THE DIETS OF CO-OCCURING ANURANS IN A SMALL SOUTH AFRICAN RIVER: ASSESSMENTS USING STOMACH CONTENTS, STABLE ISOTOPE RATIOS AND FATTY ACID PROFILES

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Likho Sikutshwa

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ABSTRACT

During their life cycle amphibians change their habitat and feeding, and are important consumers in both terrestrial and aquatic habitats. The trophic ecology of anurans has been studied intensively using gut content analysis, but very little work has been done using biochemical techniques such as stable isotope and fatty acid analyses. These biochemical techniques produce data that allow for additional inferences about the trophic ecology of anurans, as they reveal what the organisms assimilated over time rather than what was recently ingested. The investigation of the feeding ecology and trophic interactions of anurans using gut content analyses together with stable isotope and fatty acid analyses will help us to better understand their ecological roles. The objective of this thesis was to assess the feeding ecology of four anurans (*Amietia angolensis, Amietophyrnus rangeri, Strongylopus grayii* and *Xenopus laevis*) in a temperate river using a combination of gut content, stable isotope and fatty acid analyses. Frogs, tadpoles and potential food sources were sampled in two sites (upstream and downstream) in the Kowie River, Eastern Cape Province, South Africa.

Gut content analyses identified 147 prey items belonging to 12 prey orders in the stomachs of the sampled specimens. In both *A. angolensis* and *S. grayii* the most important prey category was Coleoptera, followed by Hemiptera, Diptera and Hymenoptera at both sites. Aquatic prey items (aquatic hemipterans, chirinomids and blackflies) were the most important food sources for *X. laevis* (Alimentary Index (IAi) = 6.4; 5.2; 4.2). In *Am. rangeri,* Hymenoptera was the most important prey category (IAi = 8.3). The trophic niche overlap between *A. angolensis* and *S. grayii* was biologically significant (> 0.6), and in the remaining species there was no significant trophic niche overlap. *Amietia angolensis* showed a larger trophic niche (Levin's measure; B = 7.7 and B_{st} = 0.84 downstream, and upstream B = 7.6 (B_{st} = 0.82) compared to the other species. The gut content analyses showed that frogs feed on a variety of prey items that constitutes food sources from both aquatic and terrestrial habitats. Stable isotopes indicated that aquatic derived sources contributed significantly more towards the diets of *X. laevis, A. angolensis* tadpoles and *S. grayii* tadpoles compared with the other anurans, whereas aquatic and terrestrial derived food sources contributed equally to the diets of *A. angolensis* and *S. grayii*. Increased trophic positions in *A. angolensis* and *S. grayii* occurred throughout their development. The four different species had

similar fatty acid profiles in the upstream region, and fairly similar $\delta^{13}C$ values, suggesting that they probably consumed similar food. Fatty acid profiles of anurans in the downstream region showed distinct separations among the species. Tadpoles had high levels of diatom-associated fatty acids (20:5ω3; *A. angolensis* tadpole – 8.4 %, *S. grayii* tadpole – 9.4 % upstream and downstream; 9.1 and 6.1 % total fatty acids (TFA), respectively). All four species had substantial contributions from bacterial fatty acids, and large proportions of saturated fatty acids (30.6 - 50.0 %) including those with 14 and 18 carbons, indicating that bacterial and detritus food sources played an important role in their diets. The fatty acid profiles revealed high proportions of polyunsaturated fatty acids (PUFAs) and essential fatty acids (EFAs) in all species, indicating a good quality of food and that the quality of food consumed was similar among species.

The results demonstrated the usefulness of a combination of traditional techniques (gut content techniques) and biochemical techniques (stable isotopes and fatty acid analysis) for assessing consumption and assimilation. The amphibian assemblages examined derived much of their energy from terrestrial and aquatic sources. This information will allow more precise and comprehensive assessments of trophic interactions in freshwater habitats, along with aiding in future amphibian conservation and management efforts.

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LIST OF ABBREVIATIONS

ai-	*anteiso-*
ANOSIM	analysis of similarity
BAFA	bacterial fatty acid (s)
BHT	butylated hydroxytuluene
C	carbon
CPOM	coarse particulate organic matter
DM	dry mass
EFA	essential fatty acid (s)
FA	fatty acid (s)
FAME	fatty acid methyl ester
GC	gas chromatography
GCA	gut content analyses
GC-MS	gas chromatography-mass-spectrometry
HPFA	higher plant fatty acid (s)
i-	*iso-*
MUFA	monounsaturated fatty acid (s)
N	nitrogen
n-MDS	non-metric multidimensional scaling
PCA	principal component analyses

PUFA	polyunsaturated fatty acid (s)
SD	standard deviation
SIA	stable isotope analysis
SIAR	stable isotope analysis in R
SIMPER	similarity percentages
SFA	saturated fatty acid (s)
SPM	suspended particulate matter
SVL	snout-vent length
TFA	total (identified) fatty acid (s)

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Now this is the hardest part- if you can be sure of one chapter of your thesis that will be read by everyone and I mean EVERYONE, well this is it! Most importantly, this thesis represents to me a beautiful struggle and journey through which I have acquired knowledge and life lessons that will forever stay with me. It has indeed been an unforgettable journey but worth it!

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DEDICATION

This work is dedicated to my mom (Lumka Sikutshwa) for believing in me and cheering me on.

"It always seems impossible until it's done"

Nelson Rholihlahla Mandela

DECLARATION

I, the undersigned declare that this thesis submitted to Rhodes University, Grahamstown, South Africa, for the degree of Master of Science and work contained herein is my original work carried out under the supervision of Dr Nicole B Richoux and Dr Dan Parker, and unless cited has not been submitted to any other University for any degree.

1 GENERAL INTRODUCTION

1.1 FOOD WEB ECOLOGY

Food webs portray trophic interactions among species or individuals in an ecosystem (Post 2002) and they are composed of multiple food chains or linear routes to the top predators (Pimm 2002). Because they give insight into population dynamics, community ecology (Polis & Strong 1996; Wilbur 1997), and ecosystem level processes (DeAngelis 1992), food webs are a central concept in ecology. Winemiller (1990) described food webs as being spatially and temporally variable. According to Polis et al. (1997) and Nowlin (2007), food webs are influenced by the surrounding landscapes, and by the movement of nutrients, energy, and organisms within and across habitats. Food web dynamics may be influenced by abiotic and biotic factors, including changes in trophic structure, energy and nutrients (Bengtsson & Martinez 1996).

1.2 STABLE ISOTOPES IN TROPHIC ECOLOGY

Food webs track the flow of energy through a community. Direct feeding observations, gut content analysis, stable isotope analysis, and fatty acid analysis can be used in food web ecology to understand links between resources and consumers. Stable isotopes provide information on broad influences of diet and trophic relationships. Ecologist are increasingly using stable isotopes to describe, quantify and understand food web structure and function in marine (Davenport & Bax 2002), freshwater (Cabana & Rasmussen 1994), estuarine (Alfaro et al. 2006), and terrestrial (Sanzone et al. 2003) ecosystems. Stable isotopes are naturally abundant and can effectively trace energy flow within and between ecosystems. Carbon isotopic signatures provide information about the original sources of carbon from primary producer to consumer (Post 2002; Layman et al. 2012), because different energy sources often show distinct carbon ratios with little fractionation up the food chain (Schriever & Williams 2013). Stable nitrogen ratios are generally used as reliable indicators of consumer trophic position as they predictably increase through trophic transfer up the food chain (Cabana & Rasmussen 1994; Vander Zanden & Rasmussen 1999) in a food web. Trophic position can be described as a continuous measure of diet estimating an organism's place within a food web.

Gut content analysis and direct feeding observations have been used in studies of food web dynamics to describe the diet and trophic position of individuals and populations, from which inferences about their ecological roles can be made (Newsome et al. 2007). However, these techniques have some inherent limitations, such as requiring large sample sizes, and that they provide only snapshots of what the organism has recently ingested (Schriever & Williams 2013). Stable isotope analysis can be used independently and /or complementary with other more traditional techniques (Jefferson & Russell 2008), because stable isotopes integrate not only what food was ingested but also what has been assimilated in the tissue over longer periods (Peterson & Fry 1987). It is possible to determine an organism's ecological role by integrating information from stable isotope and stomach content analyses.

1.3 FATTY ACID SIGNATURES IN TROPHIC ECOLOGY

Like stable isotope signatures, fatty acid analysis provides data on assimilated food rather than recently ingested food by consumers. Since fatty acids (FA) can be traced through several trophic levels, they provide information about the prey sources and productivity at the base of the food web (Ruess et al. 2004). According to Arts et al. (2009), FAs are required for maintaining the structure and function of cell membranes and are therefore vital for growth and reproduction. Since some FAs cannot be efficiently synthesized by animals but are important for proper functioning of organs, these must then be obtained from their diet (Olsen 1999; Parrish 1999). These essential fatty acids (EFAs; 20:4ω6, 20:5ω3 and 22:6ω3) are produced by autotrophs and tend to be specially conserved in food webs (Arts et al. 2009). This retaining and transference of specific components through the food chain permits the use of FAs as biomarkers. Tocher (2003) reported that dietary FAs like ω3- and ω6-forms can be converted by some consumers into longer chain FAs with more double bonds. Because of these changes it can be difficult to assign specific FAs to specific food sources. Hence, the ideal biomarkers are those synthesized at the base of a food web that continue without major changes into higher trophic levels (Napolitano et al. 1997; Dalsgaard et al. 2003). Different primary producers and heterotrophs have distinct FA profiles and these can be used as signatures to describe feeding interactions (Graeve et al. 1997; Budge et al. 2006). As a result, FAs can be used to determine trophic interactions and the sources of lipids assimilated over a period of time (Graeve et al. 1994; Dalsgaard et al. 2003)

Fatty acid analysis has been successfully used in many ecological studies to aid in the determination of organism diets in a variety of environments (Iverson et al. 2004) including freshwater (RamíRez et al. 2009), marine (Budge et al. 2006; Beck et al. 2007; Iverson et al. 2007), and terrestrial (Ruess et al. 2005) systems. Various possible food sources have specific FAs which are ingested and assimilated by consumers (Kharlamenko et al. 2008).

Because both traditional methods (e.g. stomach content analysis) and biomarker approaches (e.g. fatty acid and stable isotope analyses) have some limitations when used independently, FA biomarkers are better used in combination with other techniques (Alfaro et al. 2006). A limitation to the stomach content analysis is that it reflects recently ingested food, there may be biases from differential digestion rates of different prey, and there is a high probability of inaccurately reflecting long term dietary patterns (Gillespie 2013). One of the disadvantages of stable isotopes is the variation in trophic shift that usually takes place among consumers which may result in bias when defining the contributions of food sources to consumer diets (McCutchan et al. 2003). Some of the disadvantages of the FA technique are the non-specific nature of most FAs as few organisms or group of organisms have unique FAs (Garrett & Grisham 1999). Furthermore, the processes for lipid digestion and FA metabolism may vary (Garrett & Grisham 1999). However, these three approaches can be used to provide complimentary information on food sources and feeding dynamics of organisms in different ecosystems.

Thesis overview

Amphibians influence ecosystem processes in freshwater systems through their feeding activities (Wissinger et al. 1999; Davic & Welsh. 2004; Whiles et al. 2006; Regester et al. 2008). A study by Gibbson et al. (2006) illustrated that a single 10ha wetland produced > 360,000 metamorphic amphibians in one year, suggesting that energy and nutrient subsidies may be greater than previously realized in some habitats. Such high abundances, together with the voracious appetites of many amphibian species and their high conversion efficiencies (Grayson et al. 2005), enable them to exert a substantial influence on the flow of energy and nutrients through aquatic and terrestrial food webs. However, there are knowledge gaps regarding the ecological roles that amphibians play in connecting the aquatic and terrestrial habitats. Given the lack of information on the energy flow between aquatic and terrestrial habitats via amphibian populations, and the continued development of biomarker techniques for assessing diets and trophic status, I performed

my study using several amphibian species as models to investigate amphibian trophic positioning at a freshwater/terrestrial interface.

