variations in micronutrient absorption, usage and storage efficiency in amphibians (McWilliams, 2008). Furthermore, practical experience has proven the importance of micronutrients in amphibian husbandry. Mineral and vitamin deficiencies are common causes of pathology in captive amphibians, indicating the need for further research (McWilliams, 2008; Latney and Clayton, 2014). Overall, amphibian dietary micronutrient requirements include a positive calcium-phosphorus (Ca:P) ratio and sufficient amount of vitamins A, B1, D3 and E (Hadfield et al., 2006). The importance of many other micronutrients is still unclear (Browne, 2009).

When formulating nutrient recommendations for species where nutrient requirements are unknown, one must consult published data on “related species”. These related species can be comparable in environment, life stage, metabolism or feeding habits (Ferrie et al., 2014). For example, based on the similarities between these two classes of animals, nutritional needs of amphibians are often extrapolated from research done on reptiles (Attard, 2013; Latney and Clayton, 2014). Additionally, the National Research Council (NRC), which provides open source data on the nutrient requirements for numerous animal species (Latney and Clayton, 2014), suggests other model species for amphibians (Ferrie et al., 2014). Cats and dogs can function as models for terrestrial carnivorous and omnivorous amphibians, fish for aquatic carnivorous and omnivorous amphibians and rats for basic omnivorous amphibians. Poultry may serve as a classic model for the nutritional needs of egg laying animals (Ferrie et al., 2014). Ferrie et al. (2014) joined all these recommendations to form a basic guideline for amphibian nutrition requirements (Table 3). This guideline, in combination with nutritional profiles of feeder insects, can then be used to formulate basic diets for amphibians, taking into account species variability. Ferrie et al. (2014) do stress that the values presented in Table 3 are, most likely, suitable amounts and thus do not represent absolute minimum requirements. True minimum nutritional requirements can only be determined through empirical research (Ferrie et al., 2014) and, studies on herptile nutrient minimum requirements are scarce (Latney and Clayton, 2014). This being said, nutritional recommendations for amphibians based on the requirements of endotherms should be interpreted with care, as these are most likely an understatement. Since ectotherms have lower energy requirements, thus taking in less food compared to endotherms, diets for amphibians should be nutrient denser; meaning these diets should have higher nutrient densities per unit of energy (Finke, 2002).

5.1. PROTEIN

As most amphibians are carnivorous (insectivores), with diets predominantly consisting of invertebrates, these animals tend to have high protein requirements. Generally, amphibian dietary protein contents will vary between 30 % and 60 % ME (Donoghue, 1998; McWilliams, 2008). This means that dietary protein will represent 30-60 % of the daily calorie intake of these animals (McWilliams, 2008), thus proving a valuable energy source. Protein generates approximately 16.7 kJ ME/g (Barker et al., 1998; Donoghue, 1998).

Table 3. Preliminary recommendations for nutrient intake of amphibians post-metamorphosis, based on NRC recommendations for dogs and cats, fish, poultry, and rats, as found in Ferrie et al. (2014).

	Amphibian recommendation (adult)[a]		Nutrient content of typical commercial feeder insects[b]					
Nutrient		Primary NRC model species	Adult crickets	Roaches	House flies	Mealworm larvae	Super-worms	Soldier fly larvae
Crude protein (%)[c]	44.4	Fish	58.5	47.4	85.8	36.4	32.5	35.1
Arginine (%)	2.6	Fish	3.6	3.5	5.3	1.9	1.6	2.5
Glycine (%)[d]	0.9	Poultry	3.0	3.1	3.7	2.0	1.6	1.8
Histidine (%)	0.8	Fish	1.4	1.4	2.5	1.2	1.0	1.2
Isoleucine (%)	1.2	Fish	2.7	1.9	3.5	1.8	1.5	1.5
Leucine (%)	1.9	Fish	5.9	3.0	5.4	3.9	3.2	2.4
Lysine (%)	2.6	Fish	3.1	3.2	5.5	2.0	1.7	2.4
Methionine (%)	0.8	Fish	0.9	0.8	2.5	0.5	0.3	0.7
Met + cysteine (%)	1.3	Fish	1.3	1.2	3.1	0.8	0.6	0.9
Phenylalanine (%)	1.1	Fish	1.9	1.9	3.4	1.3	1.1	1.5
Phe-tyrosine (%)	2.2	Fish	4.8	5.5	7.5	4.0	3.4	3.9
Threonine (%)	1.3	Fish	2.1	2.0	3.3	1.5	1.3	1.4
Tryptophan (%)	0.4	Fish	0.4	0.4	1.0	0.3	0.3	0.6
Valine (%)	1.4	Fish	3.1	3.1	4.8	2.1	1.7	2.6
Taurine (%)[e]	0.1	Cat	0.4	0.0	0.7	0.2	0.0	0.0
Crude fat (%)[f]			19.4	25.0	8.3	26.1	29.2	28.1
Linoleic acid (%)	[g]		6.5	5.4	1.8	6.8	5.4	3.4
Linolenic acid (%)	[g]		0.2	0.2	0.2	0.3	0.2	0.1
Arachidonic acid (%)	[g]		0.06	0.10	0.02	0.0	0.0	0.0
Calcium (%)	0.6	Rat	0.1	0.1	0.3	0.0	0.0	1.9
Phosphorus (%)	0.3	Rat	0.8	0.4	1.6	0.6	0.4	0.0
Sodium (%)	0.2	Cat	0.4	0.2	0.6	0.1	0.1	0.0
Magnesium (%)	0.04	Cat	0.09	0.06	0.35	0.16	0.08	0.00
Potassium (%)	0.4	Cat	1.0	0.6	1.3	0.7	0.5	0.0
Chloride (%)	0.1	Cat	0.7	0.4	0.8	0.4	0.3	0.2
Copper (ppm)	12	Dog	17.7	19.7	56.2	11.9	5.9	8.0
Iodine (ppm)	1	Fish	0.6	0.7	0.0	0.3	0.0	0.5
Iron (ppm)[h]	97	Poultry	55.1	37.0	544.7	40.1	27.2	133.6
Manganese (ppm)	14	Fish	32.8	6.5	115.9	10.1	7.1	124.0
Selenium (ppm)[i]	0.3	Cat	0.5	0.7	6.5	0.5	0.2	0.6
Zinc (ppm)	18	Fish	191.4	81.6	373.9	101.2	50.7	112.7
Ascorbic acid (ppm)[j]	23	Fish	85.6	0.0	0.0	23.3	19.8	0.0
Biotin (ppm)	1	Fish	0.5	0.9	3.0	0.6	0.6	0.7
Choline (ppm)	1,889	Dog	4,334	2,017	2,471	3,588	2,866	2,207
Folic acid (ppm)	1	Fish	4.3	2.8	7.9	3.1	1.1	5.4
Niacin (ppm)	44	Cat	109.6	109.4	394.3	79.2	53.3	142.4
Pantothenate (ppm)	23	Fish	65.6	92.4	194.4	51.0	32.0	77.2
Pyridoxine (ppm)	7	Fish	6.6	7.7	7.4	15.8	5.3	12.0
Riboflavin (ppm)	8	Fish	97.3	39.0	336.4	15.8	12.4	32.5
Thiamin (ppm)	12	Fish	1.1	2.2	49.2	4.7	1.0	15.4
Vitamin B_{12} (μg/kg)	39	Dog	153.2	591.8	26.1	9.1	6.9	111.9
Vitamin A (IU/kg)[k]	2,914	Fish	0.0	0.0	0.0	0.0	0.0	0.0
Vitamin D3 (IU/kg)[l]	1,111	Rat	0.0	482	434	0.0	0.0	200
Vitamin E (IU/kg)[m]	88	Fish	56.2	0.0	192.6	0.0	12.7	18.5
Vitamin K (ppm)	2	Dog	NA[n]	NA	NA	NA	NA	NA

”[a]All recommendations are based on a diet containing 16736 kJ digestible energy/kg, and on a dry matter basis (DMB). These recommendations were created by tabulating the recommendations for the species noted above, and then (1) using the recommendation for the most appropriate species if apparent, (2) using the median recommended intake if no species model was notably appropriate, (3) using the highest or lowest recommended intake of all species if deficiency or toxicity were a concern. [b]All nutrient values are standardized to 16736 kJ digestible energy/kg, and on a dry matter basis (DMB). [c]Higher protein intakes associated with reduced developmental abnormalities (Martinez et al., 1994; Venesky et al., 2012; Martins et al., 2013). [d]Essential for uric acid production. [e]Limited data on essentiality of taurine in amphibians. [f]Excess dietary fat may result in food intake limitations, which may reduce intake of other nutrients. [g]Recommendations for linoleic, linolenic, arachidonic acid, EPA, and DHA are not made at this time due to substantial variability in the recommendations for other species, but are likely all essential. Additionally, levels of some fatty acids in insects may be modified due to their diet composition during growth and development. [h]Nucleated red blood cells. [i]Risk of toxicity. [j]Ability to synthesize not documented in all amphibians. [k]Appropriate form and source of vitamin A still being examined. Coated preformed vitamin A may not be available for consumption by insects due to particle size (Attard, 2013). [l]UV-B exposure has been shown to impact bone mineralization [Verschooren et al., 2011], and amphibians have capacity for endogenous synthesis of vitamin D3 [Holick, 1995; Michaels et al., 2014]. [m]Recommendation proportional to omega-3 content of diet. [n]NA, not analyzed.”

Apart from an energy source, dietary protein is commonly evaluated by its amino acid composition (Barker et al., 1998; Latney and Clayton, 2014). Yet, essential amino acid requirements for amphibians are still unknown (Oonincx and Dierenfeld, 2012) and amino acid profiles of wild invertebrates are deficient (Ferrie et al., 2014). Additionally, full amino acid profiles of many of the feeder insects have never been presented (Barker et al., 1998; Oonincx and Dierenfeld, 2012; Latney and Clayton, 2014). However, methionine and cystine seem to be the first limiting amino acids in captive invertebrate diets (Finke, 2002; 2003; 2013; Latney and Clayton, 2014; Finke, 2015). In general, the total crude protein value of most feeder insects exceeds the NRC's nutritional minimum requirements for rats and domestic carnivores. For this reason, feeder insects should provide an adequate source of protein for amphibians (Oonincx and Dierenfeld, 2012; Latney and Clayton, 2014). Granting, this statement should be interpreted with caution as data on digestibility and bioavailability of insect protein is limited. Not all protein, such as the protein content of the exoskeleton of invertebrates, may be readily available for most amphibians (Latney and Clayton, 2014).

5.2. FAT

Fat or lipids are, quantitatively and qualitatively, important components of amphibian diets (Browne, 2009). Fat is, just like protein, used as an energy source and will provide around 37.7 kJ ME/g (Barker et al., 1998; Donoghue, 1998; McWilliams, 2008). The fat content of an insectivore diet will approximately range between 40 % to 70 % ME (Donoghue, 1998; McWilliams, 2008) or 10 % to 30 % on a fresh weight basis (Browne, 2009). In addition, dietary fat plays an important role in the absorption of fat-soluble vitamins and provides essential fatty acids (Latney and Clayton, 2014). As the dietary requirements for a lot of essential fatty acids are rather variable among species, specific amphibian dietary requirements have yet to be formulated (Oonincx and Dierenfeld, 2012; Ferrie et al., 2014). Nonetheless, amphibian diets should have balanced amounts of saturated and unsaturated fatty acids (Browne, 2009). Generally, insects are high in the essential C18 fatty acids; oleic acid (18: 1), linoleic acid (18:2) and linolenic acid (18:3) (Finke, 2002; Browne, 2009; Finke, 2013), even though studies on insect fatty acid composition are scarce (Latney and Clayton, 2014).

Commonly, crude fat values of commercial insect diets exceed the optimal fat requirement for rats and domestic carnivores (Oonincx and Dierenfeld, 2012; Latney and Clayton, 2014). This should be kept in mind (in the context of captive amphibian nutrition) as excess amounts of fat in the diet may lead to obesity or imbalance in other nutrients due to caloric dilution (Browne, 2009).

5.3. CARBOHYDRATES

Insectivore diets will generally contain a small proportion of carbohydrates (all types). For this reason, dietary carbohydrates are assumed to play a minimal role in the energy provision of amphibians (McWilliams, 2008). Therefore, rates of gluconeogenesis are believed to be relatively high in these animals (Donoghue, 1998).

For short-term nutritional support, carbohydrate and fiber contents should not exceed 5 % in amphibian diets (Hadfield et al., 2006). Additionally, diets containing excessive amounts of carbohydrates have been shown to lead to intestinal blockage in captive individuals (McWilliams, 2008). Next, little to no fermentation is believed to occur in the GIT of amphibians (Donoghue, 1998). Regardless, the effects of digestible carbohydrates in these animal's diets are unknown (McWilliams, 2008).

5.4. MINERALS

Available information on amphibian dietary mineral requirements is often limited to calcium. However, as mentioned above, amphibian mineral requirements can vary heavily among species, thus so can those for calcium (McWilliams, 2008). In rats, the model species for basic omnivorous amphibians, the maintenance requirement for calcium is 5 g/kg. Most feeder insects do not come close to this value, suggesting the need for calcium supplementation in captive amphibian diets (Finke, 2002; 2003; Oonincx and Dierenfeld, 2012; Finke, 2013; Latney and Clayton, 2014).

While absolute mineral requirements are variable, calcium (Ca) and phosphorus (P) requirements do exhibit a fixed optimal ratio. The optimal Ca:P ratio for vertebrate insectivore diets should be 1.5:1 (Bernard et al., 1997; McWilliams, 2008). In rats, the minimal Ca:P ratio is 1.66 (Latney and Clayton, 2014).

In contrast to calcium, phosphorus requirements of amphibians are easily reached with an insect based diet. Nevertheless, insect prey items will often have an inverse Ca:P ratio, further emphasizing the need for calcium supplementation for captive amphibians (Finke, 2002; 2003; Oonincx and Dierenfeld, 2012; Latney and Clayton, 2014). Insects will contain around 1.5 to 3.7 g/kg of phosphorus, exceeding the minimal requirement for rats: 3 g/kg (Latney and Clayton, 2014).

Finally, data regarding the amphibian dietary requirements of other minerals as well as trace elements is lacking. However, most feeder insects are believed to have suitable levels of copper, iron, magnesium, zinc, sodium, potassium and chloride for amphibians (Barker et al., 1998; Finke, 2002; Oonincx and Dierenfeld, 2012).

Side note:
While this study focusses on dietary nutrient provisions of amphibians, some nutrients, like calcium, can also be absorbed through alternative pathways, for example; through the integument or the gills of amphibians (Stiffler, 1993).

5.5. VITAMINS

Vitamin A is a very versatile vitamin which comes in many different forms, including; retinol, retinal, 3-hydroxyretinal and retinyl esters (Latney and Clayton, 2014). Amphibians are not able to synthesize these molecules, therefore these micronutrients should be provided in their diet (Densmore and Green, 2007; McWilliams, 2008; Browne, 2009; Ogilvy et al., 2012). Research on the nutritional content of feeder insects revealed these to have inadequate amounts of vitamin A for domestic carnivores, leading to believe commercial insect diets to be vitamin A deficient (Barker et al.,1998; Finke, 2002; Oonincx and Dierenfeld, 2012; Finke, 2013). Invertebrates will average around 300 mg/kg or 1000 IU/kg of retinol (Latney and Clayton, 2014). In contrary, considerable amounts of vitamin A can be found in mammalian livers, immature rodent prey and fish oils (Browne, 2009; Li et al., 2009). Active vitamin A can additionally be formed through conversion of the provitamin; beta-carotene (Latney and Clayton, 2014), although it is unclear if these precursors are essential dietary nutrients for amphibians (Densmore and Green, 2007; McWilliams, 2008; Browne, 2009; Li et al., 2009). Some literature does recommend the supplementation of beta-carotene to prevent nutritional disease in captive amphibians (Browne, 2009). Finally, adequate dietary fat contents are required for the absorption of this fat-soluble vitamin (Latney and Clayton, 2014).

Most diets will have more than adequate amounts of vitamin B1 or thiamine. However, thiamine can be deficient in specific diets containing high quantities of thiaminase, an enzyme which breaks down vitamin B1 (Densmore and Green, 2007). High amounts of this enzyme can, for example, be found in some insect species which are therefore thiamine deficient (Finke, 2013). For captive amphibians, diets consisting of whole, frozen fish will often need thiamine supplementation in order to counter thiaminase activity (Wright, 2001).

In amphibians, vitamin D3 or cholecalciferol can be supplied through the diet or can be synthesized in the animal’s skin (Antwis and Browne, 2009; Browne et al., 2009). Vitamin D3 should be present as such in the diet, as amphibians are not able to convert the provitamin; ergocholecalciferol (vitamin D2) into cholecalciferol, contrary to mammals. Research has shown that, in order to prevent nutritional deficiencies in mammals, diets should include 25 mg/kg or 1000 IU/kg of vitamin D3 (Latney and

Clayton, 2014). Many feeder insects do not reach these requirements, therefore, commercial insect diets are thought to be vitamin D3 deficient (Finke, 2002; 2013; Latney and Clayton, 2014). Generally, inclusion of fish in the diet will result in adequate vitamin D provision (Wright, 2001). High amounts of vitamin D3 can, for example, be found in goldfish (McWilliams, 2008; Browne, 2009). On the other hand, in many amphibian species, provitamin D3 (7-dehydrocholesterol) is converted to previtamin D3 by way of a photobiosynthetic process requiring ultraviolet B (UVB) radiation. This previtamin can then be transfigured into cholecalciferol, thus providing an alternative vitamin D3 source. Nontheless, not much is known on the dietary and/or photobiosynthetic vitamin D3 dependency of amphibians as both requirements are quite variable between species (Browne et al., 2009; Latney and Clayton, 2014). For captive amphibians, exposure to UVB radiation resembling the natural conditions of that specific species, in combination with dietary supplementation of cholecalciferol, is recommended (Latney and Clayton, 2014).

Lastly, invertebrate based diets contain adequate amounts of vitamin E or a-tocopherol, meeting the requirements for rats. Most insects have suitable quantities of this vitamin. As vitamin E is a fat-soluble vitamin, availability is dependent on the amount of dietary fat. In diets containing less than 10 % fat, a content of 27 IU/kg or 18 mg/kg is essential (Latney and Clayton, 2014). On the other hand, because this vitamin functions as an antioxidant, and insect diets are high in unsaturated fats, higher levels of vitamin E are recommended for insectivores (Finke, 2002; 2003; Oonincx and Dierenfeld, 2012; Finke, 2013). For this reason, dietary requirements for rats would be an underestimation of the amphibian requirements (Finke, 2002; 2013).

6. FEEDING MANAGEMENT OF ADULT AMPHIBIANS

Depending on the overall size of the individuals of a particular species, amphibians will consume invertebrates, fish, reptiles, other amphibians and even small birds and mammals (Clayton, 2005; Hadfield et al., 2006; McWilliams, 2008). Cannibalism can also come to pass, therefore, in order to prevent this phenomenon, housing amphibians of the same species by general size is recommended (McWilliams, 2008). Overall, amphibians are opportunistic feeders (Browne, 2009) and, for most species, the bulk of their diet will consist of a wide variety of insects (Wright, 2001; Hadfield et al., 2006; Latney and Clayton, 2014).

Captive amphibians should be fed prey items that provide complete nutrition, when given in the right amount. Furthermore, it is important that these prey items are given in a suitable manner and are the appropriate size for the captive individual. Generally, amphibians tend to go after live, moving prey (McWilliams, 2008; Browne, 2009), although, some animals can be fooled by artificially making immobile prey move around (McWilliams, 2008). Healthy amphibians should show immediate interest in live prey. Classic response is triggered by visual stimuli and includes orientation, approach and capture of the prey item (Hadfield et al., 2006). Contrasting, various aquatic species target prey by scent and will also ingest immobile (dead) prey (Browne, 2009). Aquatic amphibians can therefore be fed packaged frozen fish food, and some species have been known to eat pallet food (Wright, 2001).

Amphibians in captivity are usually fed a small variety of prey (Bernard et al., 1997; Finke, 2002; 2003; McWilliams, 2008; Browne, 2009; Oonincx and Dierenfeld, 2012; Attard, 2013; Finke, 2013; Ferrie et al., 2014) and, generally, don’t get fed on a daily basis. When food is provided, these animals will receive a limited number of large prey items, which contrasts with wild amphibian diets, that often consist of 100s to 1000s of smaller prey daily. For this reason, feeding strategies used for captive amphibians may need to be reconsidered (McWilliams, 2008). Furthermore, feeding amphibians a large variety of prey items provides greater nutritional value as invertebrate prey species will contain different amount and types of nutrients (McWilliams, 2008; Browne, 2009; Ferrie et al., 2014). For example, a lot of the worms and beetle larvae used as feeder insects are high in fat, low in amino acids and have an inadequate Ca:P ratio (McWilliams, 2008; Latney and Clayton, 2014). Additionally, insect larvae are high in energy, resulting in caloric dilution. Consequently, a diet consisting primarily of such larvae would be borderline deficient (Bernard et al., 1997; Finke, 2002; Ferrie et al., 2014). Other feeder insects like flies, ants, crickets and locust are usually low in calcium and vitamin A and tend to have an inverse Ca:P ratio. Some examples of nutrient contents of invertebrate prey are found in Table 4. To ensure a fully balanced diet, the deficiencies in invertebrate prey items should be compensated by simultaneously feeding complementary prey. Additionally, feeding a large variety of invertebrate prey will also increase activity and welfare of the animals (McWilliams, 2008). Regardless, this is often easier said than done, as in most cases only a limited amount of feeder insects species will be commercially available in a specific geographical region. For example, research has shown that American and European organizations heavily rely on just a handful of commercially available feeder-insect-species to feed amphibians (Ferrie et al., 2014). Invertebrate prey items most commonly fed to amphibians are; ants (various genera), blackflies (*Musca* spp), blackworms (*Lumbriculus* spp.), bloodworms (*Chironomidae larvae*), brine shrimp (*Artemia* spp.), crayfish (various genera), crickets (*Gryllus* spp., *Acheta* spp.), earthworms (*Lumbricus* spp.), fruit flies (*Drosophila hydei* and *Drosophila melanogaster*), glass shrimp (*Palaemonectes* spp.), locusts (*Melanoptus* spp.), mealworms (*Tenebrio* spp. larvae), silkworms (*Bombyx* spp. larvae), springtails (*Collembola* spp.), superworms (*Zophobas* spp. larvae), tubifex worms (*Tubifex* spp.), water fleas (*Daphnia* spp.), whiteworms (*Enchytraeus* spp.) and waxworms (*Galleria* spp. or *Achroia* spp. larvae). Besides invertebrates, varied species of fish, like guppies (*Poecilia reticulate*), mollies (*Poecilia sphenops*), goldfish (*Carassius auratus*) and smelt (*Osmeridae*) are also fed to captive amphibians. Additionally, mammalian prey items can include adult or newborn rats or mice (McWilliams, 2008).

Tabel 4. Examples of nutrient contents of invertebrate prey, as found in Donoghue (1998).

Food item	**Water %**	**AF**	**DM**	**Prot**	**Fat**	**Carb**	**Calcium**	**Phosph**
Acheta domesticus, Domestic cricket	62.0	1.9	4.8	50.0	44.0	6.0	0.2	2.6
Tenebrio molitor, Mealworm larvae	58.0	2.1	5.0	37.0	60.0	3.0	0.1	1.2
Galleria mellonella, Waxworm larvae	63.0	2.1	5.7	27.0	73.0	0.0	0.1	0.9

Abbreviations: Prot, protein; Carb, carbohydrate; Phosph, phosphorus; AF, as fed (wet) basis; DM, dry matter basis. Values expressed in percentage (%).

While wild insect diets offer amphibians complete nutritional support, balanced captive bred insect diets are often still deficient in some (micro)nutrients (Bernard et al., 1997; McWilliams, 2008; Attard, 2013). Generally, a balance captive bred invertebrate diet will provide adequate fat, protein, amino acids, most B-vitamins and most minerals, except for calcium (Donoghue, 1998; Finke, 2002; 2003; Ferrie et al., 2014). This calcium deficiency results from invertebrates lacking a calcium-rich skeleton, instead, these animals have a chitinous (amino-cellulose) exoskeleton, containing non-protein nitrogen (Donoghue, 1998). Noteworthy: some invertebrates, like the soldier fly larvae (*Hermetia illucens*) and the pillbug or wood louse (*Porcilio scaber*) hold a calcium-rich exoskeleton (Oonincx and Dierenfeld, 2012; Finke, 2013), and thus do contain adequate amounts of calcium for amphibians (Oonincx and Dierenfeld, 2012; Finke, 2013; Latney and Clayton, 2014). For this reason, these would be interesting prey items to include in amphibian diets (Oonincx and Dierenfeld, 2012; Ferrie et al., 2014) although, further research on digestibility of these insect's exoskeleton is needed (Oonincx and Dierenfeld, 2012; Latney and Clayton, 2014). Other nutrient deficiencies seen in balanced captive bred insect diets concern vitamins A, D and E, thiamin, omega-3 fatty acids and carotenoids (Finke, 2002; 2003; Li et al., 2009;

Finke, 2013; Ferrie et al., 2014; Finke, 2015). Addressing these deficiencies is of great importance to supply adequate nutrition to captive amphibians (Browne, 2009). Wild invertebrate prey items obtain these essential micronutrients through their natural diet, resulting in variable concentrations of these nutrients in their tissues. Contrary, commercially bred insects are fed low-cost diets with a focus on growth and reproduction, disregarding the nutrient content of the insects (Finke, 2015). Furthermore, wild invertebrate prey items will provide essential micronutrients (and calcium) through their gut content (Finke, 2002; Browne, 2009; Oonincx and Dierenfeld, 2012; Ferrie et al., 2014) and even material adhering to the exoskeleton of these insects can add to their nutritional value (Finke, 2002; Ferrie et al., 2014). Commercially bred insects, on the other hand, are often starved before being sold (Donoghue, 1998), therefore, these insects should be fed a complete diet before being given to amphibians. This is called "gut loading" and ensures good nutritional value (McWilliams, 2008; Attard, 2013; Ferrie et al., 2014; Latney and Clayton, 2014). Hence, the predator will ingest the nutritive value of the prey item and its gut content (Finke, 2003; Attard, 2013). Gut loading or dietary supplementation of feeder insects is probably the best current method for providing adequate amounts of micronutrients to captive amphibians (Browne, 2009). Store bought insects should customarily be fed fruit and vegetables for about two days prior to feeding out (Donoghue, 1998). Generally, gut loading for 24 to 74 hours will lead to adequate nutrient values in the given insects (Ferrie et al., 2014). Furthermore, insects can be fed a diet containing heightened concentrations of specific nutrients, in order to compensate for present deficiencies. Research has proven that gut loading with a diet containing increased calcium levels can successfully elevate the calcium content of feeder insects (Strzelewicz et al., 1985; Allen and Oftedal, 1989; Anderson, 2000; Finke, 2002; 2003), and so, prevent calcium deficiencies in captive insectivores (Wright, 2001; McWilliams, 2008). This concept can also be applied for other micronutrients, for example; Oonincx and Van der Poel (2011) have shown that adding carrots and wheat bran to the diet of feeder insects will increase their beta-carotene and retinol levels.

Gut loading is effective for most nutrients, as long as the specified diet is taken in by the insects and contains sufficient amounts of the required nutrient(s) (Ferrie et al., 2014). Besides, this practice has successfully been applied within the captive nutritional management of other animal species (Finke, 2003; Ferrie et al., 2014). On the other hand, the nutrients present in the invertebrates' GIT should be accessible and digestible for the predator. In some cases, the invertebrates' exoskeleton prevents access to the gastro-intestinal contents of these prey items (Latney and Clayton, 2014).

Another commonly applied method used to combat nutrient deficiencies resulting from feeding typical commercial insect diets is so called "dusting" (Wright, 2001; Donoghue, 1998; McWilliams, 2008; Latney and Clayton, 2014). Applying this practice, insects are coated with a supplement powder, shortly before being fed out. In this way, the desired nutrients adhere to the insect's exoskeleton (Wright, 2001; Finke, 2003; Ferrie et al., 2014; Latney and Clayton, 2014). For example, feeder insects can be "dusted" with ground calcium carbonate in order to prevent calcium deficiency (McWilliams, 2008). Commonly applied supplement powders are not restricted to calcium, and will contain a range of micronutrients often deficient in feeder insects. Such powders might include: calcium, vitamin A, B1, D3, and E. Frequently used (herpetological) supplement brands by researchers around the globe are; RepCal ®, Dendrocare ®, Rock Solid Herpetoculture ® and VetArk ® (McWilliams, 2008). Alternatively, some ground supplement tablets for human consumption can also be used (Wright, 2001; Browne, 2009).

Powdering of insects seems effective but this method does holds some flaws. First of all, the final dose ingested by the animal is not known as an exact dose size for dusting insects is not described (McWilliams, 2008) and, adherence of the powder to the insects is variable (McWilliams, 2008; Ferrie et al., 2014). The amount of powder adhered to the insect is influenced by the physical characteristics of the insect's exoskeleton and the relative surface area of the given insect (Ferrie et al., 2014).

Secondly, due to insect grooming behavior, time between dusting and the actual ingestion of the insects by the predator is crucial for the effectiveness of this method (Bernard et al., 1997; Wright, 2001; Latney and Clayton, 2014). For example, house crickets can remove up to half of the adhered powder within minutes of dusting (Li et al., 2009). Next, competition between animals may also result in variable supplement consumption (McWilliams, 2008). Low powder intake may therefore still lead to nutrient deficiencies and excess intake may cause nutrient toxicities (Donoghue, 1998). Finally, dusting insects is not a suitable method for the nutritional supplementation of animals living in high humidity environments nor of aquatic species (Li et al., 2009; Ferrie et al., 2014).

Other method for enhancing the nutrient content of feeder insects do exist. For example, elastic-skinned feeder insects can be injected with the desired micronutrients prior to feeding out (Browne, 2009). Another example is to subject commercially bred feeder insects to specific environmental conditions and/or feed them specific diets during their growth period. This results in elevated amounts of specific nutrients within the (adult) insect’s tissues. Application of such method has led to the development of a variety of commercial feeder insect strands with heightened levels of beta-carotene, vitamin E, and omega-3 fatty acids (for example: Vitabugs ®) (Ferrie et al., 2014). A recent study conducted by Finke, (2015) showed enhanced beta-carotene (or related molecules), vitamin E and omega 3 fatty acids concentrations in such nutrient-heightened feeder insects, with vitamin E contents resembling those of wild insects. This study also confirmed that this method can be applied to alter the fatty acid composition of feeder insects, as previously reported in other animals (Finke, 2015).

To end, whole adult vertebrate prey offer more complete and balanced nutrition compared to invertebrates (Donoghue, 1998; McWilliams, 2008) and therefore, rarely need supplementation (Wright, 2001). A bony skeleton will provide adequate amounts of calcium, phosphorus and magnesium to the predator (Donoghue, 1998). Other minerals and vitamins can be obtained from the liver, kidneys, thyroid and the pancreas. Adult vertebrate prey provides high quality protein, and necessary energy is obtained from fat and protein (Donoghue, 1998; McWilliams, 2008).

Contrary to adult vertebrates, neonatal or juvenile vertebrate prey can be deficient in calcium or other micronutrients (Donoghue, 1998; McWilliams, 2008).

Summary:

1) Captive amphibian diets consist mostly of a small variety of commercial feeder insects.
2) Feeding amphibians a large variety of insect species will provide greater nutritional value.
3) Generally, insect diets are deficient in; calcium and vitamins A, B, D and E.
4) Gut loading and dusting are suitable methods for increasing the nutrient value of feeder insects.

7. NUTRITIONAL DISEASE

As mentioned before, overall, amphibians are a challenging class of animals to maintain under *ex situ* conditions, and till this day not much is known on amphibian nutritional requirements. For these reasons, the majority of the disease seen in captive amphibians are in some way related to their husbandry and management. Furthermore, nutritional diseases are no uncommon sight among captive amphibians. Overall, these diseases are caused by dietary deficiency and, to a lesser extent, dietary excess (Densmore and Green, 2007; Ferrie et al., 2014). Additionally, most of the nutritional disorders seen in captive amphibians can be linked to the feeding of commercially available insect diets, which are low in fat-soluble vitamins, like vitamin A, and have inadequate Ca:P ratios (Ferrie et al., 2014).

One of the most common dietary pathologies affecting amphibians in captivity is so called metabolic bone disease (Densmore and Green, 2007; Ferrie et al., 2014). This nutritional disease is caused by vitamin and mineral imbalances (Densmore and Green, 2007; McWilliams, 2008), and has been reported in numerous amphibian species, ranging over all three orders (Densmore and Green, 2007). In most cases, metabolic bone disease is caused by insufficient calcium intake or improper Ca:P ratios. Other etiologies include inadequate vitamin D3 and UVB provision, or excess vitamin A intake. Clinical signs result from insufficient bone mineralization and may include; abnormal posture and locomotion, bone deformities, pathological fractures, tetany, anasarca, dropsy and subcutaneous edema. With the help of radiology, alterations in bone density can also be observed (Densmore and Green, 2007; McWilliams, 2008; Ferrie et al., 2014).

Another common nutritional disease in captive amphibians is hypovitaminosis A (Densmore and Green, 2007; Latney and Clayton, 2014). As the name would suggest, this disease is the manifestation of an insufficient amounts of vitamin A, and is believed to be the result of inadequate dietary provision of this micronutrient. Vitamin A plays an important role in the bone metabolism and, additionally, serves various functions concerning the epithelium of amphibians (Densmore and Green, 2007; McWilliams, 2008). In adult amphibians, externalization of hypovitaminosis A can include; lethargy, wasting, reduced growth, bone defects, epithelial hyperplasia, squamous metaplasia and keratinization of mucosal epithelium, dermal ulcerations, and so called “short tongue syndrome” (Densmore and Green, 2007; McWilliams, 2008; Latney and Clayton, 2014). This last syndrome represents the inability of affected animals to utilize their tongues for prey-capture, secondary to the most classic representation of hypovitaminosis A: metaplasia of the lingual and oral mucosa (Densmore and Green, 2007; McWilliams, 2008; Ferrie et al., 2014; Rodríguez and Pessier, 2014). Finally, compromised epithelial function, as a result of this vitamin deficiency, leaves affected individuals increasingly vulnerable to secondary pathogens and infections (Latney and Clayton, 2014; Rodríguez and Pessier, 2014). Furthermore, this vitamin deficiency is also believed to cause poor reproductive success and low tadpole survival rates, making hypovitaminosis A an important problem in CBPs (Ferrie et al., 2014; Rodríguez and Pessier, 2014).

Other examples of nutritional deficiencies include various neurological and musculoskeletal pathologies, resulting from vitamin B deficiencies (especially thiamine) (Densmore and Green, 2007; McWilliams, 2008; Ferrie et al., 2014), secondary nutritional hyperparathyroidism, which can be caused by a variety of nutritional deficiencies and excesses, hypovitaminosis D, beta-carotene deficiency and iodine deficiency (McWilliams, 2008).

8. MATERIAL AND METHODS

Study site

Fieldwork for this research was carried out on the shores of the Titicaca lake, from October 2016 till January 2017. Lake Titicaca is located in the South American Andes, in the endorheic Altiplano basin, at an altitude of 3,810 m above sea level (De La Riva, 2005; Schimmel, 2015; IUCN, 2017). The lake itself is situated between Peru (to the west) and Bolivia (to the east) (Figure 3), and covers about 8,300 square km (De La Riva and Reichle, 2014; UNESCO, 2017). Length of the lake measures approximately 180 km and the width measures approximately 70 km (Schimmel, 2015). Lake Titicaca can be divided into two major parts, i.e. “Lago Mayor” (or “Lago Grande”), the deep, main basin, and “Lago Pequeño” (or “Lago Menor”), the smaller, shallow basin. Mean depth of the lake reads at 107 m (Dejoux et al., 2012; Genova, 2011), and maximum depth measures around 285 m (Dejoux et al., 2012). Water temperature varies between 11 and 17 ˚C (Muñoz-Saravia et al., 2018b) and water visibility varies between 5.3 and 10.5 m (Genova, 2011; Schimmel, 2015). Because of its geographical location, Lake Titicaca is situated in a “tropical zone”. Regardless, resulting from the lakes high altitude position, this lake is subjected to a mountainous climate, with high luminous intensity, low temperatures and low air humidity (Dejoux et al., 2012). Further specifications regarding various parameters of Lake Titicaca can be found in the additional information in the appendix.

Monthly trips were made to “Isla de la Luna” (16°2'42.87"S latitude, 69°4'2.34"W longitude) on the Bolivian side of the lake, in the Manco Kapac Province of La Paz department (Figure 3). Samples were taken on the south shore of the island, ranging from 16° 2'40.15"S latitude, 69° 4'10.84"W longitude to 16° 2'46.08"S latitude, 69° 3'47.47"W longitude (Figure 4). The south shore of the island was selected for this research as this area has a high abundance of *T. culeus,* both adult and juveniles (personal comment Arturo Muñoz-Saravia). Furthermore, this area possesses a rich diversity in benthic habitats, ranging from rocky, sandy and heavily vegetated bottom-structure, as well as all possible combinations of the previous. Most common vegetation includes *Myriophyllum elatinoides*, *Elodea potamogeton, Potamogeton* spp. *Schoenoplectus californium*, *Zannichellia palustris* and *Ruppia maritima* (Muñoz-Saravia et al., 2018a, unpublished data).

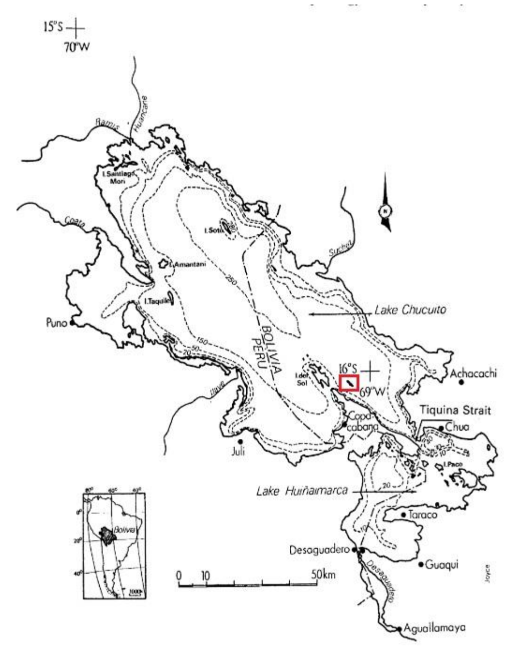


Figure 3. Illustration of the Titicaca Lake, as found in Dejoux et al. (2012). The red square contains Isla de la Luna, on the Bolivian side of the lake.