The overall aims of my study were to:

1. assess the ontogenetic dietary shifts of several frog species in a temperate river using stable isotopes and fatty acids
2. assess how the feeding activities of the frogs contribute to cross-habitat subsidies by determining their dietary compositions.

For the first aim, I hypothesized that there is an increase in trophic position as tadpoles metamorphose into adult frogs, and also changes in the relative importance of diet items. I hypothesized that the adult frogs are generalist predators and ingest a wide variety of prey of which terrestrial invertebrates make up a large proportion of the diet.

For the second aim, I hypothesized that there is a transfer of energy between aquatic and terrestrial habitats and that frogs utilize resources from both aquatic and terrestrial habitats, thereby creating an important link between aquatic and terrestrial ecosystems.

The structure of the thesis:

Chapter 2 includes the description of the study area and the methods and materials used. The feeding ecology and dietary overlap of the frog species found in the Kowie River were explored in Chapter 3. Chapter 4 explores the dietary shifts from tadpoles to adult frogs using stable isotopes, and Chapter 5 explores the trophic relations of amphibians using fatty acid analysis, with the general aim towards assessing the role of amphibians in linking aquatic and terrestrial habitats. Chapter 6 includes the general discussion and conclusions.

2 GENERAL METHODS AND MATERIALS

2.1 STUDY AREA

The study was conducted in the Kowie River, South Africa (Figure 1). The river originates south of Grahamstown (33º 36' 11" S; 26º 54' 10" E) and flows in a southeasterly direction for approximately 70km, with an average depth of 2.6 m (Whitfield 2000). The climate is warm and temperate with average annual minimum and maximum temperatures of 12.7º C and 23.3ºC, respectively (Whitfield 2000). Whitfield (2000) described a bimodal rainfall pattern occurring during summer and autumn for this region. The river is a temperate permanently open system with a catchment area of about 580 km^2 (Watling & Watling 1983).

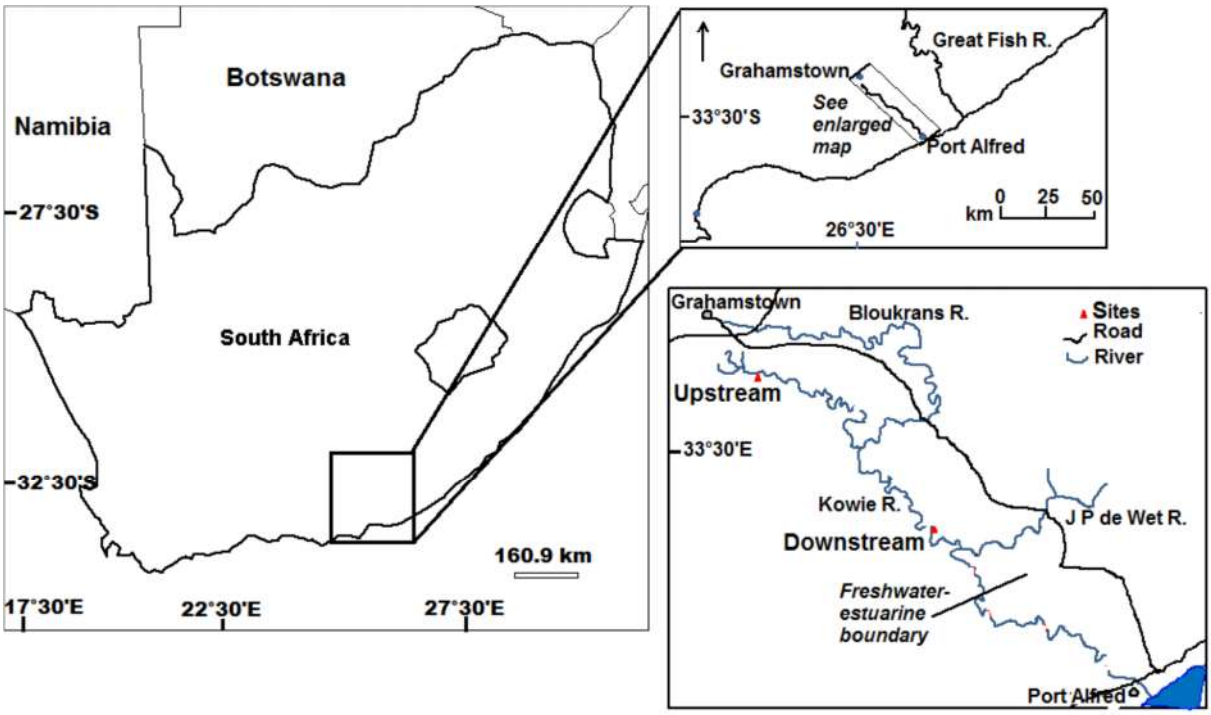


Figure 2.1 Map showing the Kowie River and its location in South Africa (Dalu et al. 2014)

The collections of frogs and tadpoles were made from two locations within the Kowie River (Figure 1); upstream (33º 20' 59.2" S; 26º 33' 37.6" E) and downstream (33º 30' 16.0" S; 26º 44' 40.9" E). Sampling took place monthly between November 2012 and June 2013. Collections were made from each site twice per month. Sites were selected based upon their accessibility and habitat characteristics. The first site was located upstream, with a mean depth of 34.4 ± 7.7 cm and stream width of 1.5 ± 0.36 m, and was surrounded by dense alien vegetation (*Latana camara* and *Eucalyptus* spp.). The second site was downstream and was surrounded mostly with natural vegetation (with *Vachellia karroo* being the dominant plant species) in the form of low-growing trees above a ground flora of grass and scattered herbs. The downstream site had a mean depth of 64.8 ± 8.2 cm and stream width of 11.3 ± 4.9 m. Both sites had a narrow band of dense *Cyperus* sp. alongside the water edges.

2.2 AMPHIBIAN AND INVERTEBRATE COLLECTION

2.2.1 Ethics statement

The capture and euthanasia of frog specimens was granted clearance by the Rhodes University Ethical Standards Committee (ethical clearance number ZOOL-15-2012).

2.2.2 Amphibian collections

A prospective survey was conducted in early November 2012 to confirm the presence of frogs in the Kowie River. Following confirmation of frog presence, tadpoles and adult *Amietia angolensis, Strongylopus grayii, Amietophyrnus rangeri* and *Xenopus laevis* were collected from each sampling site. At least three individuals per species per site were collected for processing. The study sites were actively searched for frogs. Collections started two hours after sunset (as most anurans are nocturnal) and were done with the aid of headlamps and sweep nets. Specimens were randomly searched for in pools, along pools and stream edges, under decaying logs near the river, and under leaf litter on the river bank (i.e. all potential microhabitats close to the river). Tadpoles were collected during the day by dip-netting standardized to 15 minutes per site (Conradie et al. 2011).

In addition to active searches, traps were used to aid in collection of purely aquatic specimens (*Xenopus laevis*). Three array design drift-fences made from green shade cloth and two open-ended

funnel traps (Drechsler et al. 2010) (Figure 2) were deployed at the sites for 72 hours. The length of the fence was 3 m and it was attached to 0.5 m high metal stack rods with cable ties.

Figure2.2 A drift fence with two open-ended funnel traps (designed after Willson & Gibbons 2010).

2.2.3 Frog census

To confirm the presence of frog species in the study area, recording of frog calls was done at night during sampling occasions, roughly within the period of 19:00 - 23:00. A multi-function stereo recorder (model: Olympus WS-811) was used to record frog calls. A microphone protector was placed over the recorder's microphone to protect it from moisture and reduce the interference of wind. The recordings were filtered using Raven Lite 1.0 software to cut out noise. Calls from the recordings were identified using an audio compact disc (Du Preez, & Carruthers, 2009).

2.2.4 Prey sampling

Potential basal food sources (benthic algae, suspended particulate matter, epiphyton) for tadpoles were sampled in a concurrent study (T. Dalu, published in Bergamino et al. 2014) at both sampling locations. Adult frogs are generalist feeders with opportunistic foraging behaviours (Santos et al. 2004). The main prey items in the adult diet typically consist of invertebrates including annelids,

arachnids, molluscs and arthropods (Santos et al. 2004; Najera-Hillman et al. 2009), so these potential prey items were sampled in concurrent studies (S. Moyo and L. Chari, unpublished data) at both sites. Any comparisons of tracer data in the amphibians that I collected to potential prey utilized the data derived in these companion studies.

2.3 LABORATORY TECHNIQUES

2.3.1 Morphology and sample treatment

Measurements of the snout vent-length (SVL) were taken from each frog specimen using Vernier calipers (nearest 0.01 mm), as well as total body length for tadpoles. All frogs were identified to species level and tadpoles to family level using a field key (Du Preez, & Carruthers 2009). Specimens were euthanized in an aqueous solution of buffered tricaine methanesulfonate (MS-222) (Kupfer et al. 2005; Conradie et al. 2011; Schriever & Williams 2013). Muscle tissue from the thigh was cut from each specimen using fine forceps and scissors. The frog muscles and tadpole (tail muscle) samples were placed in separate aluminium foil envelops, labeled and stored at -80 ^{o}C. Frog muscle tissues and tadpole tails were used for both stable isotope and fatty acid analyses. Samples were freeze dried (VirTis Bench Top 2K) for 48 hours and homogenized into fine powder with a mortar and pestle.

2.3.2 Gut Content Analysis (GCA)

Recent ingestion by adult frogs was assessed through stomach contents analysis. Stomachs were removed through a longitudinal abdominal incision, preserved in 10% formalin and later transferred to 70% ethanol (França et al. 2004; Rodrigues et al. 2004). Prey items were counted and identified to the lowest taxonomic level possible (order and family where possible, although some items like Hymenoptera were identified to family; ants were identified to Formicidae, hence all subsequent mention of Hymenoptera excludes ants) using a dissecting microscope and a key (Picker et al. 2004). Depending on its stage of development and the habitat in which it occurred, each prey type was classified as terrestrial, aquatic, or both (Gerber & Gabriel 2002). Larval aquatic insects were classified as aquatic, while flying adults were considered terrestrial. Individual adult frogs were sexed by examination of the gonads (Rodrigues et al. 2004).

2.3.3 Stable Isotope Analysis (SIA)

Approximately 1 mg of homogenized sample tissues was weighed using a Mettler Toledo XP205 balance with a precision of 0.00001 g and placed in tin capsules. Analysis of stable isotopes was carried out on a Europa Scientific 20-20 IRMS linked to ANCA SL prep unit at Iso Environmental cc, Grahamstown. For internal calibration, standards (beet sugar and ammonium sulfate, and a certified protein standard Casein which was calibrated against IAEA-CH-6 and IAEA-N-1) were processed. Stable isotopes values were reported in delta notation, $\delta^{13}C$ or $\delta^{15}N$, using the equation $\delta^{15}N$ or $\delta^{13}C = [(R_{sample}/R_{standard})-1] *1000$, where R is ^{13}C or ^{15}N and R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios of the sample and standard, respectively.

2.3.4 Fatty acid Analysis

Lipid Extraction- Lipid extraction was done using methods based on Folch et al. (1957) (with modifications by N.B. Richoux; see Bergamino & Richoux 2014). Frozen samples were freeze dried for 24 to 48 hours, and manually ground into fine powder with a mortar and pestle. Samples were weighed (approximately 50 - 60mg) into 15 ml test tubes and a known quantity of standard (19:0) was added to each sample. Tubes were topped with 2 ml of chloroform ($CHCl_3$) with 0.01% butylated hydroxytoluene, flushed with a gentle stream of nitrogen (N_2), sealed with Teflon tape and stored at -20 ºC. One ml of ice-cold methanol (MeOH) was added to each sample, then the sample was vortexed, sonicated for 4 minutes in an ice bath, and vortexed again. The samples were covered with a gentle stream of N_2 and stored at -20 ºC for 24 hours. The contents were then carefully decanted through a pre-rinsed cotton wool plugged pipette wetted with 2:1 $CHCl_3$:MeOH. The test tube was rinsed twice with 1 ml of 2:1 $CHCl_3$:MeOH. A solution of 0.9% potassium chloride (KCl; 1.5 mL) was added to the second test tube. Samples were vortexed for 30 seconds and centrifuged for 3 minutes at 3000 rpm, after which the top aqueous layer was removed and discarded. The KCl step was repeated once with 0.5 ml of 0.9% KCl and 0.5 ml of MeOH. Anhydrous sodium sulfate ($NaSO_4$) was added to each tube and contents were then poured through a pipette plugged with cotton wool and $NaSO_4$. The tubes were rinsed twice with $CHCl_3$. The extract in the third tube was then concentrated to dryness under a gentle N_2 stream and covered with 0.5 ml of dried $CHCl_3$. All tubes were topped with N_2, sealed with Teflon tape and stored at -20 ºC.

Column chromatography- Methods from Budge et al. (2006) were used during the column separation procedure. Neutral lipids were separated from polar lipids by column separation. All solvent solutions used during column chromatography were dried with $NaSO_4$ and dried stock solutions of MeOH and $CHCl_3$, 98:1:0.5 $CHCl_3$: MeOH: formic acid and methylene chloride (DCM) were prepared. Pipettes plugged with glass cotton wool used during the lipid separation were ashed in a muffle furnace for 5 hours at 450 ºC. Silica gel (0.8 g) was placed in each pipette and it was activated by placing the pipettes in an oven at 100 ºC for an hour. These were cooled for at least 30 minutes before use. Six ml of MeOH were passed carefully through the column into a waste beaker. As the last of the MeOH reached the top of the silica gel, 6 ml of $CHCl_3$ was added to the column. The previous step was repeated using 3 ml of 98:1:0.5 $CHCl_3$: MeOH: formic acid, and when the solvent was 1.5 cm above the silica gel, the waste beaker was switched with a 15 ml vial and 3 ml of 98:1:0.5 $CHCl_3$: MeOH: formic acid was passed through. When the solvent mixture reached the silica gel, the sample extract was added and rinsed through with $CHCl_3$. The resultant extract was the neutral lipid fraction of the sample, which was evaporated to dryness and topped with 1.5 ml of DCM. All samples were covered with nitrogen gas and sealed with Teflon tape until fatty acid methyl ester (FAME) synthesis.

FAME synthesis- FAMEs were prepared following methods modified from Budge et al. (2006). The lipid extracts suspended in 1.5 ml DCM were topped with 3 ml of Hilditch reagent (1.5 ml of concentrated sulfuric acid added to 100 ml of dried MeOH), covered with N_2, sealed with Teflon tape, vortexed, and placed in an oven at 100 ºC for one hour. The test tubes were then cooled to room temperature and 3 ml of hexane was added followed by 1 ml of milliQ water. Samples were vortexed and centrifuged for 5 minutes at 3000 rpm. After centrifugation, the top layer was removed and kept in a second test tube, after which the hexane step was repeated once using 1 ml of hexane. Two ml of milliQ water was added to the pooled extraction, and samples were vortexed and centrifuged for 2 minutes. Following centrifugation the top layer was removed to a third tube and anhydrous $NaSO_4$ was added to each sample, gently shaken and allowed to settle. This extract was then evaporated down to approximately 2 ml under a gentle N_2 stream and transferred to a 2 ml vial. The tubes were rinsed with 1 ml of hexane and added to the 2 ml vial. The extract was concentrated to dryness under a stream of N_2 and covered with 0.5 ml of hexane. All vials were flushed with N_2 and sealed with Teflon tape until injection into the gas chromatograph (GC).

Fatty acid composition of each sample was determined using an Agilent 7890A GC fitted with a ZB Waxplus 320 column and a flame ionization detector. With the oven set at 150 ºC, 1 µl of each FAME sample was automatically injected (inlet temperature set at 250 ºC). The oven temperature was increased to 225 ºC at 2.5 ºC min^{-1} after 5 minutes for a total run time of 40 minutes. Helium was the carrier gas. The peaks were integrated using Chemstation. Peak identities were confirmed using Agilent 7000 GC/MS-QQQ together with a NIST MS library, and by comparing retention times of known external standards (Supelco, 37 component FAME mix, marine PUFA no. 1). Each fatty acid was measured as a proportion of the total identified fatty acids (% TFA). Fatty acids were reported in the shorthand form x: aωb, where *x* is the number of carbon atoms in the acyl chain, *a* is the total number of double bonds, and *b* is the position of the first double bond from the methyl end of the molecule.

3 DIETARY PATTERNS OF FOUR ANURAN SPECIES FOUND ALONG THE KOWIE RIVER EASTERN CAPE, SOUTH AFRICA

3.1 INTRODUCTION

Anurans are commonly described as generalist predators with opportunistic foraging behaviour (Santos et al. 2004). Adults are predators of invertebrates, including annelids, centipedes, millipedes, arachnids and especially insects (Toft 1981; Duellman 1994; Santos et al. 2004), while juveniles are detritivores and herbivores (Altig et al. 2007). Larger frogs may sometimes eat small vertebrates such as fish, birds, rodents, and other frogs (Duellman 1994). Some frogs, however, show some degree of diet specialization (Simon & Toft 1991). For example, many dendrobatid and some bufonid species are myrmecophagous (specialized for eating ants), and they consume ants in higher proportions than what is found in the surrounding environments (Toft 1980a, 1981).

Energy from invertebrates (mostly detritivores) to higher trophic levels can be channeled through amphibians, making these organisms important linking components of ecosystems (Burton & Likens 1975; Cogalniceanu et al. 2001). Detailed dietary information is crucial for the understanding of anuran life histories, the position of anurans in the trophic network, and the ecological role of anurans in different ecosystems (Anderson et al. 1999; Cogalniceanu et al. 2001). Animal diets are usually confirmed by direct observation of their feeding habits and by analysis of stomach content and faeces (Dalerum & Angerbjörn 2005). Because these data provide information only on food consumed during a short period of time, they provide limited results in some cases (Ruess et al. 2004). Despite these limitations, stomach content analysis may provide valuable quantitative measurements of what was ingested. In addition, this technique may be used to derive information on the taxonomy and size composition of diets and predatory-prey interactions in complex systems where species consume a diversity of prey items. For example, Santos et al. (2004) used stomach content analysis to study the feeding habits of six anuran species in a rainforest fragment in Northern Brazil and found that the species showed generalist feeding behaviours. Similarly, França et al. (2004) examined trophic and spatial niches of two large species of *Leptodactylus* in Southeastern Brazil and identified a diverse range of prey consumed by these two species. Prey included subaquatic organisms to terrestrial species, indicating that the anurans were generalist and/or opportunist predators

(França et al. 2004). In another study by Guidali et al. (2000), analysis based on stomach contents was used to determine the diet and trophic niche overlap of two ranids (*Rana dalmatina* and *R. synkleton esculenta*). These researchers identified a greater trophic niche breadth in *R. dalmatina* than *R. synkleton esculenta*, although both species were designated as opportunistic predators (Guidali et al. 2000).

Stomach content data were gathered to analyze the feeding habits of four anuran species inhabiting the Kowie River. The common platanna (*Xenopus laevis*) is ubiquitous in sub-Saharan Africa and commonly restricted to aquatic habitats (natural or man-made; Du Preez & Carruthers 2009; Measey et al. 2012). The common river frog *Amietia angolensis* and the clicking stream frog *Strongylopus grayii* (family Pyxcephalidae) live in close proximity to water on the banks of slow-flowing streams and other permanent water bodies. The raucous toad *Amietophrynus rangeri* favours terrestrial habitats. The main objectives were to determine the interspecific differences in the type, quantity and relative importance of particular food items consumed by the anuran populations.

3.2 MATERIALS AND METHODS

3.2.1 Study area, sample collection and sample treatment

Detailed descriptions of the study area, sample collection and sample treatment are provided in Chapter 2.

3.2.2 Data analysis

3.2.2.1 Quantifying diet variation

The diet was quantified as the frequency of occurrence (*%F*), which is the number of stomachs containing a specific prey item as a percentage of all sampled stomachs, and numerical abundance (*%N*), which represented the abundance in the percentage of a prey item in relation to the total abundance of all stomachs (Hyslop 1980). These parameters were used to estimate the importance of each prey item in the diet of a frog population based on the Alimentary Index (IAi) according to the formula (Kawakami & Vazoler 1980);

$$\text{IAi} = \%F \times \%Ni / \Sigma\ (\%F \times \%Ni) \times 100)$$

To estimate the trophic niche and breadth, I used the Levin's measure (B and $B_{st} = B$ standards; Krebs 1989; França et al. 2004), calculated as:

$$B = 1/(\sum_i^n p_i^2)$$

where p = the proportion of the total food item in food category i and n = the number of food categories. Niche overlap was calculated using the symmetrical measure first used by Pianka (1973):

$$\hat{O}_{jk} = \frac{\sum_i^n P_{ik}P_{ij}}{\sqrt{\sum_i^n P_{ij}^2 \sum_i^n P_{ik}^2}}$$

where $\hat{O}_{jk}$ = Pianka's measure of niche overlap between species j and species k, P_{ij} = the proportion of resource i of the total resources used by species j, P_{ik} = the proportion of resource i of the total resources used by species k, and n = the total number of resource states. The value of $\hat{O}_{jk}$ varies between 0 (no common resources) and 1 (perfect overlap). The values of the overlap index greater than 0.60 were considered biologically significant (Labropoulou & Eleftheriou 1997).

3.3 RESULTS

Among 82 adult frog individuals collected (Table 3.1), seven had stomachs up to 25% full, ten were 50% full, 12 were partially full (75%), and 23 specimens had full stomachs (100%). Twelve individuals had no prey in their stomachs, and nine specimens had food items in advanced stages of digestion and were not identifiable. A total of 147 prey items belonging to 12 prey orders was identified (Table 3.2 & 3.3). The prey items in the stomach contents of *Amietia angolensis* and *Strongylopus grayii* consisted of aquatic and terrestrial invertebrates. Araneae, Diptera, Coleoptera, Hemiptera, and Hymenoptera were the most frequent food items (%F 16.2; 15.3; 11.2; 10.2 and 9.1, respectively) in the stomach contents of *A. angolensis* upstream and constituted the most important food items in the diet of *A. angolensis* (IAi = 2.0; 2.6; 2.8 and 1.1, respectively). Similarly, downstream, Araneae, Diptera, Coleoptera, Hemiptera, and Hymenoptera were the most frequent prey items in the stomach contents of *A. angolensis* (%F = 12.5; 13.2; 14.3; 11.4, and 9.7, respectively). The most important food items in the diet of *S. grayii* upstream were Coleoptera, Hemiptera, Araneae, and Hymenoptera (Formicidae) (IAi = 5.7; 1.7; 1.6, and 1.6, respectively). Downstream, the most frequent food items constituting the most important food items in diet of *S. grayii* were Coleoptera, Diptera,

Araneae, and Hemiptera (Table 3.3). Orthoptera, Odonata, Blattodea, and Collembola were also conspicuous prey in the diets of *A. angolensis* and *S. grayii* (Table 3.2 & 3.3). In *Amietophyrnus rangeri*, all prey items were terrestrial invertebrates, and only aquatic invertebrates were identified in the stomach contents of *Xenopus laevis* (Table 3.2 & 3.3).