Sampling trips of three to five days were made in the last week of each month for two purposes. Firstly, potential prey items of *Telmatobius culeus* were captured for nutritional analysis. Secondly, benthic traps were set and emptied in order to determine potential prey item availability overall (at the specified location) and according to depth.

Sampling of potential prey items for nutritional analysis

Invertebrates were captured using a hand-held net with fine mesh (0.5 mm), swiping among the bottom of the lake and among the vegetation at a depth ranging from 0.1 m up to 1.5 m. Contents of the nets were emptied in designated polypropylene containers (1250 ml), keeping the captured animals on water. Additionally, rocks were collected and immediately washed in 10 l buckets filled with water, in order to capture benthic species. Water from the buckets was then poured through a sieve (0.5 mm mesh) and captured animals were kept on water, in designated containers. Following capture, invertebrates were separated, by hand, to the lowest taxonomic level possible, based on visual characteristics, and then dried in open air. Finally, dried fish were obtained from local fishermen. Fish were caught off shore in the above mentioned sampling area using gill nets, and dried in open air on the beach of the island. All dried samples were stored per species in 50 ml polypropylene centrifuge tubes with cap.

Estimation of potential prey items availability

In order to estimate potential prey availability according to depth, five sets of benthic traps were set on the southern shoreline of Isla de la Luna in Bolivia. Traps were set at: 1) 16° 2'41.03"S latitude, 69° 4'7.37"W longitude, 2) 16° 2'41.90"S latitude, 69° 4'4.29"W longitude, 3) 16° 2'42.72"S latitude, 69° 4'0.68"W longitude, 4) 16° 2'42.54"S latitude, 69° 3'56.59"W longitude, 5) 16° 2'43.61"S latitude, 69°

3'53.20"W longitude (Figure 4). Each set consisted of three traps set at a depth of 0.5 (A), 1 (B) and 1.5 (C) meters respectively. Water depth was determined using a scaled rope with a weight at the end, and traps were set in place by freediving.

Figure 4. Illustration of Isla de la Luna, Bolivia. A-B : Represent the outer edges of the sampling area on the south shore of the island. 1-5: Represent the locations of the five sets of benthic traps set in order to catch macro-invertebrates. Illustration made with Google Earth Pro®.

Traps had a cylindrical shape and were constructed out of mesh wire (steel, diameter: 2 mm), with a mesh size of 3 cm. The top of the traps could be opened to insert and remove rocks, while the bottom of the traps was sealed off. The traps were 50 cm in length with a diameter of 25 cm (Muñoz-Saravia et al., 2018a, unpublished data) (As per illustration: design and some pictures of said used traps are added in the illustrative material in the appendix).

Rocks collected on the waterline of Isla de la Luna were inserted into the traps before closing off. Traps were then put into place at their respected depths and position. Once a month, traps were removed and emptied. Using a sturdy net with small mesh (0,5mm), traps were fished up manually and brought to shore. The traps were then opened and the rocks washed in a bucket. After having cleaned all the rocks, traps were filled up again and put back in place.

Invertebrate and fish caught in the traps were collected out of the buckets, similar to the above mentioned method. Wet samples from each trap were then stored on alcohol (96 % ethanol) and brought to the laboratory in the Museo de Historia Natural Alcide d'Orbigny, Cochabamba, Bolivia. There, for each trap individually, potential prey items were separated by hand, to the lowest taxonomic level possible, based on visual characteristics, counted and dried in open air. Finally, dried samples were stored per species, per trap in 5 ml polypropylene centrifuge tubes.

Nutritional analysis

Dried samples were shipped to Belgium and sent to independent laboratories for nutritional analysis. For the determination of ash contents and mineral composition of the potential prey items, a subset of the samples was sent to ECOCHEM: Laboratory of Analytical Chemistry and Applied Ecochemistry, Department of Analytical and Physical Chemistry, Coupure Links 653, 900 Gent, Belgium (http://ecochem.ugent.be). For the determination of macronutrient contents of the potential prey items, a subset of the samples was sent to the Laboratory for Animal Nutrition and Animal Product Quality, Department of Animal Sciences and Aquatic Ecology, Ghent University, Campus Coupure Links 653 - block F, B-9000 Gent, Belgium (https://www.ugent.be/bw/asae/en/research/lanupro). Finally, crude protein values were obtained using nitrogen content data from potential prey items of the Titicaca water frog, originating from a similar study (Muñoz-Saravia et al., 2018a, unpublished data).

Subsamples of the potential prey items were oven dried at 60 ˚C for five days before being homogenized. Proximate analysis was carried out: ash contents were determined by incinerating the samples at 550 °C for four hours in a muffle furnace, as described by Bogut et al. (2007). Next, fat content was determined by applying the Soxhlet method. Finally, crude protein (CP) was calculated applying Kjeldahl’s x 6.25 method, using the Kjel-Foss on nitrogen content data obtained by Muñoz-Saravia et al. (2018a, unpublished data).

Sample preparation for mineral analysis consisted of microwave digestion with 10 ml HNO3 in a closed vessels, followed by filtration of the residue. Samples were analyzed for Na, K, P, S, Ca, Mg, Cu, Fe, Mn, and Zn concentrations, using inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian Vista MPX radial, Varian, Palo Alto, CA, USA) and Se and Hg concentrations via inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, Perkin Elmer, Sunnyvale, CA, USA).

Gross energy was determined by bomb calorimetry.

Additionally, crude fiber (CF) was calculated after obtaining nutritional values of the prey items and was done applying: *100 % -CP % -fat % -Ash %*. Finally, the Ca:P ratio was calculated by dividing the calcium values by the phosphorus values of the prey items.

Calculation of dietary nutrient profile of *Telmatobius culeus*

Nutrient content data from potential prey items was combined with gut content data of *Telmatobius culeus* obtained by Muñoz-Saravia et al. (2018a, unpublished data), in order to generate the dietary nutrient profile of the wild diet of the Titicaca water frog. Firstly, the index of relative importance, based on mass of ingested prey items (IRI m), as calculated by Muñoz-Saravia et al. (2018a, unpublished data), was configured into proportional values. The total sum of the IRI m values was set at 100 % and percentages for each prey species was then back-calculated (see Table 2). Next, these proportional values were converted as to represent amounts in grams per kilogram (g/kg) of nutrients. Going from percentages (1/100) to g/kg (1/1000), proportional IRI m values were multiplied by 10. These final proportional values were then used as a weighting factor assigned to each type of prey item in order to generate intake figures for each nutrient (in g/kg). Thus, each nutrient from each prey item was multiplied by the proportional value of relative importance of that particular prey item. The resulting values were then summed up for each different nutrient component analyzed in this study to get the final proportional nutrient ingestion of the frogs. Additionally, contributions of each prey item, for each nutrient, were divided by the total calculated ingestion of that nutrient in order to show the relative contribution of each prey item. All above mentioned calculations were executed using Microsoft® Office 365 Excel.

Theoretical nutrient recommendations

Based on the proportional dietary nutrient profile obtained in this research, theoretical nutrient recommendations of *Telmatobius culeus* for CP, fat, ash and gross energy (GE) were deduced. This was done by firstly, proportionally, redistributing excess ash in the diet of the frogs to the originally calculated CP and fat contents respectively. For GE, excess ash was subtracted from the total weight of the calculated diet (1 kg) and the GE originally determined in this research assigned to the remaining portion of the diet. Next, the weight of the diet was set back to 1 kg and the GE proportionally increased to match such a diet. For more insight on the reasoning behind this calculation please consult the discussion.

Statistical analysis of prey item availability by depth

Data obtained from the benthic traps was organized in a spreadsheet using Microsoft® Office 365 Excel. Furthermore, average prey item counts (per species and all species together) were determined overall (average of all months and all depths) and per depth (average of all months per depth separately).

Next, using Rstudio® and R 3.4.2® software, potential prey item availability per depth was determined. First, obtained data was tested for normality and homogeneity of variance (Bartlett test). The data in this research was found to be non-homogenic and groups not to have equal variance. For these reasons, data was logarithmically transformed (using log10 +1). By doing so, homogeneity of variance was achieved for all prey items with the exception of *Biomphalaria* sp. and *Corixidae*. Next, a Kruskall-Wallis test was carried out for the comparisons of means of non-normally distributed data. The correlation between trap depth (A: 0.5 m, B: 1 m and C: 1.5 m) and prey item occurrence (number of potential prey items) was analyzed. First of all, the Kruskall-Wallis test was carried out for the overall data (all potential prey items together); next, the same was done for each species of potential prey item separately. Additionally, for each Kruskall-Wallis test showing a significant effect of depth on occurrence, a Dunn test was carried out as post hoc analysis in order to determine which trap depths were significantly different from one another.

9. RESULTS

Potential prey items

A total of 12 types of potential prey items were captured during the fieldwork of this study. Nine invertebrate types were collected, including two species of snails. Obtained invertebrates were: *Austrelmis s.l.* (larval and adult form analyzed separately), nonbiting midges or lake flies: *Chironomidae* (larval form), water boatmen: *Corixidae,* flatworms: *Girardia* sp., leeches: *Helobdella s.l.,* crustaceans: *Hyalella* spp., gastropods: *Heleobia* sp. *and Biomphalaria* sp., and caddisflies: *Trichoptera* (larval form). Three species of fish were obtained: pupfish: *Orestias ispi and Orestias luteus,* and pencil catfish: *Trichomycterus rivulatus*.

A list of the potential prey items captured during this study, together with the obtained amounts (weight expressed in dry matter), is given in Table 5.

Tabel 5. Potential prey items caught on the shoreline of Isla de la Luna, Bolivia, with their amounts expressed in dry matter (DM) weight:

NR	Prey (common name)	Prey	Mass (g)
1	Riffle Beetles	*Austrelmis s.l.*	3.1
2	Riffle Beetles	*Austrelmis s.l.* larvae	1.15
3	Nonbiting Midges	*Chironomidae* larvae	0.05
4	Water Boatmen	*Corixidae*	1.31
5	Flatworms	*Girardia* sp.	2.03
6	Leeches	*Helobdella s.l.*	4.81
7	Crustaceans	*Hyalella* spp.	> 30
8	Gastropods	*Heleobia* sp.	5.32
9	Pupfish	*Orestias ispi*	>10
10	Pupfish	*Orestias luteos*	>10
11	Gastropods	*Biomphalaria* sp.	0.10
12	Pencil Catfish	*Trichomycterus* sp.	>10
13	Caddisflies	*Trichoptera* larvae	2.61

Note: A total of 2 g (DM) of each species was required for nutritional analysis. Due to scarcity of certain prey items, this value was not reached for all potential prey items.

Additionally, as per illustration, some pictures of said potential prey items are added under illustrative material in the appendix.

Nutritional analysis

Crude protein (CP), fat, ash, gross energy and minerals composition of potential prey items was determined. For minerals: calcium (Ca), magnesium (Mg), phosphorus (P), sulfur (S), potassium (K), sodium (Na), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), selenium (Se) and mercury (Hg) contents were analyzed.

Nutritional composition of each potential prey items species analyzed in this study can be found in the additional results the appendix.

Unfortunately, insufficient amounts of *Austrelmis s.l.* larvae, *Anisanculu*s sp. and Chironomidae larvae were collected in order to conduct full nutritional analysis. Therefore, some data concerning these invertebrates is missing.

Nutrient contents per potential prey item caught on the shores of Isla de la Luna varied considerably. First of all starting with CP, most of the potential prey items had rather high CP values, with the exception of *Heleobia* sp. and *Biomphalaria* sp. Highest CP values were noted for *Helobdella s.l.*, followed by *Austrelmis s.l.* and *Girardia* sp. For fat, again, potential prey items contained rather high values with the exception of the two snail species. Here, highest fat contents were found in *Trichomycterus* sp., containing substantially higher contents of this nutrient compared to the other examined prey items. Remaining prey items consisted of approximately the same amount of fat. For ash, both snails, *Heleobia* sp. and *Biomphalaria* sp., contained substantially higher quantities of this nutrient as compared to other prey items. Besides these snails, *Hyalella* spp. also contained rather high amounts of ash. Next, GE contents of prey items varied more substantially. Overall, prey items where a high amount of ash was found were low in GE. Thus, *Heleobia* sp. and *Biomphalaria* sp. ranked the lowest, followed by *Hyalella* spp*.* Highest GE contents were reported for *Trichomycterus* sp.. For Ca, *Biomphalaria* sp. contained the highest value, followed by *Hyalella* spp. Interestingly, *Heleobia* sp. had the second lowest Ca content of all investigated prey items. Moving on to Mg, *Chironomidae* and *Hyalella* spp. seem to contain higher amounts of this element as compared to other prey items. For Zn and Fe, overall, benthic species were found to contain the highest amounts of these metals. Contents of other minerals varied more substantially between different prey items and no clear pattern could be identified. For more detail regarding the nutrient content of each prey item please consult the additional results in the appendix.

Calculation of dietary nutrient profile of *Telmatobius culeus*

Gut content analysis for 44 *Telmatobius culeus* frogs, together with the IRI m values as reported by Muñoz-Saravia et al. (2018a, unpublished data), as well as the proportional IRI m (%) values, can be found in Table 2. Prey item intake values derived from the of the above mentioned research revealed *Hyalella* spp. to be the most ingested prey item (according to mass and count) by the Titicaca water frog, followed by *Heleobia* sp. (according to count)*.* The prey item representing the next highest count in the gut of the frogs was *Biomphalaria* sp.*,* already accounting for a much lower fraction of the prey intake of these frogs. Other prey items were found to represent a much lower fraction of the ingestion of the Titicaca water frog. Notwithstanding, *Orestias ispi* was reported to represent the second highest prey item mass in the frog’s gut, but only represented a marginal fraction of the ingested prey items of this frog species. Reported IRI m values revealed *Hyalella* spp., *Heleobia* sp. and *Biomphalaria* sp. to determine the bulk of the nutrient intake of the Titicaca water frog.

Dietary nutrient profile of *Telmatobius culeus*

The nutrient profile of the wild diet of *Telmatobius culeus* can be found in Table 6.

These values revealed the wild diet of these frogs to contain moderate amounts of CP, average amounts of fat and extremely high ash contents, together with a moderately low GE content. Furthermore, high values were found for most examined minerals, with the exception of P, K, Cu and

Mn. This also translated into a high Ca:P ratio. Furthermore, Zn, Fe and Hg contents were found to be extremely high.

Table 6. Dietary nutrient profile of *Telmatobius culeus*:

Nutrient	Ingestion	Unit	% Missing Data
CP	34	%	0.35
Fat	21	%	0.35
CF	0.1	%	0.35
Ash	45	%	0.35
GE	9908	kj/kg	0.35
Ca	82	g/kg	0.35
Mg	1.7	g/kg	0.35
P	3.0	g/kg	0.35
S	6.3	g/kg	0.35
K	2.4	g/kg	0.35
Na	3.4	g/kg	0.35
Cu	11	mg/kg	0.35
Fe	618	mg/kg	0.35
Mn	21	mg/kg	0.35
Zn	1446	mg/kg	0.35
Se	1246	µg/kg	0.35
Hg	93	µg/kg	0.35
Ca:P	27:1	/	0.35

Abbreviations: CP: Crude Protein, CF: Crude Fiber, GE: Gross Energy, Ca: Calcium, Mg: Magnesium, P: Phosphorus, S: Sulfur, K: Potassium, Na: Sodium, Cu: Copper, Fe: Iron, Mn: Manganese, Zn: Zinc, Se: Selenium, Hg: Mercury, Ca:P: Calcium-Phosphorus ratio.

Note 1: Insufficient samples of *Austrelmis s.l.* larvae, *Anisanculus* sp. and *Chironomidae* larvae were collected during the fieldwork of this study in order to conduct full nutritional analysis of these prey items. For this reason, a certain amount of data is missing for optimal intake calculations. A, percentage of “missing data” is added to the table as to get an idea of the possible deviation of these reported values from the actual nutritional intake of the Titicaca water frog.

Note 2: Certain nutrient concentrations of certain prey items were below the detection limit of the analytical method applied in this study; Copper levels of *Chironomidae* larvae, *Heleobia* sp., *Orestias ispi*, *Biomphalaria* sp. and *Trichoptera* larvae were below the detection limit of 10 mg/kg. Mercury levels of *Hyalella* spp. and *Biomphalaria* sp. were below the detection limit of 65 µg/kg. In these cases half of the detection limit (5 for Cu and 32.5 for Hg) was used as the nutrient value of these prey items in the calculations of the nutrient intake of the Titicaca water frog.

Contribution of each prey item to the dietary nutrient profile of *Telmatobius culeus*

A representation of the contribution of each prey item, for each nutrient determined in this research, to the dietary nutrient profile of the Titicaca water frog, is given in the additional results in the appendix. For macronutrients, *Hyalella* spp. contributed the most to the nutrient intake of the frogs, followed by *Heleobia* sp. Next, *Biomphalaria* sp. and *Orestias ispi* had approximately equal shares in the macronutrient intake of *T. culeus.* Finally, *Austrelmis s.l.* and *Trichoptera* contributed substantially less to the CP and fat intake of these frogs. For gross energy, again, *Hyalella* spp. accounted for the largest fraction in the diet of the Titicaca water frog. Overall contributions of other prey items were substantially less compared to this. For ash, *Heleobia* sp. was responsible for the bulk of this nutrient in the wild diet of *T. culeus*, followed by *Hyalella* spp*.* and *Biomphalaria* sp. which accounted for the remaining ash content. Other prey items contributed negligibly regarding this nutrient. For minerals, Ca, Mg, S, K, Na, Cu, Fe and Mn contents were mostly determined by *Hyalella* spp., while *Heleobia* sp. delivered most of the P, Zn, Se and Hg to the intake of *Telmatobius culeus*. For more detail regarding the contribution of each prey item to a specific nutrient in the wild diet of the Titicaca water frog, please consult the additional results in the appendix.

Theoretical nutrient recommendations

Theoretically deduced nutrient recommendations for CP, fat, ash and GE of the Titicaca water frog can be found in Table 7.

Theoretical nutrient recommendations for the frogs were shown to include high amounts of CP and fat and a considerably lower ash content. Gross energy contents of these theoretical recommendations was average.

For clarification regarding these theoretical recommendations please consult the discussion.

Table 7. Theoretical nutrient recommendations for *Telmatobius culeus*:

Nutrient	Theoretical recommendation	Unit
CP	53	%
fat	33	%
ash	13	%
GE	14588	kJ/kg

Note: These values were deduced based on the nutrient intake figures calculated in this research. As it is not known how much of the wild diet of the Titicaca water frog is actually assimilated, these calculated theoretical nutrient recommendations are somewhat speculative. For clarification please consult the discussion.