Table 3.1 Total number of specimens in each species (and family) of frogs collected from the upstream and downstream sites

Species	**Habitat**	**Number of frogs**	
		upstream	**downstream**
Bufonidae			
Amietophyrus rangeri	Terrestrial	0	4
Pipidae			
Xenopus laevis	Aquatic	10	9
Pyxicephalidae			
Amietia angolensis	Semi-aquatic	20	17
Strongylopus grayii	Semi-aquatic	11	11
TOTAL		**41**	**41**

Diet composition varied among the four frog species analyzed. *Xenopus laevis* collected upstream consumed dipterans (Chirinomidae and Simuliidae), Hemiptera, and Ephemenoptera (mayfly larvae), with mayflies, aquatic hemipterans, chirinomids and blackflies constituting the most important food items in the diet (IAi = 6.4; 5.2; 4.2, and 3.7, respectively). Downstream, the *X. laevis* specimens had Ephemenoptera, Simuliidae and Chirinomidae as the most important prey in the diet (IAi = 9.6; 7.1 and 5.0, respectively), aquatic hemiprerans and coleopterans, and Baetidae were also noticeable prey in the diet (IAi: 2.3; 2.1, and 1.3, rescpectively). *Amietophrynus rangeri* fed mostly on terrestrial insects, with Formicidae being the most frequent food item in the stomach contents (*%F* = 30.0) followed by Hymenoptera (non-Formicidae) (*%F* = 20.0) and beetles (*%F* = 16.7), and the alimentary index showed these food items to be an important food source for this species (IAi: 8.3; 3.4, and 2.2, respectively). Ingestion of plant material (such as twigs, small leaves, grass, etc.) was recorded in 21 stomachs (8 at the downstream site and 13 at the upstream site) of *A. angolensis* and *S. grayii*. This plant material made up a small percentage of each stomach (~2-4%). In *X. laevis*, some stomachs had traces of sediment (<5%) and algae (<3%).

Table 3.2 Alimentary Index (IAi) of the stomach contents of the frogs collected upstream along the Kowie River. SVL= Snout vent-length

		A. angolensis			*S. grayii*			*X. laevis*		
n		15			9			10		
Mean SVL (mm)		49.14			35.00			42.29		
Mean mass (g)		15.81			6.52			11.56		
Prey items	*Common names*	*%F*	*%N*	IAi	*%F*	*%N*	IAi	*%F*	*%N*	IAi
Araneae	Spiders[t]	16.21	12.50	2.03	22.30	7.14	1.59	0.00	0.00	0.00
Coleoptera-A	Beetles[a]	11.20	25.00	2.80	0.00	0.00	0.00	16.25	15.63	2.54
Coleoptera-B	Beetles[t]	10.25	4.16	0.43	20.05	28.57	5.73	0.00	0.00	0.00
Diptera	Flies	15.32	4.17	2.6	9.30	7.14	0.66	0.00	0.00	0.00
Chironomidae[a]		0.00	0.00	0.00	0.00	0.00	0.00	10.23	8.96	4.23
Simulidae[a]	Blackflies	0.00	0.00	0.00	0.00	0.00	0.00	11.32	6.63	3.69
Hemiptera-A	Bugs[a]	9.09	4.17	0.38	7.21	7.15	0.52	19.63	26.27	5.16
Hemiptera-B	Bugs[t]	10.21	8.33	0.85	11.72	7.14	1.67	0.00	0.00	0.00
Hymenoptera		9.09	12.50	1.14	5.40	6.35	0.68	0.00	0.00	0.00
Formicidae	Ants[t]	2.58	4.17	0.11	10.82	14.29	1.55	0.00	0.00	0.00
Lepidoptera	Moths[t]	0.00	0.00	0.00	2.30	7.14	0.16	0.00	0.00	0.00
Odonata	Damselflies[t]	8.62	12.50	1.08	11.60	7.15	0.83	0.00	0.00	0.00
Orthoptera										
Gryllacridae	Grasshoppers[t]	7.43	12.50	0.93	4.70	6.14	0.34	0.00	0.00	0.00
Ephemenoptera	Mayflies	0.00	0.00	0.00	0.00	0.00	0.00	27.23	23.56	6.42
Baetidae	Small minnow mayflies[a]	0.00	0.00	0.00	0.00	0.00	0.00	16.66	13.65	2.27
Blattodea	Cockroaches[t]	7.14	8.54	1.85	0.00	0.00	0.00	0.00	0.00	0.00
Collembola	Springtails	6.14	3.59	0.85	0.00	0.00	0.00	0.00	0.00	0.00
Diplura		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jaygidae	Diplurans[t]	8.09	4.15	0.65	0.00	0.00	0.00	0.00	0.00	0.00

The letters [a] – represent aquatic larvae and [t] – and terrestrial adults. %N numerical percentage, %F frequency of occurrence.

Table 3.3 Alimentary Index (IAi) of the stomach contents of the frogs collected downstream along the Kowie River. SVL= Snout vent-length

		A. angolensis			*S. grayii*			*X. laevis*			*Am. rangeri*		
n		9			10			7			4		
Mean SVL (mm)		50.95			35.90			44.43			37.00		
Mean mass (g)		20.73			7.73			14.14			6.62		
Prey items	*Common names*	*%F*	*%N*	IAi	*%F*	*%N*	IAi	*%F*	*%N*	IAi	*%F*	*%N*	IAi
Araneae	Spiders[t]	12.51	15.29	1.91	11.11	8.96	1.45	0.00	0.00	0.00	0.00	0.00	0.00
Coleoptera- A	Beetles[a]	8.26	13.69	1.13	13.89	7.41	1.50	11.88	17.50	2.08	0.00	0.00	0.00
Coleoptera- B	Beetles[t]	14.32	5.00	0.72	21.05	12.50	3.84	0.00	0.00	0.00	16.67	18.75	3.44
Diptera	Flies	13.15	10.95	1.44	15.21	9.26	2.06	0.00	0.00	0.00	0.00	0.00	0.00
Chironomidae[a]	Chironomids	0.00	0.00	0.00	0.00	0.00	0.00	12.77	7.97	5.01	0.00	0.00	0.00
Simulidae[a]	Blackflies	0.00	0.00	0.00	0.00	0.00	0.00	23.77	29.06	7.12	0.00	0.00	0.00
Hemiptera- A	Bugs[a]	5.86	8.50	0.50	6.67	5.56	0.54	16.67	13.95	2.33	0.00	0.00	0.00
Hemiptera-B	Bugs[t]	11.43	10.01	1.14	10.25	8.33	1.25	0.00	0.00	0.00	0.00	0.00	0.00
Hymenoptera		9.65	10.35	1.05	5.68	4.51	0.35	0.00	0.00	0.00	20.00	9.99	2.20
Formicidae	Ant[t]	6.13	5.01	0.31	4.25	3.70	0.25	0.00	0.00	0.00	30.01	25.00	8.25
Lepidoptera	Moths[t]	5.59	4.95	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Odonata	Damselflies[t]	0.00	0.00	0.00	12.11	9.09	1.61	0.00	0.00	0.00	5.00	5.43	0.30
Orthoptera		6.98	10.92	0.76	0.00	0.00	0.00	0.00	0.00	0.00	8.33	6.70	0.61
Gryllacridae	Grasshoppers[t]	6.12	5.30	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ephemenoptera	Mayflies	0.00	0.00	0.00	0.00	0.00	0.00	35.43	26.99	9.62	0.00	0.00	0.00
Baetidae[a]		0.00	0.00	0.00	0.00	0.00	0.00	12.25	10.60	1.30	0.00	0.00	0.00
Blattodea	Cockroaches[t]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Collembola	Springtails	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diplura	Diplurans[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jaygidae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

The feeding intensity of the frogs was estimated by the number of prey individuals per stomach. *Strongylopus grayii* had the highest number of prey items per stomach (average of 3.5 prey items per stomach). The lowest number of prey items per stomach was observed in *Am. rangeri*, with an average of 2.1 prey items per stomach. *Amietia angolensis* and *S. grayii* had 2.4 and 3.2 prey items per stomach, respectively. The trend for the proportion of empty stomachs was high for the upstream site and it increased from February (50%) for *X. laevis* to March – June (100%).

Values of diet overlap varied from 0, when no food type was shared, to 1, when there was the same proportional use of all food resources. Although there is no critical level with which overlap values can be compared, Labropoulou & Eleftheriou (1997) suggested that values higher than 0.6 show biological significance. The niche overlap between *A. angolensis* and *S. grayii* was high at the upstream ($\hat{O}_{jk}$ = 0.46) and downstream locations ($\hat{O}_{jk}$ = 0.66, Tables 3.4 and 3.5), as they had the highest prey diversities. At the downstream site, the trophic niche overlap was low for *Am. rangeri* and *X. laevis* ($\hat{O}_{jk}$ = 0.21, Table 3.5), while upstream the niche overlap was low for *S. grayii* and *X. laevis* ($\hat{O}_{jk}$ = 0.21, Table 3.4). *Xenopus laevis* and *Am. rangeri* had the lowest niche overlap. A very low degree of overlap existed between *X. laevis* and the other three anuran species, *A. angolensis, S. grayii* and *Am. rangeri*.

Table 3.4 Trophic niche overlap among the frog species sampled at the upstream site. Values >0.6 show biologically significant overlap (Labropoulou & Eleftheriou 1997).

	A. angolensis	*S. grayii*	*X. laevis*
A. angolensis	1.00	0.46	0.16
S. grayii		1.00	0.20
X. laevis			1.00

Table 3.5 Trophic niche overlap among the frog species sampled at the downstream site. Values in bold indicate biologically significant overlap (>0.6) (Labropoulou & Eleftheriou 1997).

	A. angolensis	*S. grayii*	*X. laevis*	*Am. Rangeri*
A. angolensis	1.00	**0.66**	0.31	0.36
S. grayii		1.00	0.34	0.51
X. laevis			1.00	0.21
Am. rangeri				1.00

The trophic niche breadth of *A. angolensis* (Levin's measure) was B = 7.7 (B_{st} = 0.84) downstream, and upstream it was B = 7.6 (B_{st} = 0.82); these niche breadth and standardized niche breadth measures were larger than those of all other species from both sites (Table 3.6). The species that consumed fewer prey such as *X. laevis* and *Am. rangeri* had smaller trophic niche breadths (Table 3.6).

Table 3.6 Trophic niche breadth of four frog species found within the Kowie River. *B = Levin's measure of niche breadth and B_{st} = Levin's standardized niche breadth.*

Species	**Downstream**		**Upstream**	
	B	**Bst**	**B**	**Bst**
A. angolensis	7.68	0.84	7.58	0.82
S. grayii	4.17	0.79	8.93	0.99
X. laevis	2.53	0.76	4.05	0.35
Am. Rangeri	2.36	0.68	-	-

3.4 DISCUSSION

Several researchers have categorized anurans as opportunistic general foragers that consume a wide variety of invertebrate prey (França et al. 2004; Maneyro et al. 2004; Santos et al. 2004; Dietl et al. 2009; Hothem et al. 2009). *Amietia angolensis* and *Strongylopus grayii* in the Kowie River had a wide diet diversity made up of both aquatic and terrestrial invertebrates, while *Xenopus laevis* had stomach contents consisting only of aquatic invertebrates, and *Amietophyrnus rangeri* fed only on terrestrial invertebrates. Anuran species consume invertebrate prey with notable variations in behaviour (cryptic or conspicuous; slow moving or highly mobile insects) and developmental stage (larval or adult; França et al. 2004; Santos et al. 2004; Dietl et al. 2009). Such variations in prey selection suggest differences in the surrounding habitat, which is the case for *A. angolensis* and *S. grayii* in the Kowie River. These anurans were observed and collected on the water edges and banks of the stream during the field work, and they were associated with the vegetation, water (since aquatic insects were identified in the stomach contents) and soil (given the ingestion of terrestrial hemipterans). These findings support those from previous studies (França et al. 2004; Santos et al. 2004) that identified large ranges in prey type in several amphibians. França et al. (2004) found a large range of prey (including subaquatic and terrestrial organisms) used by two coexisting species of anurans (*Leptodactylus latrans* and *Leptodacytlus labyrinthicus*). These authors also detected differences in microhabitat utilization by the two species, which might have been enough to allow coexistence. *Leptodactylus latrans* was found in a greater variety of habitats, whereas *Leptodacytlus labryinthicus* was restricted to water bodies with standing water and hence had a narrow trophic breadth (França et al. 2004). According to Forstner et al (1998), there is a relationship between diet composition of a frog species and the microhabitat used, as different environments shelter various types of prey.

A relatively wide niche breadth was measured in *A. angolensis* in the Kowie River, reflecting a broader diet range compared to other species which consumed fewer numbers of prey. The observed differences in trophic niche breadth among the frog populations in the Kowie River may be related to their spatial distribution. *Amietia angolensis* occurred in a greater variety of habitats, and had a wide trophic niche (7.58 upstream and 7.68 downstream), whereas *X. laevis* was restricted to water bodies and standing water and had a narrower trophic niche (4.05 upstream and 2.50 downstream). The similarity in diets among some populations (such as *A. angolensis* and *S. grayii* sp.) may be a result of a wide variety of prey available in the Kowie River and to the coarse food categories used in the dietary analysis. Differences between the

microhabitat utilized by the consumer, type of prey exploited, and time of activity have provided evidence that competition for food can be avoided in some habitats (Lima & Magnusson 1998). In this study in the Kowie River, microhabitat variations among the species could occur at relatively small spatial scales even though they fed within a few meters from each other (Table 3.1). For example, Van Sluys & Rocha (1998) reported two syntopic frog species (*Dendropsuphus* sp. and *Pseudopaludicula* sp.) in the Amazon varied in their feeding habits as a result of their body size, microhabitat use and activity time. The researchers observed that when active, individuals of *Pseudopaludicula* sp. were found partially submerged at the lake borders, whereas *Dendropsuphus* were found predominantly on *Nymphae* sp. leaves on the banks of the lake (Van Sluys & Rocha 1998).

From the insect orders identified as prey in the present study, Diptera and Hemiptera were consumed by almost all of the frog species. Other invertebrates such as Araneae, Coleoptera and Hymenoptera were also consumed frequently. This result is similar to that of a study by Santos et al. (2004), who found Odonata, Coleoptera, Hymenoptera, and Araneae were consumed by almost all six anuran species analyzed. In this present study, some prey items could not be identified, as a result some insect orders may have been underestimated as prey items. Several studies have identified zoobenthos and zooplankton to make up a principal part of the diet of some frogs, especially *X. laevis* (McCoid & Fritts 1980; Measey 1998; Bwong & Measey 2010). In the Kowie River, these prey categories were not identified in the stomach contents of *X. laevis*. *Amietophrynus rangeri* appears to fit into the category defined by Toft (1980b) as an "ant-specialist" given the high intake of ants by all individuals examined. Researchers have described terrestrial and aquatic insects as preferential prey items for frog genera such as *Hyla, Phyllomedusa, Leptodactylus* and *Physalaemus* (Toft 1980b, 1981; Van Sluys & Rocha 1998; Anderson et al. 1999; Cogalniceanu et al. 2001; Santos et al. 2004). Hothem et al. (2009) evaluated the diets of three sympatric anuran species (*Pseudacris regilla*, *Rana boylii*, and *Lithobates catesbeianus*), and based on the stomach content analysis of all the species, *L. catesbeianus* was the only one having equal preferences for both terrestrial and aquatic prey. In another study, Stojanova and Mollov (2008) looked at the diet and trophic niche overlap of *Rana arvalis* (the moor frog) and *Rana temporaria* (the common frog) from Poland and identified the most important prey categories as Coleoptera, Hemiptera, Hymenoptera, Diptera, and Arachnida. The authors observed that both frogs consumed terrestrial prey almost exclusively (Stojanova & Mollov 2008).

The vegetation material found in some adult frog stomachs in the Kowie River was probably unintentionally consumed while the animals were foraging (for example, see Hirai & Matsui 1999; Solé et al. 2005; Dietl et al. 2009). Still, the idea that frogs may possibly select plant resources as food items needs to be considered. According to Anderson et al. (1999), anurans may actively select vegetation because it may aid in the removal of intestinal parasites, make available roughage to assist in crushing up arthropod exoskeletons, or provide nutrients and added sources of water. More information on the ingestion of vegetation by amphibians will enhance our understanding of behavioural patterns. For example, the occurrence of stamens, seeds and leaves in the guts of some specimens from the Kowie River showed that vegetated areas are used as foraging territories in addition to potential reproductive sites. Herbivory in frogs (especially in tropical frogs) has become more frequently reported in recent years (Santos et al. 2004).

Prey availability in the environment (a variable that was beyond the scope of this study) was not incorporated in any of the parameters used here. Resource availability is not easy to measure and has rarely been incorporated into anuran diet studies (but see Cogalniceanu et al. 2001; Whitfield & Donnelly 2006). Researchers have recently illustrated that small insectivores can be very selective, discriminating actively among prey taxa (Simon & Toft 1991; Toft 1995). Furthermore, anurans can change their diet as they grow (Schriever & Williams 2013). Mites and collembolans are usually the smallest arthropods available to anurans, prey of intermediate sizes include ants, beetles, bugs and termites, whereas the largest arthropod items are orthopterans, spiders, and lepidopterans (Lima & Magnusson 1998; Santos et al. 2004). The most important prey categories in general include coleopterans, hemipterans and dipterans, and these were consumed frequently by almost all the frogs collected in the Kowie River. The coleopterans and dipterans are relatively abundant and occur in a wide range of habitats, and as such are readily available for predators (Stojanova & Mollov 2008). The importance of these two insect groups as dominant prey for frogs in the Kowie River was confirmed by their frequent occurrence in stomach contents. Additional important prey included non-insect invertebrates such as Arachnida.

To my knowledge, this study represents the first report on the feeding habits of anurans in the Kowie River, Eastern Cape Province, South Africa. The sampling period and sample size provided preliminary estimations of the diets in several populations, and the general prey compositions for *A. angolensis* and *S. grayii* that were similar to published reports of similar species elsewhere in the world. In addition, results from this study also support published

literature on the diets of *X.laevis* and *Am. rangeri,* as *Xenopus* was showen to have a diet mainly composed of almost entirely of aquatic prey while for *Am. rangeri* terrestrial prey constituted its diet. A key finding was that both *A. angolensis* and *S. grayii* species utilized a large prey range represented by both aquatic and terrestrial insects.

4 USE OF STABLE ISOTOPES TO ASSESS POTENTIAL HABITAT CONNECTIONS VIA AMPHIBIAN DIETS

4.1 INTRODUCTION

The complex life cycles of amphibians and the different habitats they inhabit makes them unique among tetrapods. Amphibians are significant consumers in both terrestrial and aquatic habitats (Najera-Hillman et al. 2009), and the life history cycle of most anurans includes dramatic ontogenetic changes in morphology, physiology and behaviour (Enriquez-Urzelai et al. 2013). Amphibians are important in connecting terrestrial and aquatic systems (Regester et al. 2005; Whiles et al. 2006), as they change their habitat and foraging through migrations and developmental shifts. Energy and materials from the terrestrial ecosystems are transferred via amphibian eggs and embryos, whereas late stage tadpoles transfer aquatic energy to terrestrial systems when they metamorphose into adults (Trakimas et al. 2011). A valuation of the trophic relationships and ecological roles of this group is necessary to enhance our understanding of the transfer of energy between habitats (Houlahan et al. 2000; Trakimas et al. 2011).

Knowledge on the diets of the various life stages of amphibians, including tadpoles and adults, is valuable to demonstrate the role of this group in linking habitats. Direct feeding observations and stomach contents analysis reflect what the consumer has recently ingested and may not reflect long-term feeding patterns (Jefferson & Russell 2008; Trakimas et al. 2011; Gillespie 2013). Stable isotope analysis can provide a time integrated measure of trophic position and ultimate carbon sources for consumers (Vander Zanden & Rasmussen 1999).

Although the stable isotope technique is becoming increasingly useful in trophic ecology, amphibians are relatively rarely represented in the stable isotope literature (Baffico & Ubeda 2006; Gillespie 2013, but see Whiles et al. 2006; Verburg et al. 2007). An increasing number of studies on aquatic and terrestrial food webs described the trophic positions and food origins of amphibian species (Kupfer et al. 2006; Verburg et al. 2007; Araújo et al. 2009; Barnum et al. 2013). For example, in an ecosystem in which amphibians were the main vertebrate taxa, Verburg et al. (2007) used nitrogen and carbon stable isotopic signatures to examine the trophic interactions and identified tadpoles and adult amphibians as intermediate links in the aquatic and terrestrial food webs, respectively. Similarly, Kupfer et al. (2006) observed that riparian frogs (*Occidozyga lima, Phynoglossus martenssi* and *Hoplobatrachus chinensis*) were part of the terrestrial food web surrounding a river in Thailand, and placed them as second level

predators within the food web. In a study using both carbon and nitrogen isotopes, one of New Zealand's most widespread native frog (*Leiopelma hochstetteri*) was placed at an intermediate trophic level amongst predators (including *Anguilla australis*, *Rattus rattus* and *Galaxias fasciatus*), as the diet of adult *L. hochstetteri* included terrestrial invertebrates (Najera-Hillman et al. 2009). Caut et al. (2012) demonstrated stable isotopic analysis as an efficient way to evaluate variations in tadpole trophic status and the ecological role of tadpoles in freshwater ecosystems. Their study indicated evidence for plasticity in tadpole diets in different ecological scenarios (i.e. the tadpoles' responses to competitors and non-lethal presence of native and non-native predators; Caut et al. 2012). Furthermore, a stable isotope study by Trakimas et al. (2011) revealed that terrestrial carbon was present in the eggs and embryos of amphibians, thereby indicating a terrestrial subsidy to an aquatic system, and they also discovered that algal carbon was probably responsible for tadpole growth preceding metamorphosis to adults. Such results point out the dynamic nature of resource transfers at the terrestrial-aquatic boundary and the general significance of fluxes across ecosystem boundaries as facilitated by amphibians (Trakimas et al. 2011).

The general aim of this chapter was to assess the trophic ecology of four frog species in the Kowie River: *Amietia angolensis* (common river frog), *Strongylopus grayii* (clicking stream frog), *Xenopus laevis* (common platanna), and the *Amietophyrnus rangeri* (raucous toad), using stable isotope analysis. The specific aims of this investigation were to:

1) examine the isotopic composition of the four species at two study sites to describe the consumer diets,
2) assess whether ontogenetic diet shifts occurred within any species.

4.2 MATERIALS AND METHODS

4.2.1 Study area, sample collection and treatment

Detailed descriptions of the study area and sample collection are provided in Chapter 2.

4.2.2 Data analysis

Isotopic data for potential food sources were derived from concurrent studies completed at the same study locations (S. Moyo; L. Chari; unpublished data). Prey items from the terrestrial habitat were pooled together as 'terrestrial sources', and the aquatic invertebrates category

(included larval stages of some terrestrial insects) represented the 'aquatic sources'. The proportional contributions of different prey categories (aquatic and terrestrial sources) to the diets of adult frogs and tadpoles at each site were estimated using the Bayesian stable isotope mixing model (SIAR; Parnell et al. 2010). Variations in stable isotope values of both consumers and prey, as well as variation in isotopic fractionation factors, were incorporated into the SIAR mixing models (Parnell et al. 2010). Isotopic fractionation values used to run the model were 2.3 ± 0.24 ‰ for $\delta^{15}N$ and 0.5 ± 0.19 ‰ for $\delta^{13}C$. Many researchers use 3.4 as the isotopic fractionation value; however, this value is best applied to entire food webs (Post 2002). For studies on specific populations, it is best to use fractionation factors determined from similar species feeding on similar foods (Martinez de Rio et al. 2009). The results estimating the proportional contribution of each source to the consumer diet were represented as boxplots with 25, 75 and 95% Bayesian credible intervals (Parnell et al. 2010). The trophic positions (TP) of the various anurans were estimated using tissue nitrogen isotope data:

$$TP_{consumer} = 1 + (\delta^{15}N_{comsumer} - \delta^{15}N_{baseline})/F$$

where $TP_{consumer}$ is the trophic position of an individual estimated from $\delta^{15}N$, $\delta^{15}N_{consumer}$ is the nitrogen isotope signature of the consumer being evaluated, $\delta^{15}N_{baseline}$ represents the nitrogen isotopic ratio value of a primary producer (in this case, it was periphyton) and F is the fractionation of nitrogen by trophic level (Post 2002). An estimated fractionation of 2.3‰ was incorporated into each model (McCutchan et al. 2003; Schriever & Williams 2013).