Potential prey items caught in benthic traps

Potential prey items obtained in benthic traps included: *Austrelmis s.l.* (larval and adult form), nonbiting midges or lake flies: *Chironomidae* (larval form), water boatmen: *Corixidae,* flatworms: *Girardia* sp.*,* leeches: *Helobdella s.l.,* crustaceans: *Hyalella* spp.*,* gastropods: *Anisanculus, Heleobia* sp. *and Biomphalaria* sp.*,* pencil catfish: *Trichomycterus rivulatus,* and caddisflies: *Trichoptera* (larval form). All potential prey items caught by hand were also caught using benthic traps, with the exception of pupfish: *Orestias ispi and Orestias luteus.*

Overall, *Hyalella* spp. were the most abundant potential prey item caught on the shores of Isla de la Luna. The second most abundant potential prey items were *Girardia* sp., closely followed by *Austrelmis s.l.* (adult). Next, *Chironomidae* (larval form), *Austrelmis s.l.* (larval for) and *Heleobia* sp. followed. Remaining potential prey items were documented at less than 5 % of the total potential prey items acquired on the shores of the Isla de la Luna, each.

Per depth separately, *Hyalella* spp. represented the most abundant prey item available on the shores of the Titicaca Lake. *Chironomidae* (larval form) was the second most abundant prey item at 0.5 m, while *Austrelmis s.l.* (adult) and *Girardia* sp. were the second most abundant prey items at a depth of 1 m and 1.5 m respectively.

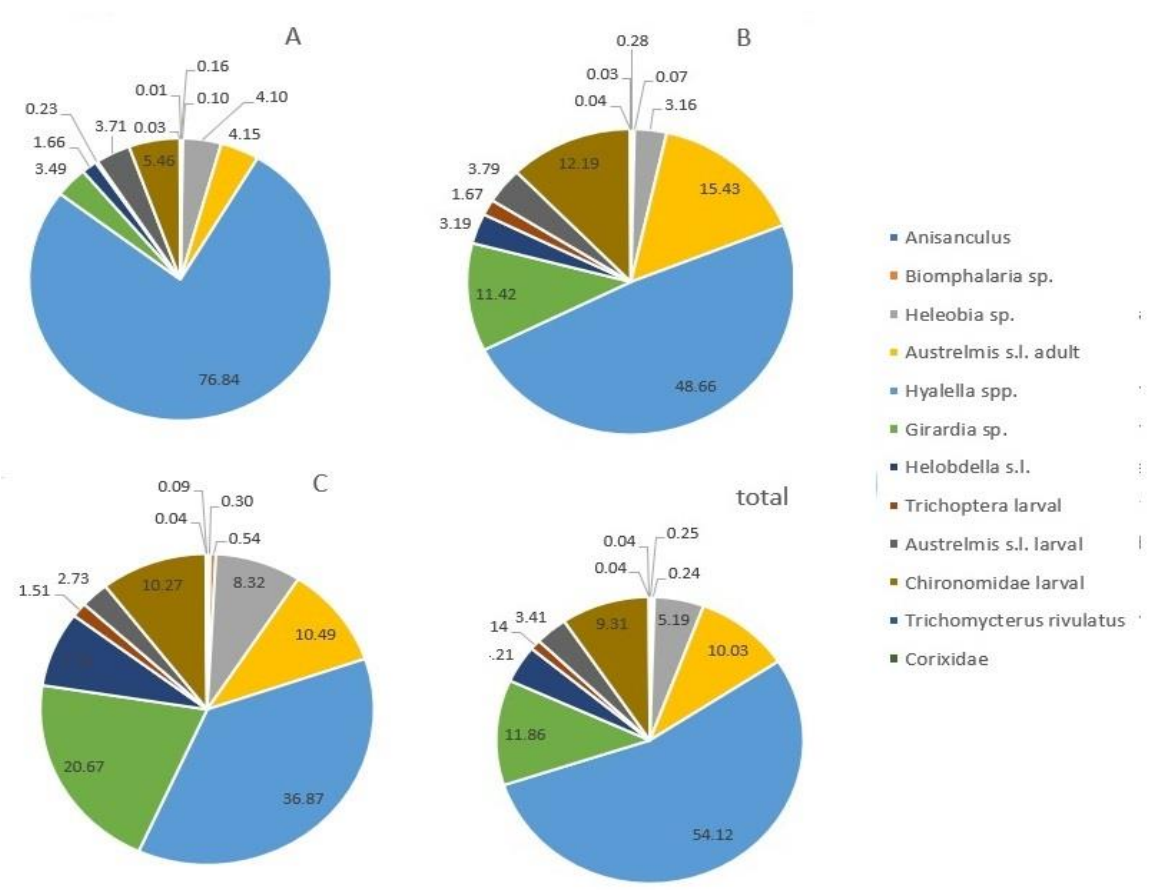
For further insight on prey item occurrence, see graph 1. Additionally, a table reporting the average count and proportional amounts of potential prey items per depth can be found in the additional results in the appendix.

Estimation of potential prey items availability per depth

Overall, mean prey item occurrence was highest at low depth and decreasing with increasing depth (A: 40.9, B: 16.4 and C: 14.3). Regardless, after logarithmic transformation, no significant effect of depth on overall prey items occurrence was found.

For analysis of prey species separately, the most significant effect of depth on prey item occurrence

Graph 1. Average proportional representation of prey items caught in benthic traps:



Average proportional count of potential prey items caught in benthic traps from October 2016 till January 2017, on the shore of Isla de la Lune, Bolivia, expressed in percentage (%), per species. A: Average proportional species count for traps at 0.5 m. B: Average proportional species count for traps at 1 m. C: Average proportional species count for traps at 1.5 m. Total: Overall average values.

Note: prey item abundance was calculated based on prey item count, not on mass.

was observed for *Hyalella* spp. (*P* < 0.001). Mean values for *Hyalella* spp. per depth were 411.9, 106.7 and 70.1, for depth "A", "B" and "C" respectively. For *Hyalella* spp., decreasing prey item occurrence was also noticed for increasing depth. Significant differences were found between depth A and B (*P* < 0.001) and between depth A and C (*P* < 0.001). Similar findings were reported for larval *Austrelmis s.l.* (Mean values: A: 20.1, B: 8.2, C: 5.0; *P* < 0.001) but, a significant difference in prey item occurrence was only noted between depths A and C (*P* < 0.001). For *Girardia* sp., reverse findings were observed, namely, mean prey item occurrence was found to increase with increasing depth (A: 16.8, B: 22.9, C: 38.7). Nevertheless, no significant effect of depth was found on prey item prevalence, although being close (*P* = 0.057, A-C: *P* = 0.050). Likely, a significant effect would have been found with a larger sample size. A significant effect of depth was also found for *Biomphalaria* sp. (*P* = 0.024), where depths B and C were shown to be significantly different from one another (*P* = 0.023). However, even after logarithmic transformation, homogeneity of variance was not achieved for this prey item. Presumably, small sample size would have been the cause of this. Mean values for *Biomphalaria* sp. did not reveal any obvious patterns (A: 0.5, B: 0.1, C: 0.9).

Interestingly, no significant effect of depth was found for *Heleobia* sp. (*P* = 0.059, A-B: *P* = 0.056), although, again, being fairly close. Mean values for *Heleobia* sp. would lead to believe these to be more prevalent in shallower water (A: 20.9, B: 6.5, C:13.7).

No significant effect of depth was found for *Austrelmis s.l., Anisanculus, Chironomidae, Corixidae, Helobdella s.l., Trichomycterus, and Trichoptera.*
Supporting graphics can be found in the appendix.

10. DISCUSSION

10.1. PREY SELECTION

As the bulk of the wild diet of the Titicaca water frog mostly consists of *Hyalella* spp., this amphipod (crustacean) largely determines the overall dietary intake of these frogs. This seems logical as *Telmatobius culeus* is believed to be an opportunistic predator (Muñoz-Saravia et al., 2018a, unpublished data) and *Hyalella* spp. was the most abundant prey item available, overall, and at all three investigated depths during this research. The prey item with the second most influence on the frog's dietary nutrient profile is *Heleobia* sp. The third most contributing prey item is *Biomphalaria* sp., representing a considerably lower fraction of the frog's nutrient intake. Other prey items were reported to be ingested in much lower quantities, contributing significantly less to the dietary intake of *Telmatobius culeus*.

All that said, as stated before, one can speculate that some potential prey items may have been underrepresented by applying gut content sampling, as some of the soft-bodied prey items, such as *Helobdella s.l.* and *Girardia* sp.*,* may have been digested prior to sampling (Muñoz-Saravia et al., 2018a, unpublished data). Accordingly, it is possible that hard-bodied prey items, such as *Hyalella* spp.*, Heleobia* sp. and *Biomphalaria* sp.*,* could have been overrepresented using this sampling method, as the shells of these animals are believed to largely remain indigested by the frogs (Muñoz-Saravia et al., 2018a, unpublished data). Consequently, one should be aware of the possible bias due to the applied method, favoring hard-bodied prey items. Additionally, other conventional dietary analysis samplingtechniques, like gastric lavage, have the same limitation (Gillespie, 2013). A possible solution to this problem may be the application of stable isotope analysis techniques on tissue samples of the Titicaca water frog. Such techniques offer a better insight on the actual assimilated ingesta of an animal and thus could be utilized to better determine the proportional prey intake of *T. culeus* in future studies (McKechnie, 2004). Stable isotope analysis has already successfully been applied on a vulnerable amphibian species; *Eurycea sosorum* (IUCN, 2017), revealing that this salamander mainly preyed on different prey items as was previously assumed. Additionally, such techniques offer a reliable alternative to invasive sampling strategies (like gut sampling), far more suitable for application in research regarding endangered species (Gillespie, 2013). All in all, this does not mean that the present data are not useful, because they do confirm the carnivorous-insectivorous feeding behavior of the frog, and for instance also demonstrate that the Ca:P ratio, a concern in captive breeding, is properly met by the natural diet. The present study is also the first attempt to determine the nutrient composition of an amphibian's wild diet, rendering crucial information for successful breeding under human care.

Proportional snail ingestion

Interestingly, abundance of both snails; *Heleobia* sp. and *Biomphalaria* sp., on the shores of Isla de la Luna, was found to be much lower than the proportional amounts found in the gut of the frogs. During this research, *Heleobia* sp. and *Biomphalaria* sp. were found to occur five and 28 times lower in the benthic traps respectively, as compared to the gut content-analysis-results. Various potential explanations for this phenomenon were hypothesized.

1) The methodological bias, favoring hard-bodied prey items; The values in the gut of *T. culeus* may, as stated above, report an overrepresentation of the actual proportional intake of these invertebrates.

Nevertheless, other hard bodied invertebrates from the Titicaca Lake, such as *Austrelmis s.l.* (adult) and *Anisancylus,* did not show this pattern, leading to believe that this bias probably is not the main reason for their high abundance in the stomach contents.

2) Annual or seasonal variations in prey item abundance; In this study, prey items were sampled during a short period of time (three months), therefore, one should be careful when drawing overall conclusions. It is entirely possible that snails like *Heleobia* sp. and *Biomphalaria* sp. could be more abundant during other periods of the year, or that by coincidence, during the sampling period, lower occurrence of these snails was present. Muñoz-Saravia et al. (2018, unpublished data) also sampled the south shore of Isla de la Luna for potential prey items, using similar benthic traps, for a duration of 20 months and reported corresponding findings as was found in this research. Moreover, Muñoz-Saravia et al. (2018a, unpublished data) even reported lower *Heleobia* sp. occurrence than was found in this study (average of 1.30 % over 20 months), leading to believe temporal variations not to be the answer after all.

3) Spatial variation in snail occurrence; For example, these differences could be due to variations in snail occurrence according to microhabitat. Captured frogs utilized for gut content analysis could not be linked to a specific soil and/or vegetation structure (Muñoz-Saravia et al., 2018a, unpublished data) and, different benthic habitats were not sampled strategically (in this research, not in the research by Muñoz-Saravia et al. (2018a, unpublished data)). Thus, it is possible that snails are more plentiful in specific microhabitats and that captured frogs originated from such snail rich habitats, explaining their high occurrence in the frog's gut. Furthermore, differences in snail abundance in the frog's gut as compared to the benthic traps might also be the result of variations in snail manifestation according to depth. As was illustrated in this study, and the study by Muñoz-Saravia et al. (2018a, unpublished data), invertebrate occurrence in the Titicaca Lake can vary substantially according to depth. For instance, this was well demonstrated by the abundance of *Hyalella* spp*.* Nonetheless, in both these studies, analysis of snail abundance according to depth did not offer an adequate explanation for the occurrence differences mentioned above. However, the maximum trap depth was 1.50 m during this research and 3 m for Muñoz-Saravia et al. (2018a, unpublished data). Therefore, perhaps, habitats below 3 m could hold higher snail densities as compared to those closer to the surface. As frogs for gut content analysis could not be linked to any specific depth (Muñoz-Saravia et al., 2018a, unpublished data) and, frogs are believed to live up to a depth of at least 20 m (Hutchison et al., 1976; De La Riva, 2005), these frogs could originate from deeper, presumably snail rich waters, offering a possible explanation for the high snail occurrence in their gut. Lastly, these differences in abundance are unlikely to be the result of regional variation in snail occurrence, as collection of frogs for gut sampling by Muñoz-Saravia et al. (2018a, unpublished data) was carried out in the same locality (south shore of Isla de la Luna).

4) Inappropriate prey item sampling methods; Possibly, benthic traps are not the optimal method for the capture of these snails and therefore, proportional values obtained with such traps may not give an accurate representation of their overall abundance. Visual investigation of the habitats on the Southern shores of Isla de la Luna by snorkeling, revealed *Heleobia* sp. to be more present on the aquatic vegetation. As the benthic traps used for both this research and the research by Muñoz-Saravia et al. (2018a, unpublished data) were solely filled with rocks, this may have been a "negative selection" towards the snails. This is remains somewhat speculative as occurrence of snails on rocks *versus* plants was not investigated. The inclusion of plant materials in the benthic traps may therefore be an interesting innovation for future sampling campaigns.

5) Active selection of snails (out of all possible prey items) by the frogs, resulting in a high percentages of *Heleobia* sp. and *Biomphalaria* sp. in their gut. Possible reasons for this selective intake are discussed in the next segment.

Summary:

1) Methodological bias.
2) Temporal variation.
3) Spatial variation.
4) Sampling method.
5) Active selection.

Hypotheses regarding the active selection of snails

1) Alternative function:

As mentioned before, the shells of *Heleobia* sp. and *Biomphalaria* sp. are not believed be digestible for the frogs, therefore, these hard shells may serve an alternative function in the digestive tract of *T. culeus.* For instance, the presence of shells could help mechanical breakdown and digestion of ingesta, similar to the function of small rocks in the stomach of birds. On the other hand, one can speculate that the presence of hard shells in the GIT of these frogs will slow down the passage of food, leading to better digestion due to longer exposure of the ingesta to digestive juices. This may also lead to extended contact with the absorptive surfaces in the gut, possibly resulting in improved nutritional absorption. Interestingly, these theories appear to be supported by the data generated in this study. For instance, the ash component of the nutrient intake of the Titicaca water frog, as calculated in this research, was extremely high. Logically, there are three possible explanations for this. Firstly, the Titicaca water frog has extremely high mineral requirements, which seems unlikely. Secondly, excess minerals in the diet of the frog are simply not absorbed by these animals. Thirdly, the bulk of the ash in the frog's diet stems from a source that is not digested and thus, the minerals that this ash represents are not taken in by the frogs. As over half of the ash in the frog's wild diet was found to derive from *Heleobia* sp. and *Biomphalaria* sp.*,* it is reasonable to assume that this ash originates from these snail's shells. Therefore, the most likely explanation for the high ash content in the frog's diet is that the minerals in these shells are not available for the frogs, and thus, above mentioned theories seem quite likely. Additionally, speculations that the shells of these snails may have a mechanical function in the gut of *Telmatobius culeus* were also hypothesized by Muñoz-Saravia et al. (2018a, unpublished data). Notwithstanding these theories being rather speculative, investigation of these hypotheses could be interesting considerations for furthered research.

2) Nutrient compensation:

Frogs may select snails for specific nutritional reasons. Gut content analysis in combination with the prey item abundance data suggest Titicaca water frogs to take in high amounts of *Hyalella* spp. because these prey items are abundant and thus easily accessible. As *Hyalella* spp. roughly represents 64 % of the frog's diet in the wild, these invertebrates will form the baseline for the frog's nutrient intake. However, a diet solely based on these crustaceans may not completely fulfil the frog's dietary needs and thus, frogs may be actively selecting other prey items, like *Heleobia* sp. and *Biomphalaria* sp., in order to acquire specific nutrients. Nevertheless, it is noteworthy to mention that both theories; firstly, that the shells of snails may have a mechanical function in the frog's gut and, secondly, that the frogs may be selecting snails for nutritional reasons, are not mutually exclusive. There is no reason to think that both theories could not be true.

Potential nutritive advantage of snails

The first nutrient that comes to mind which snails could possibly offer the Titicaca water frog is **calcium**. Rationally, snails could be a great source of Ca as their shells are mostly made up of this mineral (Aboua, 1990; Adeyeye and Afolabi, 2004). Nevertheless, as mentioned above, the shells of *Heleobia* sp. and *Biomphalaria* sp. are not believed to be digested by the frogs and, consequently, the Ca in these shells would not be readily available. Furthermore, the data generated in this research also supports the claim that *Heleobia* sp. and *Biomphalaria* sp. are not selected by the frogs for their Ca content. Firstly, *Heleobia* sp. was shown to contain rather low amounts of calcium, leading to believe the shell of this invertebrate to have high carbohydrate contents as was shown in to be the case for certain mollusk species (Jatto et al., 2010). Secondly, nutritional analysis of prey items revealed almost three quarter of the Ca in the wild diet of *T. culeus* to originate from *Hyalella* spp. and thus not from *Heleobia* sp. nor *Biomphalaria* sp*.* Accordingly, one can speculate that the exoskeleton of *Hyalella* spp. is rich in Ca and forms the main source of this mineral for the frogs. The cuticula of crustaceans has been shown to be composed of three major components, one of these being calcium carbonate (Rødde et al., 2008; Nagasawa, 2012), further supporting this claim. Illustrating this, proximate analysis of the shells of various shrimp species revealed these to be rich in Ca (Adeyeye et al., 2008; Ravichandran et al., 2009). The exoskeletons of two woodlouse species (*Porcellio scaber* and *Armadillidium vulgare*) were also shown to contain substantial quantities of this element (Becker et al., 2005). Nonetheless, not much is known on the digestibility of the exoskeleton of *Hyalella* spp., or other invertebrates, in *Telmatobius culeus*, and therefore any conclusions should be drawn with care. It is entirely possible that the Ca in the exoskeleton of these invertebrates is thus not available for the frogs. For this reason, further research on the digestibility of the exoskeleton of the Titicaca water frog's prey items, as well as the availability of nutrients in the exoskeleton of these invertebrates is needed. Furthermore, investigation into the presence of certain enzymes, like chitinase, in the gut of these frogs, as was already found in a limited number of amphibian species (Latney and Clayton, 2014), would generate much needed insight in the digestive capabilities of this species.