4.3 RESULTS

4.3.1 Stable isotope composition of food sources

Aquatic insects (Baetidae, Coengrionidae, Hydropsychidae, Simulidae) and terrestrial insects (Hymenoptera, Orthoptera), arachnids (Araneae), periphyton, suspended particulate matter (SPM) and coarse particulate organic matter (CPOM) were considered as potential food sources (Table 4.1). Based on their $\delta^{13}C$ values, the food sources were distinct from the consumers. Terrestrial leaves had the lowest mean $\delta^{13}C$ value of -28.1 ± 1.58 ‰ at the upstream site (Figure 4.5) and downstream SPM had the lowest mean $\delta^{13}C$ value of -29.6 ± 2.8 ‰. The $\delta^{13}C$ values of terrestrial sources upstream ranged from -24.8 to -26.6 ‰, with an average of -25.6 ‰, and the aquatic sources had a range of -19.2 to -28.1‰, with an average of -25.2 ‰. The other food sources at the upstream site, such as SPM and periphyton, had similar carbon and nitrogen isotopic values (Figure 4.1; Table 4.1). Baetidae, Coengrionidae, SPM and

Simulidae showed similar $\delta^{15}N$ values at the upstream site (Figure 4.1). Aquatic sources from upstream fell within a similar range of 4 to 7 ‰ in $\delta^{15}N$ values, whereas the terrestrial sources had broad $\delta^{15}N$ ranges. Downstream, $\delta^{13}C$ of terrestrial food sources ranged from -17.9 to -29.4 ‰, with an average of -21.6 ‰, whereas aquatic sources ranged from -18.2 to -29.6 ‰, with an average of -26.20 ‰. Aquatic sources (Baetidae, Simulidae, Coengrionidae and Hydropsychidae) downstream showed the highest mean $\delta^{15}N$ values (17.8 ± 1.3, 18.5 ± 1.1, 15.4 ± 0.1 ‰, respectively) but had low $\delta^{13}C$ values (Figure 4.2). The other potential food sources showed wide ranges in nitrogen isotopic values.

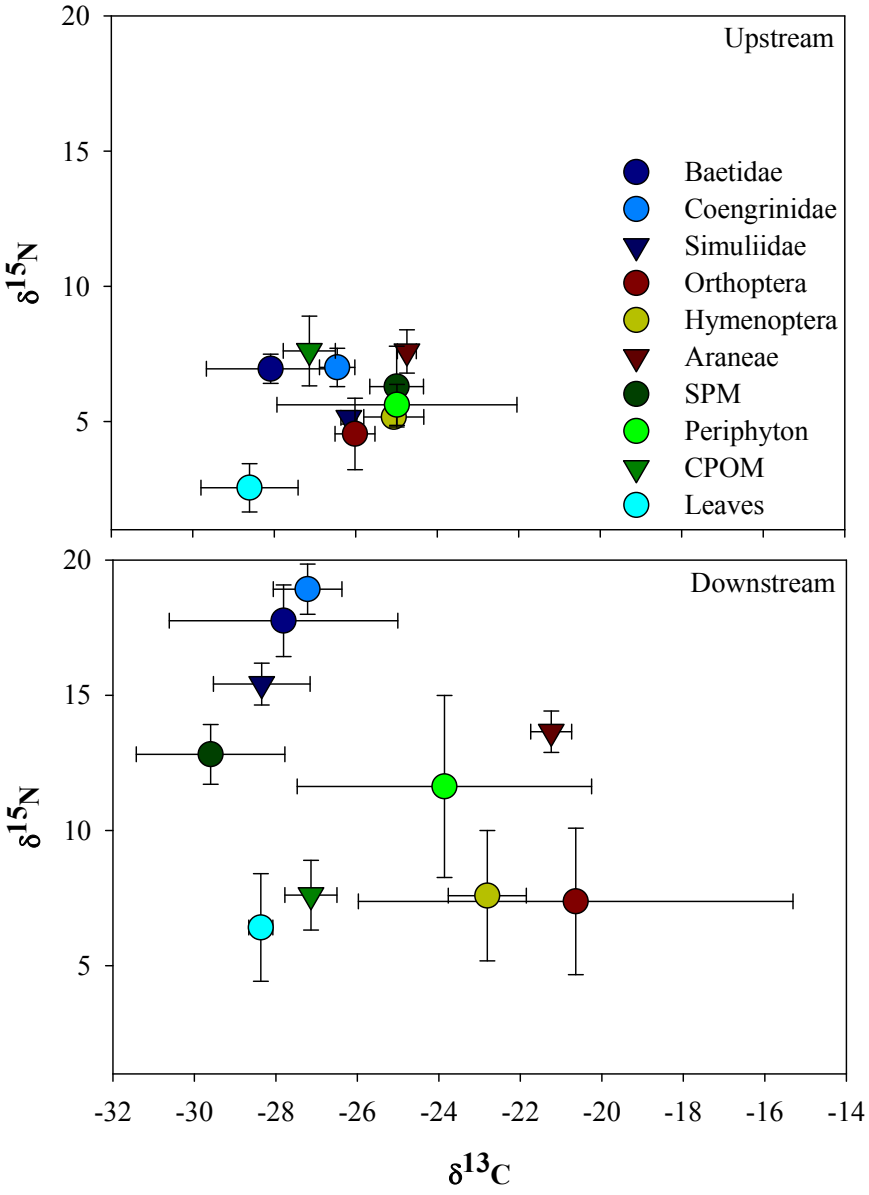


Figure 4.1 Average stable isotopes of carbon and nitrogen for the potential food source in the two sampled regions of the Kowie River.

4.3.2 Stable isotope composition of consumers

Isotopic signatures of adult *Amietia angolensis* averaged 8.3 ± 1.5 ‰ and -23.9 ± 1.3 ‰ upstream for N and C, respectively, which were similar to the isotopic values measured in this species from the downstream site (Table 4.1 & Figure 4.2). *Amietia angolensis* tadpoles had the lowest carbon isotopic values among consumers across both sites (-27.1 ± 2.1 ‰ upstream and -26.8 ± 2.0 ‰ downstream; Figure 4.3 & 4.4a and Table 4.1). The raucous toad, *Amietophrynus rangeri*, at the downstream site was the most ^{13}C-enriched consumer (-20.9 ± 1.1 ‰), followed by the clicking stream frog *Strongylopus grayii* (-23.1 ± 1.8 ‰). Common platanna *Xenopus laevis* exhibited similar $\delta^{13}C$ values upstream to those found downstream (Table 4.1). *Amietia angolensis* tadpoles had $\delta^{15}N$ values (8.9‰ upstream and 8.5‰ downstream) similar to that of the adults (8.3‰ upstream and 8.4‰ downstream) across sites. The clicking stream frog, *S. grayii,* had much higher $\delta^{15}N$ values than its tadpoles at both sites (1.8‰ higher upstream and 2.5‰ higher downstream). *Xenopus laevis* was highest in $\delta^{15}N$ values (12.3 ± 2.2 ‰) among all the consumers.

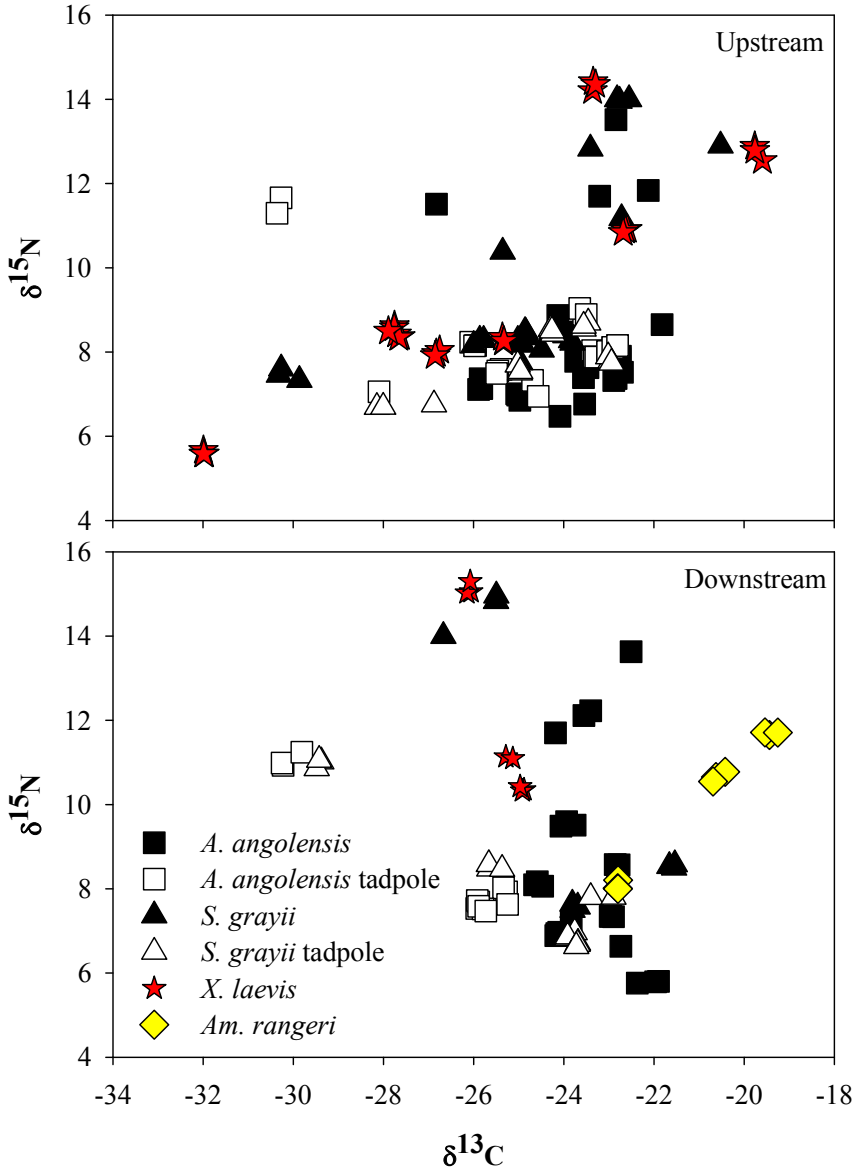
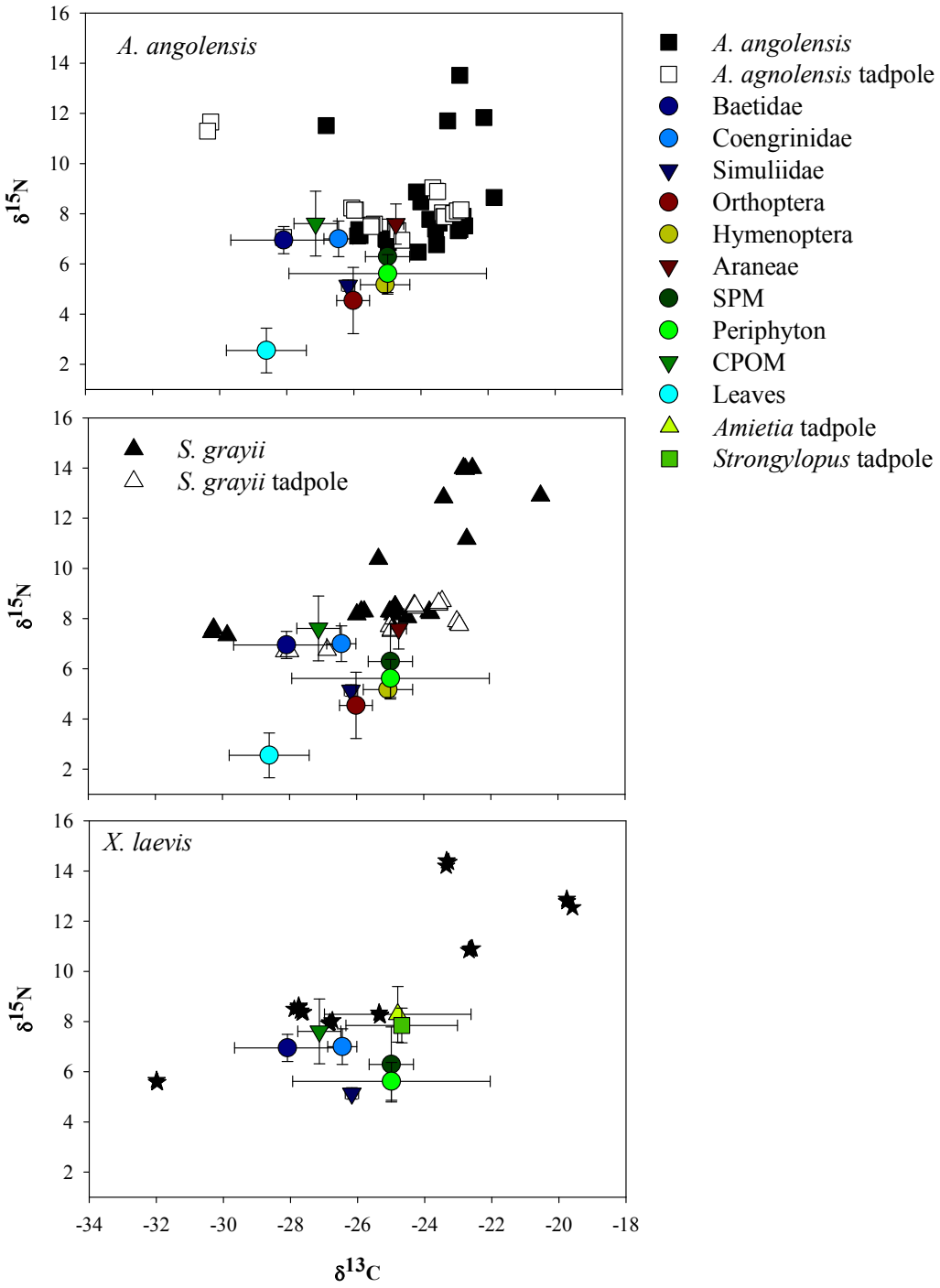