In the light of this, results of this research suggest the exoskeleton of *Hyalella* spp. not to be digestible or not entirely digestible. In this study, the wild diet of *T. culeus* was shown to contain 8.27 % of Ca, which is an extremely high figure. First of all, a considerable portion of this Ca intake originates from the shells of *Heleobia* sp. and *Biomphalaria* sp. and thus probably is not available for the frogs. Second, *Hyalella* spp. would generate a Ca content equal to approximately 6 % of the total nutrient intake of the Titicaca water frog. This figure again, is extremely high, leading to believe not all Ca in the exoskeleton of *Hyalella* spp. to be readily available and thus, the exoskeleton of these prey items not to be (fully) digestible. This partial digestibility could be a good example of the mechanical function snail shells could serve in the gut of *Telmatobius culeus*. One can speculate that mechanical action of these shells could break down the indigestible exoskeletons of *Hyalella* spp. in such a way that sufficient Ca would be released in order to meet the frog's requirements. Like this, only a portion of the Ca content of the exoskeleton of *Hyalella* spp. would be available for the frogs, which seems more likely than *T. culeus* having such high Ca requirements as the intake-figures would lead to believe. Furthermore, a similar hypothesis was also suggested by Muñoz-Saravia et al. (2018a, unpublished data) as in this research, the exoskeleton of *Hyalella* spp. was reported to get crushed in the digestive tract of the frogs, which, most probably, is the result of mechanical action of the shells of *Heleobia* sp. and *Biomphalaria* sp*.* Additionally, this hypothesis corresponds with the findings of (Dierenfeld and King, 2008), who conducted research on the availability of nutrients of the black soldier fly larvae. In this research significantly higher availability of various nutrients, including Ca, was found if these invertebrates had been crushed prior to ingestion.

Next, if the exoskeleton of *Hyalella* spp. would absolutely not be digestible it is possible that frogs are actually selecting *Heleobia* sp. and *Biomphalaria* sp. for their Ca contents after all. Moreover, it is more likely that the frogs could be choosing *Biomphalaria* sp. as a Ca source as compared to *Heleobia* sp*.,* because *Biomphalaria* sp. have Ca contents approximately 100 times higher than the latter. Personal experience also indicated the shells of *Biomphalaria* sp. to be more fragile than those of *Heleobia* sp. For this reason it is possible that Titicaca water frog are selecting *Biomphalaria* sp. as a Ca source and *Heleobia* sp*.* for the mechanical action of its shells. Shells of *Heleobia* sp. might grind up against the shells of *Biomphalaria* sp. in the gut of the frogs, releasing sufficient Ca to meet the frog's requirement. Regardless, as stated before, an alternative explanation for the vast amount of Ca in the wild diet of the Titicaca water frog would be that this excess Ca, simply, is not absorbed by the frogs and therefore, above mentioned hypotheses remain partly speculative.

When comparing the nutrient composition of *Heleobia* sp. and *Biomphalaria* sp. with that of *Hyalella* spp*.,* other minerals stand out. Moreover, snails seem to contain higher concentrations of certain minerals (**K, Na, S, Se, Zn, Fe** and **P**) and therefore, Titicaca water frogs could actively be selecting these snails in order to compensate for any of those minerals. However, this is unlikely to be the case for **sulphur**, **sodium** and **potassium**, as for these nutrients, *Hyalella* spp., overall, still represents the main nutritional source.

Heleobia sp. and *Biomphalaria* sp. seem to contain rather high amounts of **selenium**. These snails account for approximately half of the frog's Se intake and therefore, could be a great source of this element. Especially *Heleobia* sp. appears to be rich in Se, offering 2.5 times higher amounts of the elements than *Hyalella* spp. Ferrie et al. (2014) recommend a Se intake of 300 µg/kg for amphibians, while, in this research, an intake of approximately four times higher was found. As Se is toxic in excess (van Paemel et al., 2010), it seems logical that the Se in these snails is originating from their shells and thus, just like for Ca, is not readily available for the frogs. In other words, it is unlikely that frogs are selecting snails for their Se contents. Furthermore, after subtraction of the Se contents of these snails, from the overall intake of *T. culeus*, a still too high figure is obtained. The bulk of this remaining Se stems from *Hyalella* spp., proving these amphipods to also be a good source of this element. Regardless, following the chain of thoughts that was laid down in the discussion about Ca, one can imagen the Se contents of *Hyalella* spp. also not to be (completely) available for the frogs if this Se originated from the exoskeleton of these invertebrates. However, when subtracting the Se contents of *Hyalella* spp., *Heleobia* sp. and *Biomphalaria* sp. from the overall Se intake of the Titicaca water frog, a lower value is obtained as compared to the recommendations set up by Ferrie et al. (2014). For this reason, one might speculate, again, that some of the Se in the exoskeleton of *Hyalella* spp. may be available for the frogs after all and that maybe, snail shells in the gut of the frogs may play a role in this. One must not forget that invertebrates are made up of more than just an exoskeleton and that *T. culeus* could get its Se requirements from the soft body of *Hyalella* spp. The same can be said about the shells and the soft body of *Heleobia* sp. and *Biomphalaria* sp. Nevertheless, in conclusion, just like for Ca, it seems unlikely that Titicaca water frogs are selecting snails in order to fulfill their Se requirements.

Both *Heleobia* sp. and *Biomphalaria* sp. seem to be a great source of **zinc** for *Telmatobius culeus*, offering approximately 20 times higher Zn values than *Hyalella* spp. Overall, these snails account for 91.1 % of the Titicaca water frog's Zn intake, as calculated in this research. Compared to this, *Hyalella* spp. only offer a marginal amount of Zn to the frogs. However, *Hyalella* spp. still offers an amount of Zn almost seven times higher than the nutritional recommendations set up by Ferrie et al. (2014). Additionally, proximate analysis of different crustacean species revealed these to have substantial Zn contents in their soft bodies (Adeyeye et al., 2008; Oksuz et al., 2009; Ehigiator and Oterai, 2012; Zotti et al., 2016), leading to believe a considerable amount of this Zn to be available for the frogs. Thus, it is likely that *T. culeus* would reach its Zn requirements from *Hyalella* spp. alone and therefore, it is reasonable to assume that these frogs are not selecting snails in order to compensate for this element. *Heleobia* sp. and *Biomphalaria* sp. contain significantly higher **iron** concentrations as compared to *Hyalella* spp. Nevertheless, the same can be said as for Zn. Iron contents of *Hyalella* spp. can reasonably be assumed to more than cover the requirements of *T. culeus* and thus, frogs are most likely not selecting snails for their Fe.

Finally, Titicaca water frogs seem to only get 29 % of their **phosphorus** intake from *Hyalella* spp*.,* while these prey items account for nearly 64 % of their dietary ingestion in the wild*.* Furthermore, the exoskeleton of crustaceans, was shown to contain P, among other trace elements (Becker et al., 2005; Boßelmann et al., 2007; Adeyeye et al., 2008; Ravichandran et al., 2009; Nagasawa, 2012), leading to believe not all P derived from *Hyalella* spp. to be accessible for the frogs. Hence, these frogs need to supplement their diet with an additional P source. In this case, this would be *Heleobia* sp. and *Biomphalaria* sp., as most of the P intake of *T. culeus* originates from these snails. On the other hand, one can speculate, again, how much of the P in these snails is available for the frogs. It is entirely possible that most of this P is “locked in” the shells of the snails and thus also not readily available. In the light of this, research on the nutritional composition of the Giant African Snail (*Achatina folicula*) by Aboua (1990) revealed most of these snail’s P to originate from their soft bodies. Furthermore, Ehigiator and Oterai (2012) reported the flesh of the snail *Tympanotonus fuscatus* to contain higher P values as compared to their shells. For these reasons, it is reasonable to assume the bulk of the P originating from *Heleobia* sp. and *Biomphalaria* sp. to stem from their soft bodies as well. Additionally, nutritional recommendations for amphibians, set up by Ferrie et al. (2014), dictate a dietary P content which corresponds perfectly with the value calculated in this research. In conclusion, it is likely that the figure calculated in this research does not deviate much from the actual P requirements of *T. culeus* and that most of the P in the prey items analyzed in this research is readily available for the frogs. Therefore, it is possible that Titicaca water frogs are selecting snails for their P contents.

Overall, it would be interesting to determine the mineral content of the shells of *Heleobia* sp. and *Biomphalaria* sp. as compared to the overall mineral content of these snails (like was done for the Giant African Snail by Aboua (1990)). By doing so, one could find out which of the minerals these snails offer are actually available for the frogs and, in this way generate a better insight on the mineral requirements of *T. culeus*.

Moving on to macronutrients, nutrient intake calculations in this research do not point out Titicaca water frogs to actively select snails for their **protein** nor **fat** contents as in both cases, *Hyalella* spp. dictates the bulk of the intake of these nutrients.

Finally, amino acids, fatty acid and vitamin composition of the prey items of the Titicaca water frog were not investigated in this research, therefore, no deductions can be made regarding these nutrients.

Orestias ispi

Concluding the discussion about prey selection of the Titicaca water frog, there is one more prey item that is worth discoursing. During the gut content analysis carried out by Muñoz-Saravia et al. (2018a, unpublished data) *Orestias ispi* was the next most substantial prey item in the frog’s gut (according to IRI m calculations). This prey item is interesting, because these fish have great nutritional value for the frogs. Nutritional analysis of *Orestias ispi* revealed these fish to contain relatively high CP and fat values, leading to fairly high gross energy contents. Overall, these fish have the highest energy content of all prey items found to be consumed by *T. culeus.* Furthermore, fish do not have a hard exoskeleton, therefore, the majority of the nutrients from these prey items would be accessible for the frogs. Additionally, as one can logically assume, these fish are quite bigger than *Hyalella* spp., *Heleobia* sp.

or *Biomphalaria* sp. Overall, *Heleobia* sp. measures approximately 4 mm in length and 3 mm in width, and *Biomphalaria* sp. measures around 10 mm in length and 22 mm in width. *Hyalella* spp. measures anywhere from 5 to 13 mm in length. *Orestias ispi* on the other hand, measures between 40 and 75 mm in length (Dejoux, 2012), and thus, represent a considerable meal for the Titicaca water frog. The ingestion one *Orestias ispi* could therefore feed a frog for a substantial amount of time, especially because Titicaca water frogs have the lowest metabolic rate observed in any frog (Hutchison et al., 1976; Navas, 1997). Consequently, consumption of one such fish offers a significant nutritional advantage to these animals. In order to get the same amount of nutrition from above mentioned invertebrates, frogs would need to ingest, and thus hunt, a much larger amount of prey items, considerately expending energy in the process. Muñoz-Saravia et al. (2018a, unpublished data) speculated that this opportunistic consumption of *Orestias ispi,* and the benefits this holds, maybe the reason why *T. culeus* evolved certain physical characteristics (broad head, large mouth), facilitating the capture of large prey items. Considering the nutritional findings in this research, this speculation seems entirely plausible.

Consideration

Finally, one should keep in mind that this research, and the research by Muñoz-Saravia et al. (2018a, unpublished data), were conducted in a relatively small area as compared to the overall size of the Titicaca Lake. Hence, findings regarding prey selection in these studies might not be representative for *Telmatobius culeus* populations in other parts of the lake, nor for the Titicaca water frog in general.

10.2. THEORETICAL NUTRIENT RECOMMENDATIONS

In this part of the discussion an attempt was made to deduce the nutritional requirements of the Titicaca water frog based on the nutritional data generated in this research. As the dietary nutrient profile for *T. culeus*, obtained in this research, is based on the proportional prey item ingestion (index of relative importance based on mass: IRI m) of these frogs, and the digestibility of these prey items, nor availability of the nutrients in these prey items is known, one can not know how much of the wild diet of the Titicaca water frog is actually assimilated by these frogs. Furthermore, some nutrients which could be available for the frogs (possibly in excess) might not be absorbed by these animals after all. For these reasons, the nutritional data generated in this research does not represent the actual nutritional requirements of the Titicaca water frog and the deductions here represent an estimation of nutrient recommendations for this frog.

In order to evaluate the dietary data attained for the Titicaca water frog, these intake figures were compared to the dietary recommendations for amphibians, as set up by Ferrie et al. (2014). Additionally, in the interest of providing supplementary information, requirements of related species were also consulted. Dietary requirements for fish, which are used as a model species for omnivorous and carnivorous aquatic vertebrates, as stated by the NRC (1993), were used. The NRC (1993) reports the nutritional requirements for various adult fish species, therefore, average values of these requirements were utilised.

Deduction

Firstly, as mentioned above, ash values calculated in this research were extremely high, and the minerals this represents probably not readily available for the frogs. Therefore, excess ash, which is not absorbed by the frogs, and thus does not count as a nutritional requirement should be subtracted from the total ash content of the nutrient intake of the Titicaca water frog. Secondly, nutrient intake values, given in Table 6 are calculated as proportional values of a 1 kg diet (thus 1 % equals 10 g/kg).

Consequently, as we are working with proportional values, the amount of ash subtracted due to invertebrates' shells should be redistributed to the CP and fat components of the calculated intake figures in order to maintain a total of 100 % or 1 kg. For the purpose of determining the amount of ash that should be subtracted in the context of this research, the assumption was made that the shells of *Heleobia* sp. and *Biomphalaria* sp. were not digested. Furthermore, as the dry weight of the snail's shells was presumed to be substantially higher than that of their soft bodies (personal comment Arturo Muñoz-Saravia) and, the ash contents of these shell significantly higher as well, total ash contents originating from these snails was subtracted from the ash content of the nutrient intake of the Titicaca water frog. Next, as the ash content of the shells of *Heleobia* sp. and *Biomphalaria* sp. was subtracted, the Ca content of these snails could also be subtracted from the total Ca ingestion as originally calculated in this research. As stated before, the remaining Ca content, mostly originating from *Hyalella* spp., still represents a high figure. Therefore, it was speculated that most of this Ca is also not available for the frogs. In order to determine how much calcium to subtract from this intake figure, a second assumption was made. For the calculations in this study, an optimal Ca:P ratio of 1.5:1 was presumed to be accurate, as this was stated to be the optimal ratio for vertebrate insectivore diets (Bernard et al., 1997; McWilliams, 2008). As the P ingestion calculated in this research was shown to most likely represent accurate requirements for the Titicaca water frog, a P content of 0.30 % was used for the Ca recommendation calculations. This lead to an overall Ca content of 0.45 % of the theoretical nutrient recommendations of the Titicaca water frog. On the other hand, Ferrie et al. (2014) recommended a Ca content of 0.60 % of the total diet for amphibians. To be safe, the highest of these two figures was chosen to continue the calculations. Hence, the theoretical Ca requirements for *T. culeus* were set at 0.60 % of the total diet, which lead to a theoretical, accessible, Ca contribution of 0.54 % (of the total diet) for *Hyalella* spp*.* Thus, the difference between the original Ca contribution of *Hyalella* spp. (to the dietary Ca intake) and the newly determined theoretical contribution, could be subtracted from the ash component originating from *Hyalella* spp., leading to a final theoretical ash recommendation of 13 % for *Telmatobius culeus*. Other minerals, even if occurring in excess, were not subtracted from the total ash contents in this theoretical calculation, as these minerals represent a much smaller fraction of the total ash content of the wild diet of the Titicaca water frog as compared to Ca. Finally, the subtracted ash was proportionally redistributed (following the original ratio determined by the dietary nutrient profile) to CP and fat respectively. In conclusion, newly calculated theoretical CP recommendations for *T. culeus* read at 53 % and fat recommendations at 33 %.

Macronutrients

Dietary **crude protein** intake as originally calculated in this research seemed unlikely to represent realistic requirements for *T. culeus* as this figure was relatively low. Moreover, as *Hyalella* spp. represents the bulk of the diet of the Titicaca water frog and, CP contents of *Hyalella* spp. were shown to be significantly higher, higher CP requirements were expected. The reason why the originally calculated CP intake values were low is because both *Heleobia* sp. and *Biomphalaria* sp. had quite low CP contents. Nevertheless, as was discussed above, results of this research are reported as proportional values, therefore the ash in the shells of these snails was substantially lowering their proportional CP contents, leading to an overall low CP intake for *T. culeus*. Notwithstanding, newly calculated theoretical CP recommendations seem rather high as nutritional recommendations for amphibians, as set up by Ferrie et al. (2014), dictate a CP content of 44.4 %. Probably, the reason for this is because, CP contents of prey items, in this research, were calculated using the Kjeldahl's x 6.25 method. In this method, nitrogen contents of the prey items is utilized in order to generate CP values. As reported above, invertebrates have chitinous (amino-cellulose) exoskeletons, containing non-protein nitrogen (Bernard et al., 1997; Donoghue, 1998). Additionally, the cuticula of crustaceans holds proteoglycans (Nagasawa, 2012), further contributing to the non-protein nitrogen component of these prey items. Consequently, not all of the nitrogen used as a base to calculate the CP values of prey items actually originates from protein, overestimating the protein contents of these prey items. Moreover, CP recommendations mentioned above are based on the requirements of related species (made by the NRC) which, in turn, are determined based on highly purified protein (NRC, 1993). For these reasons, a CP requirement for the Titicaca water frog of roughly 45 % (as suggested by Ferrie et al. (2014)) seems more likely. When looking at the average CP requirements of fish, as reported by the NRC (1993), a requirement of 45 % seems high. However, these requirements for fish are based on highly digestible ingredients, therefore, these values represent near 100 % bioavailability. Contrasting, in this research, CP values were determined on whole body prey items and digestibility and accessibility of the protein in these prey items is not known. Probably, just like for certain minerals, not all protein in the exoskeleton of *Hyalella* spp. (the main protein source for *T. culeus*) is readily available for the frogs. Besides chitin and calcium carbonate, protein is the main component of the exoskeleton of crustacean (Nagasawa, 2012). Illustrating this, proximate analysis of the shells of different shrimp species revealed these to be composed of roughly 35 % CP (Adeyeye et al., 2008; Rødde et al., 2008; Ravichandran et al., 2009). As *Hyalella* spp. are also crustaceans (Dejoux, 2012), it is reasonable to assume these invertebrates to have substantial protein contents in their cuticula. Therefore, CP requirements for fish as dictated by the NRC (1993) probably represent an underestimation of the protein requirements of the Titicaca water frog. In this chain of thought, it would be interesting to further investigate prey item digestibility, as was stated in the discussion about prey item selection. Furthermore, research determining whether *T. culeus* possesses enzymes, like chitinase, which can break down the exoskeleton of invertebrates, would be an interesting subject for further research, as was also mentioned above. In conclusion, a CP recommendation for *T. culeus*, based on a whole body invertebrate diet, of roughly 45 % seems reasonable and rationale.

Just like for protein, the shells of *Heleobia* sp. and *Biomphalaria* sp. were lowering the proportional **fat** values of the dietary nutrient profile of *Telmatobius culeus*. As the overall fat content of *Hyalella* spp. roughly corresponds with the newly calculated theoretical recommendations of the Titicaca water frog, this does not seem an unreasonable requirement for these frogs. Contrary to protein and calcium, fat contents of the exoskeleton of crustaceans are minimal (Rødde et al., 2008; Nagasawa, 2012). Therefore, fat contents acquired in this research mostly originate from the soft body of prey items and thus, are believed to be accessible for the frogs. Additionally, when proportionally redistributing the retracted ash values of *Heleobia* sp. and *Biomphalaria* sp. to their CP and fat contents respectively, fat contents between 30-40 % are attained for these snails. Moreover, fat contents of snail shells are believed to be quite low (Aboua, 1990; Jatto et al., 2010). Thus, an overall theoretical dietary fat recommendations as calculated above seems likely. This being said, no recommendations for fat, for amphibians, were made by Ferrie et al. (2014), nor did the requirements for adult fish, set up by the NRC (1993) mention any fat requirements. For these reasons, no comparative baseline for the fat requirements of the Titicaca water frog was found and therefore, a dietary fat recommendation of 33 % remains somewhat speculative.