Figure 4.2 Carbon and nitrogen stable isotopic composition for four anurans species in the Kowie River.



4.3 Stable isotope biplots representing the individual frogs and tadpoles from the upstream site, and mean and standard deviations (error bars) of the prey items.

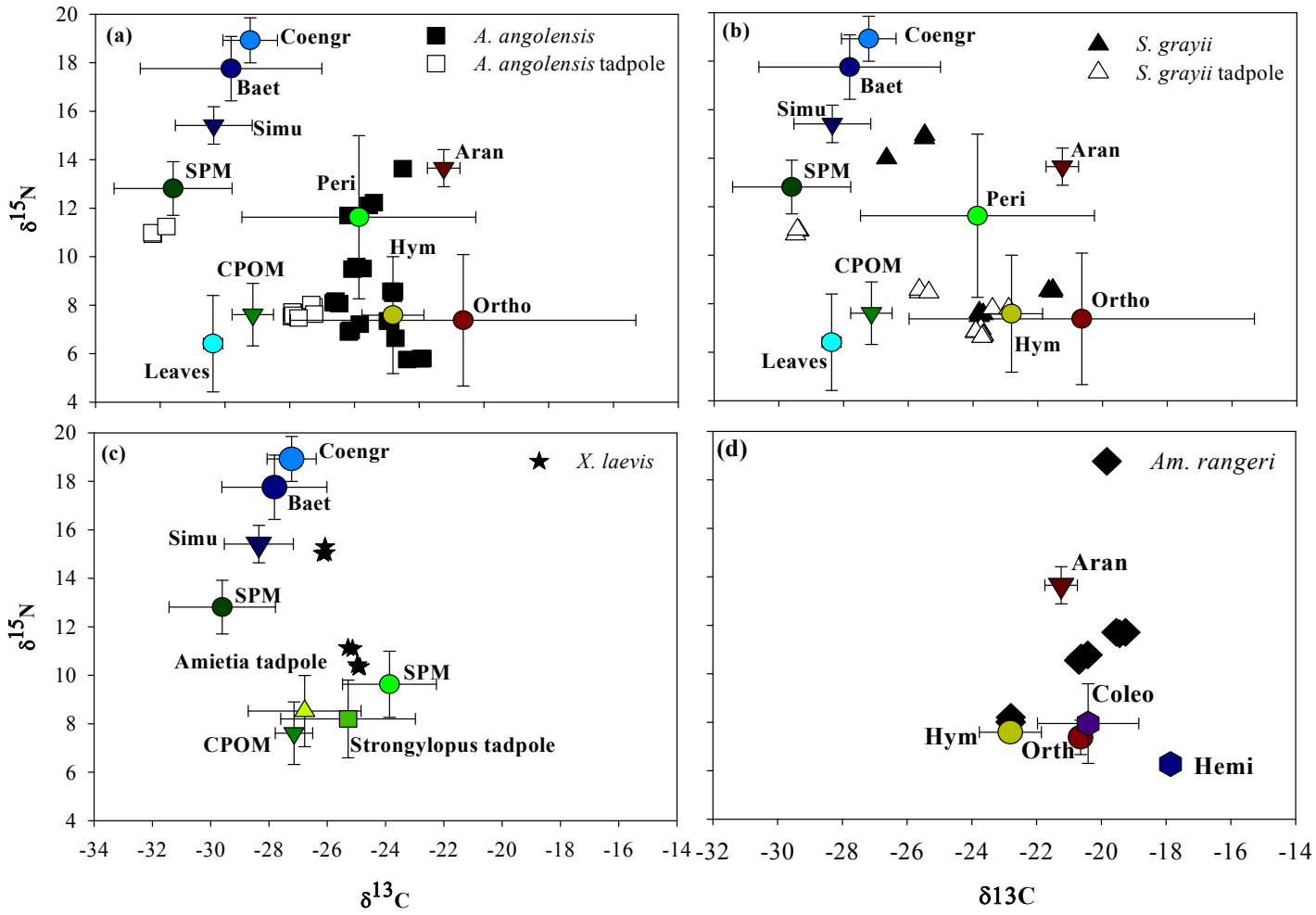


Figure 4.4 Stable isotope biplots representing the sampled individual frogs and tadpoles from the downstream site, and mean and standard deviations (error bars) of prey items. Prey items are as follows: SPM= Suspended particulate matter, Peri = Periphyton, CPOM, Aran = Araneae, Baet= Baetidae, Simu = Simuliidae, Coen = Coengrionidae, Orth = Orthoptera, Hym = Hymenoptera (T. Dalu, unpublished; L.D Chari, unpublish; S. Moyo, unpublished data).

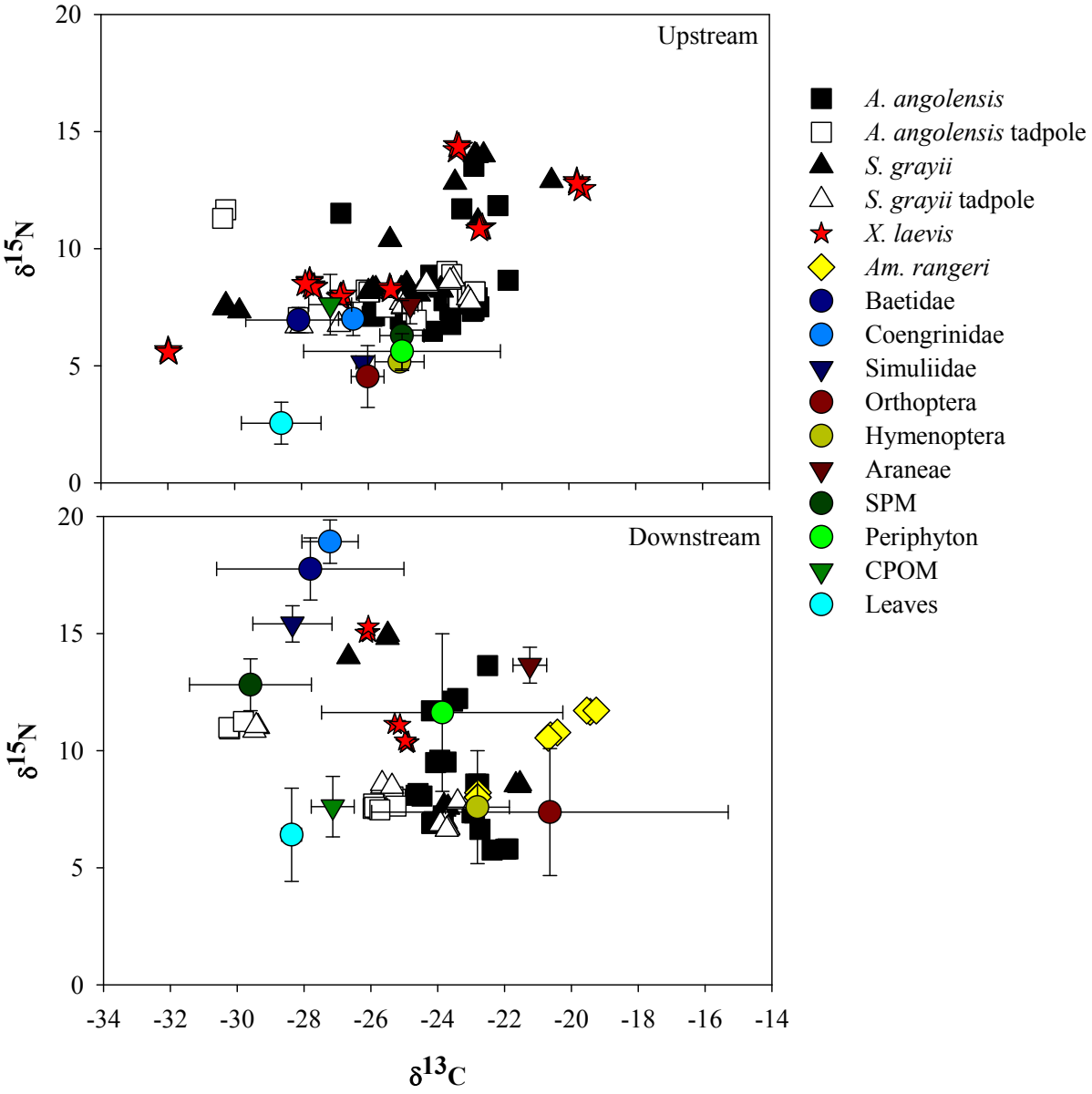


Figure 4.5 Carbon and nitrogen stable isotopic composition for all species and prey items found at the two sampled regions in the Kowie River.

4.3.3 Trophic position

Trophic positions (TP) of tadpoles and post-metamorphic adults were estimated using nitrogen stable isotope ratios, and they revealed clear differences between tadpoles and adults (Table 4.1). Tadpoles occupied a lower trophic position (*A. angolensis* tadpoles; TP = 2.3 upstream and 1.6 downstream; *S. grayii* tadpoles; TP= 2.3 upstream and 1.6 downstream), whereas adults shifted to higher trophic positions. Adults within the same species maintained relatively constant trophic positions across sites. *Xenopus laevis* and *S. grayii* occupied the highest trophic position (3.1 and 3.0, respectively), followed by *A. angolensis* (2.5 downstream; Table 4.1). Ontogenetic diet shifts were evident in *A. angolensis* and *S. grayii* based on their trophic position.