As stated above, insectivores diets should be poor in **carbohydrates** (McWilliams, 2008), and dietary contents of these nutrients should not exceed 5 % for amphibians (Hadfield et al., 2006). In this research, carbohydrate contents of the prey items of the Titicaca water frog were not determined as such, but were calculated using: *100 % -CP % -fat % -Ash %*. Based on the original nutrient intake calculations, a rather low carbohydrate content was determined, especially because, as stated above, the exoskeleton of crustaceans, like *Hyalella* spp.*,* contain substantial amount of chitin. As to provide an example for crustaceans, in Northern Shrimp (*Pandalus borealis*), exoskeleton chitin contents were determined at approximately 17-20 % (Rødde et al., 2008). Furthermore, the shells of snails were shown to contain considerable amounts of fiber (Jatto et al., 2010), further contributing to the carbohydrate contents of the Titicaca water frog's wild diet. This being said, as the hard bodies of above mentioned prey items are assumed not to be (fully) digestible for the frogs, these frogs would have quite low carbohydrate requirements after all. On the other hand, Titicaca water frogs have been shown to ingest plant material during feeding Muñoz-Saravia et al. (2018a, unpublished data). Notwithstanding, it is not known if these frogs take in plants for their nutritive value or that plants are simply accidentally taken in during prey capture. Considering this last observation, and the fact that no recommendations for carbohydrates, for amphibians, were made by Ferrie et al. (2014) and, carbohydrate contents of the prey items of *T. culeus* were not determined as such, actual carbohydrate recommendations of the Titicaca water frog remain unknown.

Gross energy

As originally determined, the wild diet of these frogs would contain 9908 kJ/kg (or 2368 kcal/kg) of gross energy. As ash does not contribute to the energy content of a diet, and ash values were substantially lowered in the context of determining the Titicaca water frog's theoretical nutrient recommendations, theoretical GE recommendations for these frogs should be higher. Following this chain of thoughts, one can assume that one kilogram of the diet of these frogs, minus the weight of the subtracted ash (320.80 g), would generate this 9908 kJ of gross energy. In other words, this energy content can be attributed to 679.20 g of the Titicaca water frog's theoretical diet. Logically, a one kilogram diet would therefore account for 14588 kJ (or 3486 kcal) of GE. Comparing this, the NRC's (1993) requirements for adult fish dictate an average dietary energy base of 13723 kJ/kg (or 3280 kcal/kg). Regardless, this value does not represent gross energy but represents the digestible energy (DE) contents of the fish's diet. Digestible energy is the energy content of a diet which is available for the animal and is measured by subtracting the remaining energy in the animal's feces from the GE contents of that diet (NRC, 1981). Rationally, GE contents of such a diet would thus be (substantially) higher and therefore, probably, surpass the gross energy contents of the Titicaca water frog's diet. This is not surprising as Titicaca water frogs have very low metabolic rates (Hutchison et al., 1976; Navas, 1997), most likely translating into low overall energy requirements. Moreover, amphibian dietary recommendations as set up by Ferrie et al. (2014) are based on even higher energy contents. These authors based basic amphibian recommendations on a 16736 kJ/kg (or 4000 kcal/kg) DE diet. Nevertheless, *T. culeus* has the lowest metabolic rate reported in any frog, and the second lowest reported in any amphibians (Hutchison et al., 1976; Navas, 1997), therefore, a theoretical GE content of 14588 kJ/kg seems acceptable.

Elements and trace elements

Overall, theoretical dietary recommendations for elements and trace elements of the Titicaca water frog were harder to deduce as the elemental dietary contents of these frog's wild diet often were magnitudes higher than any recommendations or requirement. As was speculated above, most likely, a great portion of the minerals and elements found in the wild diet of *T. culeus* are overestimations of the actual requirements of these frogs, as a large share of these minerals might not be readily available (these minerals being part of the indigestible exoskeleton and shells of invertebrates). For this reason, for the sake of this research, when unrealistically high mineral contents were found, mineral recommendations as set up by Ferrie et al. (2014) were assumed to be accurate recommendations for *Telmatobius culeus*. This being said, one should keep in mind that dietary mineral requirements for amphibians are believed to vary substantially between species due to possible physiological adaptations to specific environmental factors (McWilliams, 2008) and therefore, any conclusions drawn remain highly speculative.

As was previously conferred during this discussion, **calcium** contents initially determined for *Telmatobius culeus* were extremely high and thus, would unlikely represent accurate requirements for the frogs. Furthermore, it was deducted that the majority of this Ca, most probably, would not be readily available for the frogs. In summation, Ca recommendations as dictated by Ferrie et al. (2014) were deemed more plausible and therefore, an overall dietary Ca recommendation of 0.60 % of the total diet of *T. culeus* was assumed to be accurate. This being said, due to the unique amphibian feature of having a well-developed, calcium carbonate rich, endolymphatic sac surrounding most of the their brain (and in frogs, parts of their vertebral column) which, most probably, functioning as a Ca reserve (Hillman, 2009; Ferrie et al., 2014), it is possible this class of animals have higher overall Ca requirements than expected. As Ferrie et al. (2014) do not mention if they took this fact into consideration when determining Ca recommendations for amphibians and, these Ca recommendations were based on the requirements of rats (the model species suggested for basic omnivorous amphibians), one might speculate the Ca requirements of *T. culeus* to exceed 0.60 %. Nevertheless, it is important to concede that amphibians can take up minerals, like Ca, through the skin (Stiffler, 1993) and relatively might not need as much Ca in their diet. For this reason, it is imaginable that Titicaca water frogs would fulfill a portion of their Ca requirements separate from the diet. Moreover, water of the Titicaca Lake has been shown to be rich in minerals, including Ca (Genova, 2011; BAI, 2015a), giving additional plausibility to this theory. Future investigation into the cutaneious Ca intake of *T. culeus* would therefore be helpful in determining the actual dietary Ca requirements of these frogs. Anyhow, due to the Titicaca water frog's critically endangered status, conduction of experimental studies with *Telmatobius culeus* might be considered unethical. Finally, dietary requirements set up for adult fish by the NRC (1993) do not dictate any Ca requirements and therefore, do not offer further insight regarding possible Ca recommendations for the Titicaca water frog.

For **magnesium**, roughly the same deduction can be made as for Ca. The original dietary Mg intake figures calculated for the Titicaca water frog were substantially higher than the recommendations made by Ferrie et al. (2014) (and the requirements set up for adult fish (NRC, 1993)). Furthermore, the bulk of this Mg originates from *Hyalella* spp., which also has the highest Mg content of all prey items investigated in this research. Hence, just like for Ca, one can speculate this trace element content to originate from the exoskeleton of these invertebrates and thus, probably, not to be readily available for the frogs. Moreover, investigation of the chemical composition of other crustacean's exoskeletons revealed these to contain crystalline magnesian calcite, offering a possible expiation for the high Mg content found in *Hyalella* spp. (Becker et al., 2005; Boßelmann et al., 2007). Furthermore, Mg was also reported to be one of the overall occurring trace elements in the cuticula of crustaceans (Nagasawa, 2012). Next, research on the chemical composition of shrimp revealed their exoskeleton to have higher Mg concentrations as compared to the flesh of these animals (Adeyeye et al., 2008). Besides, when subtracting the Mg contribution of *Hyalella* spp. from the overall Mg intake of the Titicaca water frog, a new intake of 0.04 % is achieved, perfectly corresponding with the previously mentioned recommendations. In conclusion, a Mg recommendation of roughly 0.04 %, as recommended by Ferrie et al. (2014) seems acceptable for the Titicaca water frog.

Sodium intake figures of the Titicaca water frog were only slightly higher than the recommendations composed by Ferrie et al. (2014). Additionally, logically, the large majority of this Na originated from *Hyalella* spp. and *Heleobia* sp., the main ingested prey items by the frogs. Moreover, when looking at the Na contents of the different prey items of *T. culeus*, *Hyalella* spp. and *Heleobia* sp. contain significantly higher concentrations of this element as compared to the others. For these reasons, originally calculated Na intake figures seem reasonable recommendations for the Titicaca water frog. On the other hand, one can speculate, again, a portion of this mineral to originate from the hard bodies of these prey items. Furthermore, this speculation is also supported by the data generated by Aboua (1990) and Adeyeye et al. (2008), which both show higher Na contents in the hard bodies of the investigates invertebrates in those studies. As Na intake was only slightly higher than above mentioned recommendations, one can reasonably assume only a fraction of this Na to originate from the shells of *Hyalella* spp. and *Heleobia* sp. In conclusion, a Na recommendation as suggested by Ferrie et al. (2014), is assumed to be correct in the context of this research. Besides, cutaneous Na uptake by aquatic amphibians is solely believed to play a minimal role in the long term balance of this mineral (Hillman, 2009), presumably making dietary Na the main source of this mineral for the Titicaca water frog and thus, the recommendations mentioned above most likely accurate.

As was discussed above, **selenium** contents as calculated in this research were deemed unrealistically high and thus, most likely do not represent accurate requirements for the frog. Regardless, as Se functions as an antioxidant (van Paemel et al., 2010), and insect diets are high in unsaturated fats (Finke, 2002; 2003; Oonincx and Dierenfeld, 2012; Finke, 2013), Titicaca water frogs might have relatively high Se requirements. Nevertheless, fat composition of *T. culeus*' prey items were not investigated during this research, thus, no clear conclusions can be drawn regarding this. Accordingly, a Se recommendation of 0.30 ppm (parts per million or mg/kg), as recommended by Ferrie et al. (2014) was accepted in the context of this study.

No comparative values were set up by Ferrie et al. (2014) for **sulfur**, nor could any S requirements be found in the nutritional requirements for adult fish dictated by the NRC (1993). Furthermore, proximate analysis of invertebrate species do not readily report S values (Finke, 2002; Oonincx and Dierenfeld, 2012; Finke, 2013; Ferrie et al., 2014; Latney and Clayton, 2014; Finke, 2015). For these reasons, this element will not be discourse further in this discussion.

In the discussion above, originally calculated **phosphorus** contents of *T. culeus'* diet were shown to most probably represent accurate requirements for the Titicaca water frog and thus are accepted to be accurate recommendations in the context of this research.

Potassium, **copper** and **manganese** intake figures as calculated in this research seem to roughly correspond with the amphibian recommendations set up by Ferrie et al. (2014). Hence, the attained values in this study are assumed to be accurate recommendations for the Titicaca water frog. For K, a lower nutrient intake for *T. culeus* was acquired as compared to the above mentioned recommendations. However, no logical reason was found for this manifestation. As insectivorous frogs have a high dietary input of K (Hillman, 2009; Ferrie et al., 2014) and, overall, main prey items consumed by *T. culeus* contain lower K values compared to the commercially available invertebrates mentioned in Table 3, *Telmatobius culeus* is assumed to simply have lower K requirements than expected. For Cu, the dietary intake calculated for the Titicaca water frog was 10.79 ppm as compared to the 12 ppm recommendation set up by Ferrie et al. (2014). These values are fairly close, thus, no more discourse is needed regarding this element. Lastly, Mn intake for *T. culeus* were slightly higher than the value recommended by Ferrie et al. (2014). When looking at the Mn contents of the frog's prey items, and those of commercially available invertebrates in Table 3, one can see than Mn contents vary quite a lot between invertebrates. Furthermore, prey items of the Titicaca water frog containing the highest values for this element are not particularly ranked highly in the frogs prey item selection. Therefore, it is reasonable to assume that the difference between the calculated Mn intake and the recommendations set up by Ferrie et al. (2014) are due to "coincidental" variations in Mn contents between prey species and no further conclusions needs to be drawn from this manifestation.

Zinc intake as calculated in this research was extremely high and, as was discussed above, mostly stemmed from *Heleobia* sp. and *Biomphalaria* sp. Hence, just like for ash, one could speculate this Zn to originate from the shells of *Heleobia* sp. and *Biomphalaria* sp. and thus, not to be readily available for the frogs. Regardless, this value is so much higher (80 times) than the recommendations made by Ferrie et al. (2014) that natural Zn contents of the snail's shell alone would not offer a reliable explanation for this. Additionally, compared to the Zn values found in this research, nutritional analysis of other snail species revealed significantly lower figures (Aboua, 1990, Özogul et al., 2005; Fagbuaro et al., 2006; Ehigiator and Oterai, 2012). Therefore, it was speculated that the high Zn contents of these snails was the result of environmental pollution in the Titicaca Lake. This being said, comparison of the values in above mentioned studies is difficult as for most of these, nutrient contents were given on a wet weight basis or no specification regarding wet or dry weight basis was given. Anyhow, because of the tremendous difference in snail-zinc-content, it seems likely that the Zn found in *Heleobia* sp*.* and *Biomphalaria* sp. originates from anthropological sources. Furthermore, research regarding metal pollution in Lake Titicaca reported metal contamination (Cu, Zn, Cd, Hg, Pb, Co, and Fe) at all investigated sites (Monroy et al., 2014). The speculation that the high Zn content in the diet of *T. culeus* was due to pollution is also supported by the Zn values found in other invertebrates investigated in this research, which were also significantly higher than expected. For example, *Helobdella s.l.* and *Austrelmis s.l.* showed extremely high Zn contents as compared to the commercially available invertebrates mentioned in Table 3 and, to other invertebrates mentioned in proximate analysis studies (Finke, 2002; Oonincx and Dierenfeld, 2012). Interestingly, during this research, extremely high concentrations of this metal was solely found in the benthic invertebrate prey items examined, leading to believe the soil content of the Titicaca Lake to be rich in Zn, as compared to the water. Similar results were also reported by Monroy et al. (2014), who found higher metal concentrations in the benthopelagic fish species investigated during that research. Furthermore, sediments are known to be the main sink of metals in lakes and, are known to concentrate these pollutants (Monroy et al., 2014). Additionally, the deduction that invertebrates might be exposed to high Zn concentrations through sediments is also supported by water quality reports of the Titicaca Lake which, which report Zn values well below the norm (Monroy et al., 2014; BAI, 2015b; Unpublished data Arturo Muñoz-Saravia). On the other hand, Zinc soil contents were also well within acceptable limits in the research by Monroy et al. (2014). Regardless, benthic invertebrates could still be bioaccumulating Zn originating from the soil of the lake, leading to extremely high Zn intake by *Telmatobius culeus*. In conclusion, a Zn recommendation of 18 ppm, as recommended by Ferrie et al. (2014) was accepted to be true in the context of this research. This being said, Zn has been shown to play a vital role in the protection and function of the skin in humans, especially as an antioxidant (Rostan et al., 2002). Most probably, this is also true for other vertebrates (Bray and Bettger, 1990; Powell, 2000). As amphibian's skin is extremely active, utilized for the absorption of water, oxygen and other elements (Hillman, 2009), one could assume amphibians to have higher Zn requirements as compared to other vertebrates. Furthermore, Zn also acts as a protector against UV radiation (Rostan et al., 2002). As Titicaca water frogs live at high altitudes, these frogs are subjected to high UV dosage and might thus need additional Zn. Taking these facts into consideration, Zn recommendations as set up by Ferrie et al. (2014) could be an underestimation of the actual requirements of the *Telmatobius culeus*.

For **iron**, the same general deduction as for Zn can be made and thus, one can speculate the excessive iron content found in the wild diet of *Telmatobius culeus* to originate from anthropological soil contamination in the lake. In this case, there is also reason to believe recommendations as set up by Ferrie et al. (2014) to be an underestimation of *Telmatobius culeus'* Fe requirements. As mentioned in the literature-study part of this paper, Titicaca water frogs have a high hematocrit and high hemoglobin concentrations (Hutchison et al., 1976; Navas, 1997), which logically, would lead to high iron requirements (hemoglobin, a hemoprotein part of red blood cells, represents the main source of Fe in the body of vertebrates (Nielsen and Nachtigal, 1998)). However, as Fe contents of the diet of *T. culeus* were unrealistically high, actual Fe requirements remain speculative and recommendations set up by Ferrie et al. (2014) were accepted to be accurate in the context of this research.

Finally, the last element investigated during this research is **mercury**. As Hg is a toxic heavy metal, and is not considered an essential trace element for vertebrates (van Paemel et al., 2010), one cannot speak of a Hg requirement. The value calculated here, is purely informative and shows the amount of Hg pollution Titicaca water frogs have to deal with. As mercury is well known to biomagnify in the food chain, and especially animals at the top of the aquatic food chain (like frogs) are sensitive to such accumulation (Pfleeger et al., 2016), one can speculate this heavy metal to have adverse effects on this critically endangered frog species.

Conclusion

Titicaca water frogs get the bulk of their nutrients from just three prey items, i.e. *Hyalella* spp., *Heleobia* sp. and *Biomphalaria* sp*.* For these frogs, *Hyalella* spp. appears to be a great source of all nutrients investigated, with the exception of phosphorus. *Hyalella* spp. can especially be an excellent source of Ca, although it is not clear how much of this mineral is available for the frogs. The frog's active selection of snails with hard-to-digest shells suggests that these shells may serve digestive functions. Most likely, the shells of these snail would have a mechanical function in the gut of *T. culeus*. This is supported by the report that snail shells are not digested and by the extremely high ash content found in the wild diet of these frogs*.* Next, this extremely high ash content, together with the fact that the digestibility of the prey items of *T. culeus*, nor availability of the nutrients in these prey items is known, makes the determination of the nutritional requirements of this frog species hard to accomplish. This being said, theoretical dietary recommendation calculations for the Titicaca water frogs showed these to have rather high protein and fat requirements, and low carbohydrate requirements. As one could reasonably expect, gross energy contents of the diet of these frogs was shown to be somewhat on the low side. Requirements regarding elements and trace elements remain much more speculative although, for certain elements, relatively high requirements can reasonably be assumed. Regardless, nutrient data in this research does suggest these frogs to be subjected to considerable amounts of Zn, Fe and Hg, believed to originate from anthropological sources. Additionally, occasional ingestion of larger prey, like *Orestias ispi*, was shown to have great nutritional advantages for the frogs.

References:

Aboua F. (1990). Chemical composition of Achatina fulica. *Tropicultura* 8, 121–122.

Adeyeye E.I., Afolabi E.O. (2004). Amino acid composition of three different types of land snails consumed in Nigeria. *Food Chemistry* 85, 535–539.

Adeyeye E.I., Adubiaro H.O., Awodola O.J. (2008). Comparability of chemical composition and functional properties of shell and flesh of Penaeus notabilis. *Pakistan Journal of Nutrition* 7 , 741–747.

Allen W.R. (1922). Notes on the Andean frog, Telmatobius culeus (Garman). *Copeia* 108, 52–54.

Allen M.E., Oftedal O. T. (1989). Dietary manipulation of the calcium content of feed crickets. *Journal of Zoo and Wildlife Medicine* 20, 26–33.

Alroy J. (2015). Current extinction rates of reptiles and amphibians. *Proceedings of the National Academy of Sciences* 112, 13003–13008.

Amphibian Ark. (2010). Telmatobius culeus. Berkeley, California. Internet reference: http://www.amphibianark.org/telmatobius-culeus/ (consulted on 05/03/2017).

AmphibiaWeb. (2018). Amphibian Species List. University of California, Berkeley, CA, United States of America. Internet reference: https://amphibiaweb.org (consulted on 26/05/2018).

Anderson S.J. (2000). Increasing calcium levels in cultured insects. *Zoo Biology* 19, 1–9.

Angulo A. (2008). Conservation needs of Batrachophrynus and Telmatobius frogs of the Andes of Peru. *Conservation and Society* 6, 328–333.

Antwis R.E., Browne R.K. (2009). Ultraviolet radiation and Vitamin D3 in amphibian health, behaviour, diet and conservation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 154, 184–190.

Attard L. (2013). The development and evaluation of a gut-loading diet for feeder crickets formulated to provide a balanced nutrient source for insectivorous amphibians and reptiles. Thesis for the Masters of Science In Animal and Poultry Science, University of Guelph, Canada.

BAI: Bolivian Amphibian Initiative. (2015a). Rescuing Titicaca Water Frog. Internet reference: http://bolivianamphibianinitiative.org/titicaca-water-frog-project/ (consulted on 05/03/2017).

BAI: Bolivian Amphibian Initiative. (2015b). Report of water and sediment contaminates or pollutants and their possible relation with *Telmatobius culeus* die-off in April and May 2015. Internet reference: http://bolivianamphibianinitiative.org/wp-content/uploads/2015/09/6-Report-water-quality.pdf (consulted on 20/07/2018).

Barker D., Fitzpatrick M. P., Dierenfeld E. S. (1998). Nutrient composition of selected whole invertebrates. *Zoo Biology* 17, 123–134.

Barrionuevo S., Mangione S. (2006). Chytridiomycosis in two species of Telmatobius (Anura: Leptodactylidae) from Argentina. *Diseases of aquatic organisms* 73, 171–174.

Batko K. (2014). "Telmatobius culeus" (On-line), Animal Diversity Web. Internet reference: http://animaldiversity.org/accounts/Telmatobius_culeus/ (consulted on 24/03/2018).

Becker A., Ziegler A., Epple M. (2005). The mineral phase in the cuticles of two species of Crustacea consists of magnesium calcite, amorphous calcium carbonate, and amorphous calcium phosphate. *Dalton Transactions* 10, 1814–1820.

Becker C.G., Zamudio K.R. (2011). Tropical amphibian populations experience higher disease risk in natural habitats. *Proceedings of the National Academy of Sciences* 108, 9893–9898.

Beebee T.J., Griffiths R.A. (2005). The amphibian decline crisis: a watershed for conservation biology?. *Biological Conservation* 125, 271–285.

Benavides E. (2005). The Telmatobius species complex in Lake Titicaca: applying phylogeographic and coalescent approaches to evolutionary studies of highly polymorphic Andean frogs. *Herpetological Monographs* 7, 167–185.

Benavides E., Ortiz J.C., Sites Jr J.W. (2002). Species boundaries among the Telmatobius (Anura: Leptodactylidae) of the Lake Titicaca basin: allozyme and morphological evidence. *Herpetologica* 58, 31–55.

Bernard J.B., Allen M.E., Ullrey D.E. (1997). Feeding captive insectivorous animals: Nutritional aspects of insects as food. *Nutrition Advisory Group Handbook* Fact Sheet 3, 1–7.

Bishop P.J., Angulo A., Lewis J.P., Moore R.D., Rabb G.B., Garcia Moreno J. (2012). The Amphibian Extinction Crisis - what will it take to put the action into the Amphibian Conservation Action Plan? *Surveys and Perspectives Integrating Environment and Society* 5, 97–111.

Boßelmann F., Romano P., Fabritius H., Raabe D., Epple, M. (2007). The composition of the exoskeleton of two crustacea: The American lobster Homarus americanus and the edible crab Cancer pagurus. *Thermochimica Acta* 463, 65–68

Bogut I., Has-Schön E., Adámek Z., Rajković V., Galović D. (2007). Chironomus plumosus larvae-a suitable nutrient for freshwater farmed fish. *Poljoprivreda* 13, 159–162.

Bray T.M., Bettger W.J. (1990). The physiological role of zinc as an antioxidant. *Free Radical Biology and Medicine* 8, 281–291.

Browne R.K. (2009). Amphibian diet and nutrition. Amphibian Ark Science and Research, Apple Valley, MN, United States of America. http://aark.portal.isis.org/ResearchGuide/Amphibian%20husbandry/Amphibian%20diet%20and%20nutrition.pdf

Browne R., Vercammen F., Verschooren E., Antwis R.E. (2009). UV-B, Vitamin D3, and amphibian health and behaviour. AArk Science and Research. http://portal.isis.org/partners/AARK/ResearchGuide/Amphibian%20zoo%20studies/Amphibian%20UV-B%20and%20Vitamin%20D3.pdf

Burrowes P.A., De la Riva I. (2017). Detection of the amphibian chytrid fungus Batrachochytrium dendrobatidis in museum specimens of Andean aquatic birds: implications for pathogen dispersal. *Journal of Wildlife Diseases* 53, 349–355.

Clayton L.A. (2005). Amphibian gastroenterology. *Veterinary Clinics of North America: Exotic Animal Practice* 8, 227–245.

Collyns D. (2016). Scientists investigate death of 10,000 endangered 'scrotum' frogs in Peru. The Guardian. Internet reference: https://www.theguardian.com/environment/2016/oct/19/scientists-investigate-death-of-10000-endangered-scrotum-frogs-in-peru?CMP=fb_gu (consulted on 05/03/2017).

De La Riva I. (2005). Bolivian frogs of the genus Telmatobius:synopsis, taxonomic comments, and description of a new species. *Herpetological Monographs* 7, 65–101.

De la Riva I., Reichle S. (2014). Diversity and Conservation of the Amphibians of Bolivia. *Herpetological Monographs* 28, 46–65.

Dejoux C. (Ed.). (2012). Lake Titicaca: a synthesis of limnological knowledge (Vol. 68). Kluwer Academic Publishers. Dordrecht, The Netherlands. 573 p.

Densmore C.L., Green D.E. (2007). Diseases of amphibians. *Ilar Journal* 48, 235–254.

Diving Almanac & Book of Records (2018). Highest altitude dive in a submersible. Internet reference: http://divingalmanac.com/highest-altitude-dive-submersible/ (consulted on 26/05/2018).

Dierenfeld E.S., King J. (2008). Digestibility and mineral availability of Phoenix worms, Hermetia illucens, ingested by mountain chicken frogs, Leptodactylus fallax. *Journal of Herpetological Medicine and Surgery*, 18, 100–105.

Donoghue S. (1998). Nutrition of pet amphibians and reptiles. In: Seminars in Avian and Exotic Pet Medicine. Volume 7, issue 3. W.B. Saunders Company. Nutrition Support Services, Inc, Walkabout Farm, Pembroke, VA. USA, p 148–153.

Ehigiator F.A.R., Oterai E.A. (2012). Chemical composition and amino acid profile of a caridean prawn (Macrobrachium vollenhovenii) from Ovia river and tropical periwinkle (Tympanotonus fuscatus) from Benin river, Edo state, Nigeria. *International Journal of Research and Reviews in Applied Sciences* 11, 162–167.

Fagbuaro O., Oso J.A., Edward J.B., Ogunleye R.F. (2006). Nutritional status of four species of giant land snails in Nigeria. *Journal of Zhejiang University Science B* 7, 686–689.

Feder M.E., Burggren W.W. (1985). Skin breathing in vertebrates. *Scientific American* 253, 106–118.

Ferrie G.M., Alford V.C., Atkinson J., Baitchman E., Barber D., Blaner W.S., Crawshaw G., Daneault A., Dierenfeld E., Finke M., Fleming G., Gagliardo R., Hoffman E.A., Karasov W., Klasing K., Koutsos E., Lankton J., Lavin S.R., Lentini A., Livingston S., Lock B., Mason T., McComb A., Morris C., Pessier A.P., Olea-Popelka F., Probst T., Rodriguez C., Schad K., Semmen K., Sincage J., Stamper M.A., Steinmetz J., Sullivan K., Terrell S., Wertan N., Wheaton C.J., Wilson B., Valdes E.V. (2014). Nutrition and health in amphibian husbandry. *Zoo Biology* 33, 485–501.

Finke M.D. (2002). Complete nutrient composition of commercially raised invertebrates used as food for insectivores. *Zoo Biology* 21, 269–285.

Finke M.D. (2003). Gut loading to enhance the nutrient content of insects as food for reptiles: a mathematical approach. *Zoo Biology* 22, 147–162.

Finke M.D. (2013). Complete nutrient content of four species of feeder insects. *Zoo Biology* 32, 27–36.

Finke M.D. (2015). Complete nutrient content of four species of commercially available feeder insects fed enhanced diets during growth. *Zoo biology* 34, 554–564.

Frost D.R. (2017). Amphibian Species of the World: an Online Reference. Version 6.0. Telmatobiidae Fitzinger, 1843. American Museum of Natural History, New York, USA. internet reference: http://research.amnh.org/vz/herpetology/amphibia/index.php//Amphibia/Anura/Telmatobiidae, (consulted on 02/27/2017).

Genova M. (2011). Density and habitat preference of Lake Titicaca frog (*Telmatobius culeus*) ay North-West of Copacabana peninsula. Resource Ecology Group Master Thesis Report. Wageningen University, the Netherlands.

Gillespie J.H. (2013). Application of stable isotope analysis to study temporal changes in foraging ecology in a highly endangered amphibian. *PloS one* 8, e53041.

Hadfield C.A., Clayton L.A., Barnett S.L. (2006). Nutritional support of amphibians. *Journal of Exotic Pet Medicine* 15, 255–263.

Harding G., Griffiths R.A., Pavajeau L. (2016). Developments in amphibian captive breeding and reintroduction programs. *Conservation Biology* 30, 340–349.

Hedley J. (2016). Anatomy and Disorders of the Oral Cavity of Reptiles and Amphibians. *Veterinary Clinics of North America: Exotic Animal Practice* 19, 689–706.

Hillman S. S. (2009). *Ecological and environmental physiology of amphibians*. Oxford University Press. New York 469 p.

Hutchison V.H., Haines H.B., Engbretson G. (1976). Aquatic life at high altitude: respiratory adaptations in the Lake Titicaca frog, Telmatobius culeus. *Respiration Physiology* 27, 115–129.

IUCN. (2017). International Union for Conservation of Nature and Natural Resources. The IUCN List of Threatened Speacies. Internet reference: http://www.iucnredlist.org/ (consulted on 26/05/2018).

Jatto O.E., Asia I.O., Medjor W.E. (2010). Proximate and mineral composition of different species of snail shell. *Pacific Journal of Science and Technology* 11, 416–419.

Latney L.T., Clayton L.A. (2014). Updates on Amphibian Nutrition and Nutritive Value of Common Feeder Insects. *Veterinary Clinics of North America - Exotic Animal Practice* 17, 347–367.

Li H., Vaughan M.J., Browne R.K. (2009). A complex enrichment diet improves growth and health in the endangered Wyoming toad (Bufo baxteri). *Zoo Biology* 28, 197–213.

Livescience. (2017). Internet reference: http://www.livescience.com/54567-zoo-tries-to-save-strange-titicaca-frogs.html (consulted on 03/03/2017).

McKechnie A.E. (2004). Stable isotopes: powerful new tools for animal ecologists: news & views. *South African Journal of Science* 100, 131–134.

Monroy M., Maceda-Veiga A., de Sostoa A. (2014). Metal concentration in water, sediment and four fish species from Lake Titicaca reveals a large-scale environmental concern. *Science of the Total Environment* 487, 233–244.

McWilliams, D.A. (2008). Nutrition recommendations for some captive amphibian species (Anura and Caudata). *The Canadian Association of Zoos and Aquariums Nutrition Advisory and Research Group*.

Muñoz-Saravia A., Callapa G., Janssens G.P.J. (2018a). Prey selection and dietary nutrient composition of a critically endangered amphibian, the Titicaca water frog (*Telmatobius culeus*). *Unpublished*: *review process ongoing.*

Muñoz-Saravia A., Callapa G., Janssens G.P.J. (2018b). Temperature exposure and possible thermoregulation strategies in the Titicaca water frog Telmatobius culeus, a fully aquatic frog of the High Andes. *Endangered species research.* doi: 10.3354/esr00904

Muths E., Fisher R.N. (2015). An alternative framework for responding to the amphibian crisis. *Oryx* 51, 210–213.

Nagasawa H. (2012). The crustacean cuticle: structure, composition and mineralization. *Frontiers Bioscience* 4, 711–20.

Navas C.A. (1997). Thermal extremes at high elevations in the Andes: physiological ecology of frogs. *Journal of Thermal Biology* 22, 467–477.

Nielsen P., Nachtigall D. (1998). Iron supplementation in athletes. *Sports Medicine* 26, 207–216.

NRC (National Research Council). (1993). Nutrient requirements of fish. *Nutrient Requirements of Domestic Animal Series*. National Academies Press. Subcommittee on Fish Nutrition, National Research Council. Washington D.C. USA. 124 p.

NRC (National Research Council). (1981). *Nutritional energetics of domestic animals & Glossary of energy terms*. Second revised edition. National Academies Press. Subcommittee on Biological Energy, Committee on Animal Nutrition, Board on Agriculture and Renewable Recourses, Commission on Natural Recourses, National Research Council. Washington D.C. USA. 54 p.

Ogilvy V., Preziosi R.F., Fidgett A.L. (2012). A brighter future for frogs? The influence of carotenoids on the health, development and reproductive success of the red-eye tree frog. *Animal Conservation* 15, 480–488.

Oksuz A., Ozyilmaz A., Aktas M., Gercek G., Motte J. (2009). A comparative study on proximate, mineral and fatty acid compositions of deep seawater rose shrimp (Parapenaeus longirostris, Lucas 1846) and red shrimp (Plesionika martia, A. Milne-Edwards, 1883). *Journal of Animal and Veterinary Advances* 8, 183–189.

Oonincx D.G.A.B., Dierenfeld E.S. (2012). An investigation into the chemical composition of alternative invertebrate prey. *Zoo Biology* 31, 40–54.

Oonincx D.G.A.B., Van der Poel A.F.B. (2011). Effects of diet on the chemical composition of migratory locusts (Locusta migratoria). *Zoo Biology* 30, 9–16.

Oshima H., Miyazaki R., Ohe Y., Hayashi H., Kawamura K., Kikuyama S. (2002). Isolation and sequence of a novel amphibian pancreatic chitinase. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 132, 381–388.

Özogul Y., Özogul F., Olgunoglu A.I. (2005). Fatty acid profile and mineral content of the wild snail (Helix pomatia) from the region of the south of the Turkey. *European Food Research and Technology* 221, 547–549.

Pappas S. (2016). Dying Breed? Zoo Toils to Save Strange 'Scrotum Frog'. Livescience. Internet reference: http://www.livescience.com/54567-zoo-tries-to-save-strange-titicaca-frogs.html (consulted on 03/03/2017).

Pfleeger A.Z., Eagles-Smith C.A., Kowalski B.M., Herring G., Willacker J.J., Jackson A.K., Pierce, J.R. (2016). From tails to toes: developing nonlethal tissue indicators of mercury exposure in five amphibian species. *Ecotoxicology* 25, 574–583

Powell S.R. (2000). The antioxidant properties of zinc. *The Journal of nutrition* 130, 1447S–1454S.

Reichle S. (2006). Distribution, diversity and conservation status of Bolivian amphibians. PhD thesis, Rheinische Friedrich-Wilhelms-Univerität Bonn, Germany.

Ravichandran S., Rameshkumar G., Prince, A.R. (2009). Biochemical composition of shell and flesh of the Indian white shrimp Penaeus indicus (H. milne Edwards 1837). *American-Eurasian Journal of Scientific Research* 4, 191–194.

Rødde R.H., Einbu A., Vårum K.M. (2008). A seasonal study of the chemical composition and chitin quality of shrimp shells obtained from northern shrimp (Pandalus borealis). *Carbohydrate polymers* 71, 388–393.

Rodríguez C.E., Pessier A.P. (2014). Pathologic changes associated with suspected hypovitaminosis A in amphibians under managed care. *Zoo Biology* 33, 508–515.

Rostan E.F., DeBuys H.V., Madey D.L., Pinnell S.R. (2002). Evidence supporting zinc as an important antioxidant for skin. *International journal of dermatology* 41, 606–611.

Schimmel S.J.H. (2015). The abundance, recruitment and habitat preferences of the frog species *Telmatobiu*s culeus (Anura: Leptodactylidae) at Isla de la Luna, Bolivia. Internship report. Bolivian Amphibian Initiative, Museo de Historia Natural Alcide d'Orbigny, Bolivia.

Stiffler D.F. (1993). Amphibian calcium metabolism. *Journal of Experimental Biology* 184, 47–61.

Stiftung-Artenschutz. (2015). The Titicaca Water Frog *(Telmatobius culeus)* is endemic to the Lake Titicaca Basin in the high Andes of Bolivia and Peru. This critically endangered species, also known as Lake Titicaca Frog, is among the most threatened amphibian species of the world. Internet reference: http://www.stiftung-artenschutz.de/projects/titicaca-water-frog/ (consulted on 05/03/2017).

Stuart S.N., Chanson J.S., Cox N.A., Young B.E., Rodrigues A.S., Fischman D.L., Waller, R.W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science*, 306, 1783–1786.

Stuart S.N., Hoffmann M., Chanson J.S., Cox N.A., Berridge R.J., Ramani P., Young B.E. (eds.) (2008). Threatened Amphibians of the World. First edition. Lynx Edicions, Barcelona, Spain; IUCN, Gland, Switzerland; and Conservation International, Arlington, Virginia, USA.
Published as a partnership between IUCN, Conservation International and Lynx Edicions.

Strzelewicz M.A., Ullrey D.E., Schafer S.F., Bacon J.P. (1985). Feeding insectivores: increasing the calcium content of wax moth (Galleria mellonella) larvae. *The Journal of Zoo Animal Medicine* 16, 25–27.

Tapley B., Bradfield K.S., Michaels C., Bungard M. (2015). Amphibians and conservation breeding programmes: do all threatened amphibians belong on the ark?. *Biodiversity and Conservation* 24, 2625–2646.

UNESCO. (2017). Lake Titicaca. Internet reference: http://whc.unesco.org/en/tentativelists/5080/ (consulted on 02/27/2017).

Van Paemel M., Dierick N., Janssens G., Fievez V., De Smet S. (2010). Selected trace and ultratrace elements: Biological role, content in feed and requirements in animal nutrition–Elements for risk assessment. *EFSA Supporting Publications* 7, 68E.

Vitt L.J., Caldwell J.P. (2013). Evolutionary history. In: Herpetology: an introductory biology of amphibians and reptiles. Volume 4. San Diego, CA, United States of America, p 1–83.

Wells K.D. (2010). The ecology and behavior of amphibians. University of Chicago Press. Chicago, United states of America. 1148 p.

Whiles M.R., Hall R.O., Dodds W.K., Verburg P., Huryn A.D., Pringle C.M., Lips K.R., Kilham S.S., Colón-Gaud C., Rugenski A.T., Peterson S., Connelly S. (2013). Disease-driven amphibian declines alter ecosystem processes in a tropical stream. *Ecosystems* 16, 146–157.

Wright K.M. (2001). Anatomy for the clinician. In: Amphibian Medicine and Captive Husbandry. Volume 1, Krieger Publishing Co., Malabar, FL, United States of America. p 15–30.

Zotti M., Del Coco L., De Pascali S. A., Migoni D., Vizzini S., Mancinelli G., Fanizzi F.P. (2016). Comparative analysis of the proximate and elemental composition of the blue crab Callinectes sapidus, the warty crab Eriphia verrucosa, and the edible crab Cancer pagurus. *Heliyon* 2, e00075.

Appendix:

1. ADDITIONAL RESULTS

- Nutrient composition of potential prey items

Nutrient composition of potential prey items, on a dry weight basis (DM):

NR	**Prey**	**GE**	**Ash**	**CP**	**Fat**	**Ca**	**Mg**	**P**	**S**	**K**	**Na**	**Cu**	**Fe**	**Mn**	**Zn**	**Se**	**Hg**
		kJ/g	**%**	**%**	**%**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**µg/kg**	**µg/kg**
1	*Austrelmis s.l.*	19.60	/	62.00	/	3810	1640	4771	3930	1970	1152	22	478	51	2322	193	146
2	*Austrelmis s.l* larvae	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
3	*Chironomidae* larvae	/	/	/	/	7266	2118	5285	4741	1895	1236	<10	2388	59	2754	169	77
4	*Corixidae*	/	/	/	/	2775	1301	11302	9273	6578	5011	14	237	13	153	382	125
5	*Girardia* sp.	20.80	/	58.20	/	4801	1435	12542	8392	5626	1377	<10	1578	11	1157	2256	125
6	*Helobdella s.l.*	20.60	/	65.30	/	3968	1262	8510	7746	4391	5349	<10	1349	18	6762	2659	308
7	*Hyalella* spp.	13.00	29.00	43.00	28.00	93426	2159	1363	6497	2072	3501	14	457	17	192	925	<65
8	*Heleobia* sp.	3.40	79.10	15.60	5.20	3015	1009	7107	6775	3364	3740	<10	898	14	3931	2223	251
9	*Orestias ispi*	20.90	13.30	56.90	29.80	35955	1332	2483	8580	7145	2466	<10	79	8	118	1504	74
10	*Orestias luteos*	15.90	18.30	52.60	29.10	52774	1622	3291	9176	10977	3610	<10	89	7	85	1099	192
11	*Biomphalaria* sp.	3.40	77.10	12.90	10.00	302128	833	2307	2386	636	1415	<10	1139	90	3766	386	<65
12	*Trichomycterus* sp.	22.40	11.60	48.90	39.50	28088	1079	1871	6940	2514	1098	<10	188	10	196	1100	322
13	*Trichoptera* larvae	18.50	/	50.30	/	15392	1652	7785	5770	4960	2698	<10	2090	76	1613	320	1175

Abbreviations: CP: Crude Protein, GE: Gross Energy, Ca: Calcium, Mg: Magnesium, P: Phosphorus, S: Sulfur, K: Potassium, Na: Sodium, Cu: Copper, Fe: Iron, Mn: Manganese, Zn: Zinc, Se: Selenium, Hg: Mercury.

Note 1: Insufficient samples of *Austrelmis s.l.* (adult and larvae), *Anisanculus* sp., *Chironomidae* larvae, *Corixidae, Girardia* sp., *Helobdella s.l.* and *Trichoptera* larvae were collected during the fieldwork of this study in order to conduct full nutritional analysis of these prey items.

Note 2: Certain nutrient concentrations of certain prey items were below the detection limit of the analytical method applied in this study; Copper levels of *Chironomidae* larvae, *Girardia* sp., *Helobdella s.l., Heleobia* sp., *Orestias ispi*, *Orestias luteos, Biomphalaria* sp., *Trichomycterus* sp. and *Trichoptera* larvae were below the detection limit of 10 mg/kg. Mercury levels of *Hyalella* spp. and *Biomphalaria* sp. were below the detection limit of 65 µg/kg.

- Contribution of prey items, for each nutrient, to a one kilogram diet of the Titicaca water frog

Contribution of prey items to the dietary ingestion of different nutrients of the Titicaca water frog, expressed in % of the total ingestion of those nutrients:

NR	Prey	GE	Ash	CP	Fat	Ca	Mg	P	S	K	Na	Cu	Fe	Mn	Zn	Se	Hg
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	*Austrelmis* spp.	0.12	0.00	0.11	0.00	0.00	0.06	0.10	0.04	0.05	0.02	0.13	0.50	0.15	0.10	0.01	0.10
2	*Hyalella* spp.	84.20	40.93	81.59	87.19	72.78	79.55	29.14	65.95	55.04	66.10	83.30	47.49	51.56	8.52	47.63	22.36
3	*Heleobia* sp.	9.23	46.78	12.40	6.78	0.98	15.58	63.65	28.81	37.44	29.58	12.46	39.10	17.79	73.07	47.96	72.37
4	*Orestias* ispi	3.88	0.54	3.09	2.66	0.80	1.41	1.52	2.49	5.44	1.33	0.85	0.25	0.70	0.15	2.22	1.46
5	*Biomphalaria* sp.	2.38	11.75	2.64	3.36	25.41	3.31	5.33	2.62	1.82	2.88	3.21	12.78	29.48	18.04	2.15	2.42
6	*Trichoptera* larvae	0.19	0.00	0.15	0.00	0.02	0.10	0.27	0.09	0.21	0.08	0.05	0.35	0.33	0.11	0.03	1.29

Abbreviations: CP: Crude Protein, GE: Gross Energy, Ca: Calcium, Mg: Magnesium, P: Phosphorus, S: Sulfur, K: Potassium, Na: Sodium, Cu: Copper, Fe: Iron, Mn: Manganese, Zn: Zinc, Se: Selenium, Hg: Mercury.

- Contribution of prey items to the nutritional intake of different nutrients in the wild diet of the Titicaca water frog

Contribution of prey items, for each nutrient, to a one kilogram diet of the Titicaca water frog. All values expressed in dry weight (DM):

NR	Prey	GE	Ash	CP	Fat	Ca	Mg	P	S	K	Na	Cu	Fe	Mn	Zn	Se	Hg
		kJ/kg	g	g	g	g	g	g	g	g	g	mg	mg	mg	mg	µg	µg
1	Austrelmis spp.	12.14	0.00	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.30	0.03	1.44	0.12	0.09
2	Hyalella spp.	8343.03	186.11	275.96	179.70	59.96	1.39	0.87	4.17	1.33	2.25	8.98	293.29	10.91	123.22	593.64	20.86
3	*Heleobia* sp.	914.24	212.69	41.95	13.98	0.81	0.27	1.91	1.82	0.90	1.01	1.34	241.47	3.76	1057.02	597.75	67.49
4	Orestias ispi	384.26	2.45	10.46	5.48	0.66	0.02	0.05	0.16	0.13	0.05	0.09	1.45	0.15	2.17	27.65	1.36
5	*Biomphalaria* sp.	235.63	53.43	8.94	6.93	20.94	0.06	0.16	0.17	0.04	0.10	0.35	78.94	6.24	261.00	26.75	2.25
6	Trichoptera larvae	19.00	0.00	0.52	0.00	0.02	0.00	0.01	0.01	0.01	0.00	0.01	2.15	0.07	1.66	0.33	1.21
	Total	9908.30	454.69	338.21	206.09	82.39	1.74	3.00	6.32	2.42	3.40	10.79	617.59	21.16	1446.50	1246.24	93.26

Abbreviations: CP: Crude Protein, GE: Gross Energy, Ca: Calcium, Mg: Magnesium, P: Phosphorus, S: Sulfur, K: Potassium, Na: Sodium, Cu: Copper, Fe: Iron, Mn: Manganese, Zn: Zinc, Se: Selenium, Hg: Mercury.

- Average count and proportional amounts of potential prey items caught in benthic traps

Average count and proportional amounts (%), of potential prey items of the Titicaca water frog, caught in benthic traps, per depth and in total, over a three month period, on the shores of Isla de la Luna:

		A	**B**	**C**	**Total**	**A%**	**B%**	**C%**	**Total %**
1	*Anisanculus*	0.80	0.60	0.47	0.62	0.16	0.28	0.30	0.25
2	*Austrelmis s.l.*	20.67	32.93	20.33	24.67	4.15	15.43	10.49	10.03
3	*Austrelmis s.l* larvae	20.07	8.20	5.00	11.09	3.71	3.79	2.73	3.41
4	*Chironomidae* larvae	30.07	25.20	19.67	24.98	5.46	2.19	10.27	9.31
5	*Corixidae*	0.07	0.07	0.20	0.11	0.01	0.03	0.09	0.04
6	*Girardia* sp.	16.80	22.93	38.67	27.13	3.49	11.42	20.67	11.86
7	*Helobdella s.l.*	8.67	6.80	2.93	9.47	1.66	3.19	7.78	4.21
8	*Hyalella* spp.	411.87	106.73	70.07	196.22	76.84	48.66	36.87	54.12
9	*Heleobia* sp.	20.93	6.53	13.73	13.73	4.10	3.16	8.32	5.19
10	*Biomphalaria* sp.	0.53	0.13	0.93	0.53	0.10	0.07	0.54	0.24
11	*Trichomycterus* sp.	0.13	0.07	0.07	0.09	0.03	0.04	0.04	0.04
12	*Trichoptera* larvae	1.13	3.40	2.80	2.44	0.23	1.67	1.51	1.14

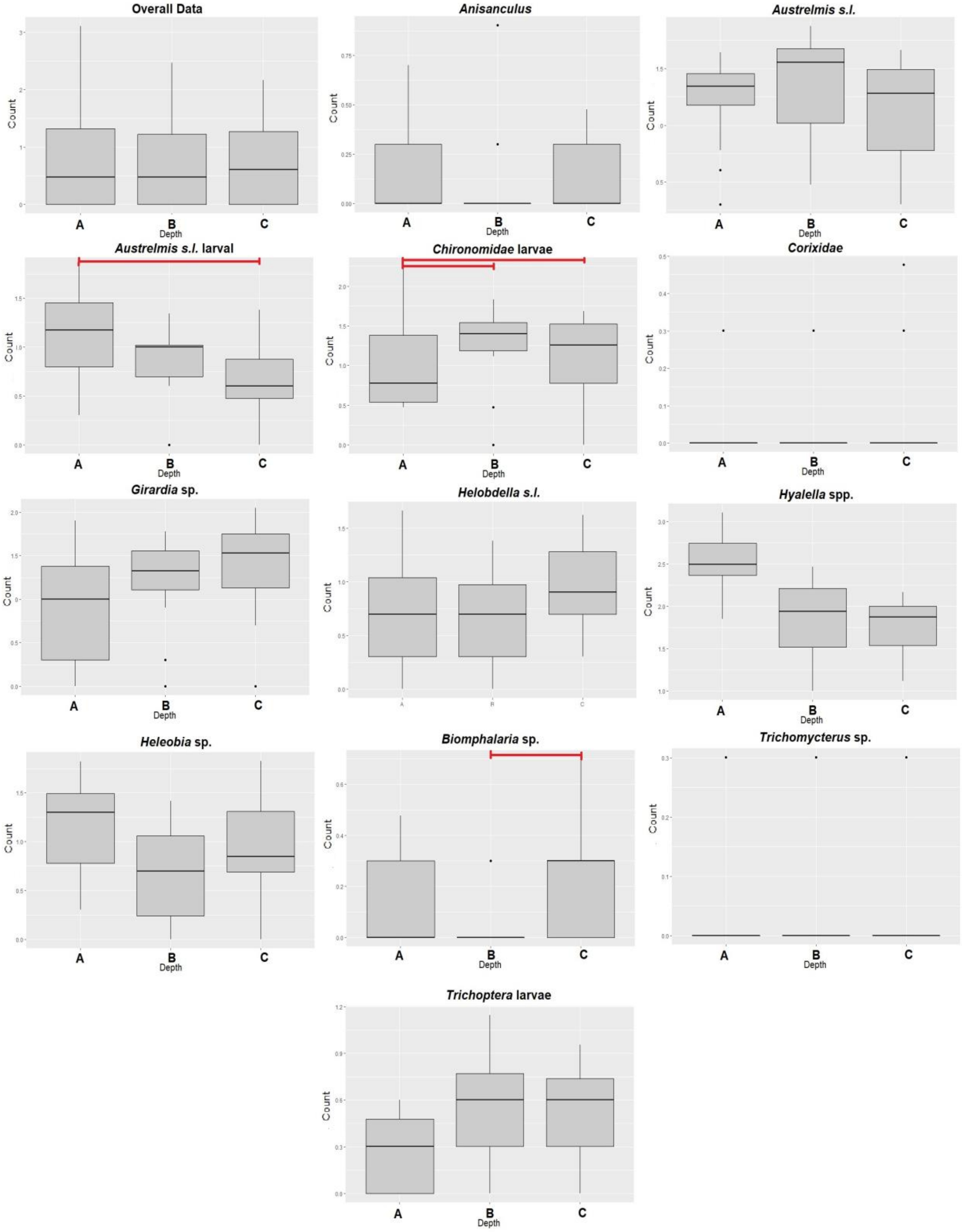
A= 0.5 m, B= 1 m, C= 1.5 m.

Note: Prey item abundance was calculated based on prey item count, not on mass.

2. SUPPORTING GRAPHS

- Boxplots of logarithmical transformed count of potential prey items per depth

Boxplots of logarithmical transformed count of potential prey items caught in benthic traps giver per depth:



A= 0.5 m, B= 1 m, C= 1.5 m.
Note: Red lines indicate significant differences between depths.

3. ILLUSTRATIVE MATTERIAL

- Pictures of potential prey items found on the shore of the Titicaca Lake

Pictures by Stéphane Knoll and Arturo Muñoz-Saravia.

NR	Common name	species	picture
1	Riffle Beetles (adult)	*Austrelmis s.l.*	
2	Riffle Beetles (larval form)	*Austrelmis s.l.*	
3	Nonbiting Midges (larval form)	*Chironomidae*	
4	Water Boatmen	*Corixidae*	

5	Flatworms	*Girardia* sp.	
6	Leeches	*Helobdella s.l.*	
7	Crustaceans	*Hyalella* spp.	
8	Gastropods	*Heleobia* sp.	
9	Gastropods	*Biomphalaria* sp.	

10	Caddisflies (larval form)	*Trichoptera*	

- Benthic-trap-design

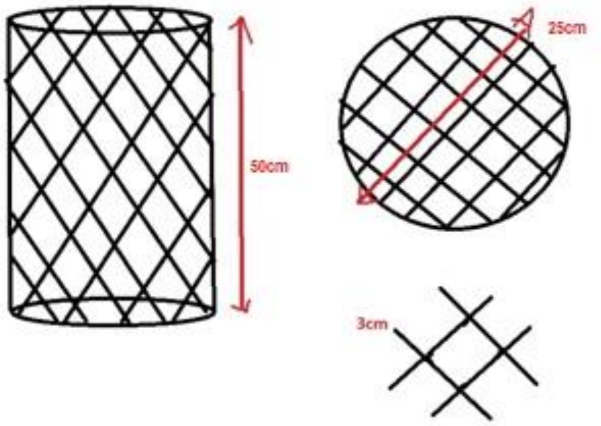


Illustration of the trap design used on the shoreline of the Titicaca Lake, Isla de la Luna, Bolivia.

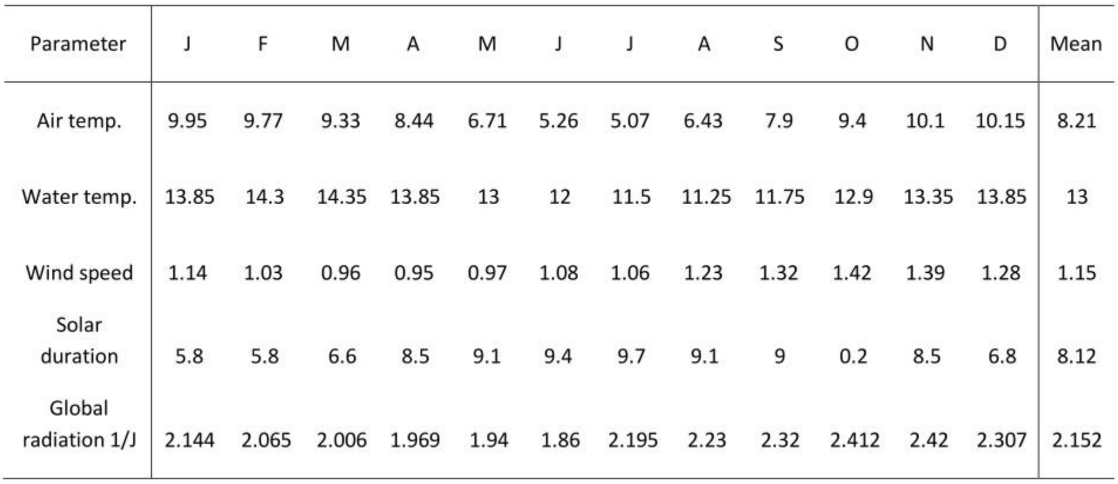
- Traps in place on the shoreline of Lake Titicaca

Illustration of traps in place on the shoreline of Lake Titicaca, Isla de la Luna, Bolivia, pictures by Stéphane Knoll.

4. ADDITIONAL INFORMATION

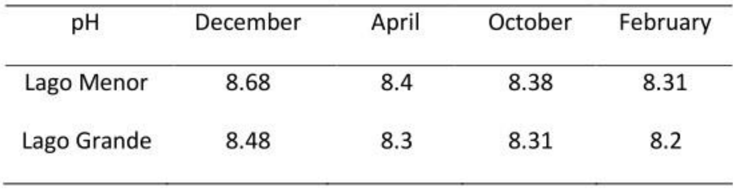
- Further specifications regarding various parameters of Lake Titicaca

Seasonal changes in air temperature, surface water temperature, wind speed, solar duration and global radiation, as found in Genova (2011).



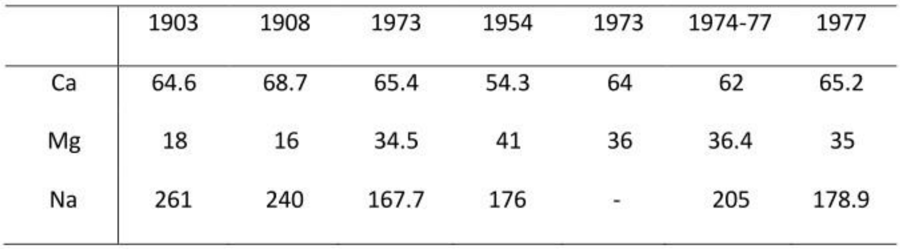
Parameter	J	F	M	A	M	J	J	A	S	O	N	D	Mean
Air temp.	9.95	9.77	9.33	8.44	6.71	5.26	5.07	6.43	7.9	9.4	10.1	10.15	8.21
Water temp.	13.85	14.3	14.35	13.85	13	12	11.5	11.25	11.75	12.9	13.35	13.85	13
Wind speed	1.14	1.03	0.96	0.95	0.97	1.08	1.06	1.23	1.32	1.42	1.39	1.28	1.15
Solar duration	5.8	5.8	6.6	8.5	9.1	9.4	9.7	9.1	9	0.2	8.5	6.8	8.12
Global radiation 1/J	2.144	2.065	2.006	1.969	1.94	1.86	2.195	2.23	2.32	2.412	2.42	2.307	2.152

Water acidity for Lago Menor and Lago Grande, Lake Titicaca, as found in Genova (2011).



pH	December	April	October	February
Lago Menor	8.68	8.4	8.38	8.31
Lago Grande	8.48	8.3	8.31	8.2

Amount of Ca, Mg and Na (mg/l) of the waters of Lake Titicaca during different years, as found in Genova (2011).



	1903	1908	1973	1954	1973	1974-77	1977
Ca	64.6	68.7	65.4	54.3	64	62	65.2
Mg	18	16	34.5	41	36	36.4	35
Na	261	240	167.7	176	-	205	178.9