Table 4.1 Stable isotopes of carbon and nitrogen (mean ± SD) for adults, tadpoles and potential food sources from both upstream and downstream locations (downstream site isotope values are presented in parentheses) (prey data from T. Dalu, unpublished; L.D Chari, unpublished data; S. Moyo, unpublished data). TP = Trophic position

	TP	$\Delta^{15}N$	$\Delta^{13}C$
Amphibians			
A. angolensis	2.3	8.29 ± 1.84	-23.86 ± 1.30
	(2.5)	(8.43 ± 2.10)	(-23.40 ± 0.81)
S. grayii	3.0	9.51 ± 2.29	-24.99 ± 2.37
	(2.8)	(10.23 ± 3.12)	(-23.10 ± 1.79)
X. laevis	3.1	9.56 ± 2.66	-25.66 ± 2.13
	(3.1)	(12.34 ± 2.18)	(-25.43 ± 0.50)
Am. rangeri	(2.6)	(10.14 ± 1.52)	(-20.93 ± 1.14)
A. angolensis tadpoles	2.3	8.85 ± 1.59	-27.10 ± 2.14
	(1.6)	(8.52 ± 1.54)	(-26.77 ± 2.02)
S. grayii tadpoles	2.3	7.69 ± 0.81	-25.72 ± 1.57
	(1.6)	(7.73 ± 0.75)	(-24.32 ± 1.08)
Aquatic sources			
Baetidae		6.95 ± 0.55	-28.09 ± 1.58
		(17.76 ± 1.33)	(-27.22 ± 2.80)
Coengrionidae		7.00 ± 0.71	-26.46 ± 0.43

	(18.92 ± 0.93)	(-27.22 ± 0.84)
Hydropsychidae	6.67 ± 0.57	-26.41 ± 0.38
	(18.49 ± 1.11)	(-27.38 ± 0.80)
Simulidae	5.14 ± 0.23	-26.17 ± 0.20
	(15.41 ± 0.77)	(-28.35 ± 1.19)
Periphyton	5.61 ± 0.76	-24.99 ± 2.94
	(3.61 ± 1.37)	(-23.86 ± 1.63)
SPM	6.29 ± 1.49	-25.00 ± 0.66
	(5.82 ± 1.11)	(-29.60 ± 2.81)
Benthic algae	4.00 ± 1.47	-19.22 ± 0.61
	(4.22 ± 1.68)	(-18.21 ± 1.32)
Terrestrial sources		
Araneae	7.59 ± 0.80	-24.75 ± 0.23
	(13.65 ± 0.77)	(-21.24 ± 0.50)
Hymenoptera	5.69 ± 0.84	-26.62 ± 0.12
	(7.59 ± 2.41)	(-22.81 ± 0.96)
Formicidae	5.17 ± 0.24	-25.07 ± 0.74
Orthoptera	4.54 ± 1.32	-26.02 ± 0.49
	(7.38 ± 2.71)	(-20.64 ± 3.33)
Leaves	2.55 ± 0.89	-28.61 ± 1.19
	(6.41 ± 1.99)	(-28.37 ± 0.30)

4.3.4 Mixing models

For the tadpoles, the SIAR models indicated that aquatic derived food sources contributed the majority of their diet (Figures 4.6 & 4.7). For *X. laevis,* the SIAR models suggested that aquatic sources contributed the majority of the diet (55% upstream and 72% downstream), with little contributions from terrestrial sources (Figure 4.8 & 4.9). By contrast, the SIAR models suggested that dietary items from terrestrial sources made up the largest proportion of the *Amietophyrnus rangeri* diet (Figure 4.9). The SIAR model indicated that terrestrially-derived resources had relatively greater nutritional contributions to *A. angolensis* than aquatic-derived food sources (Figure 4.8 & 4.9). For *Strongylopus,* the SIAR models indicated equal contributions of aquatic (48 % upstream and 49 % downstream) and terrestrial (52 % upstream and 51 % downstream) sources in their diet.

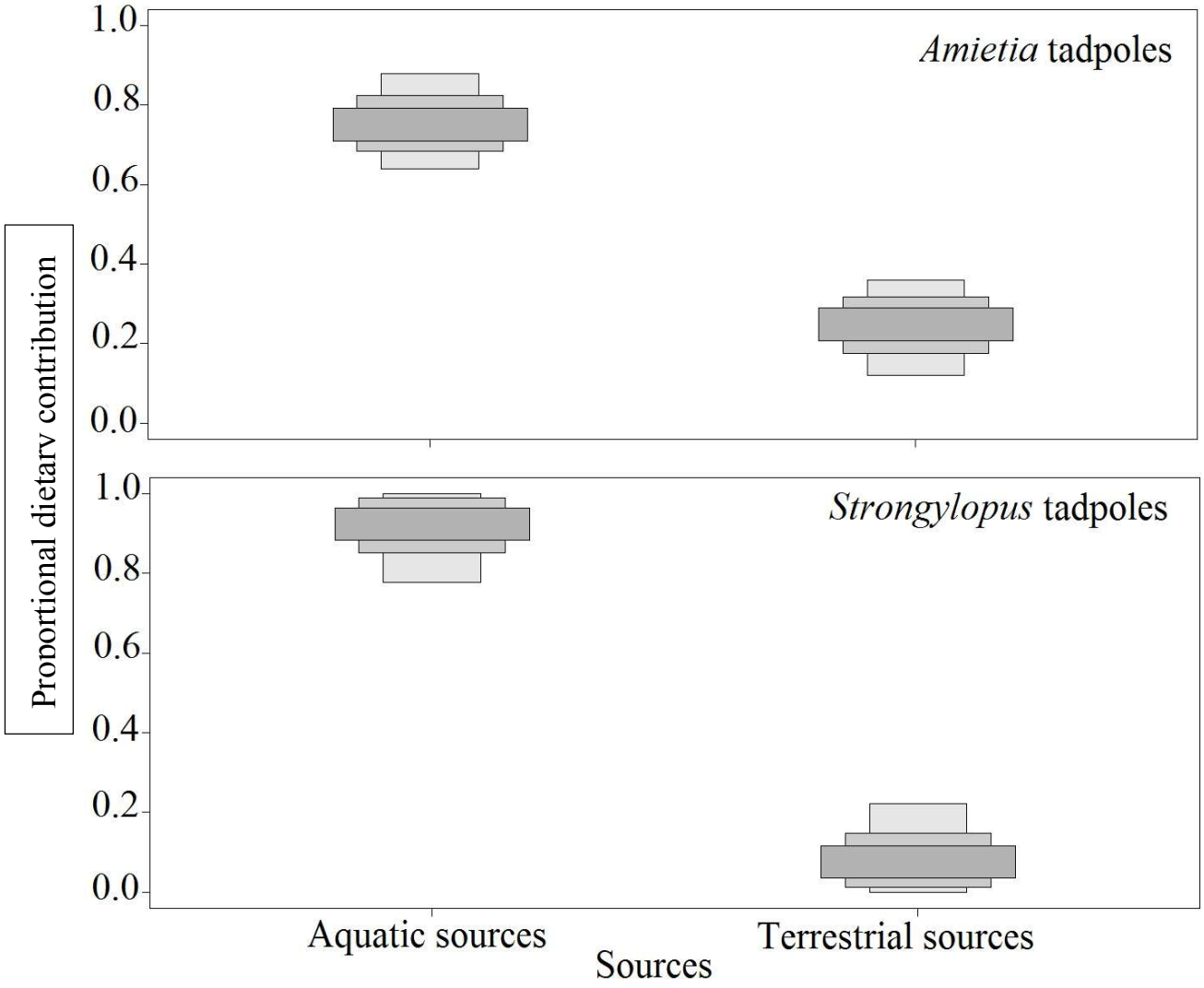


Figure 4.6 Boxplots from SIAR mixing models showing the dietary contributions of potential prey sources to the diets of the tadpoles collected from the upstream site. The dietary proportions indicate the credibility intervals at 95, 75 and 25%.

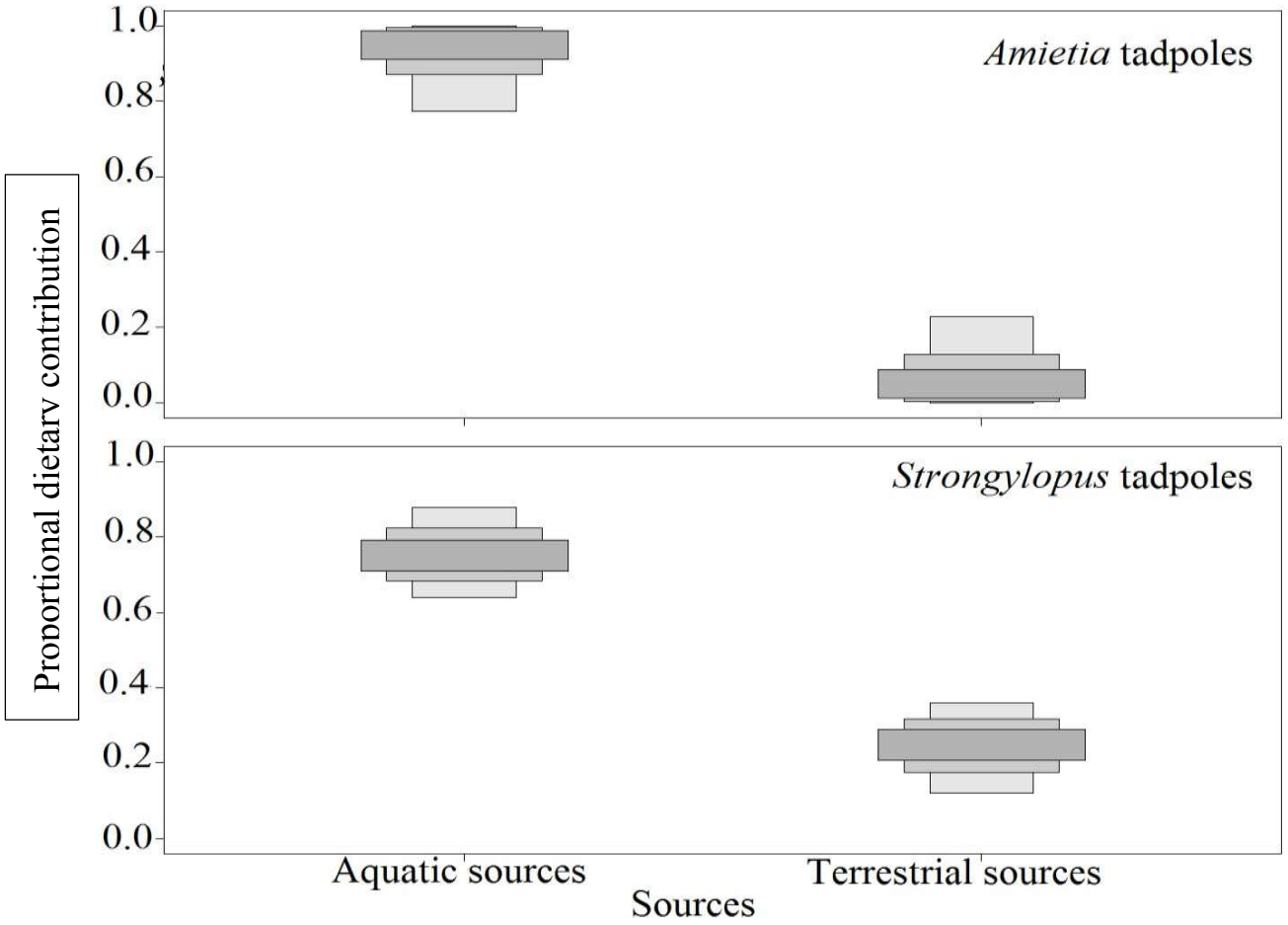


Figure 4.7 Boxplots from SIAR mixing models showing the dietary contributions of potential prey sources to the diets of the tadpoles collected from the downstream site. The dietary proportions indicate the credibility intervals at 95, 75 and 25%.

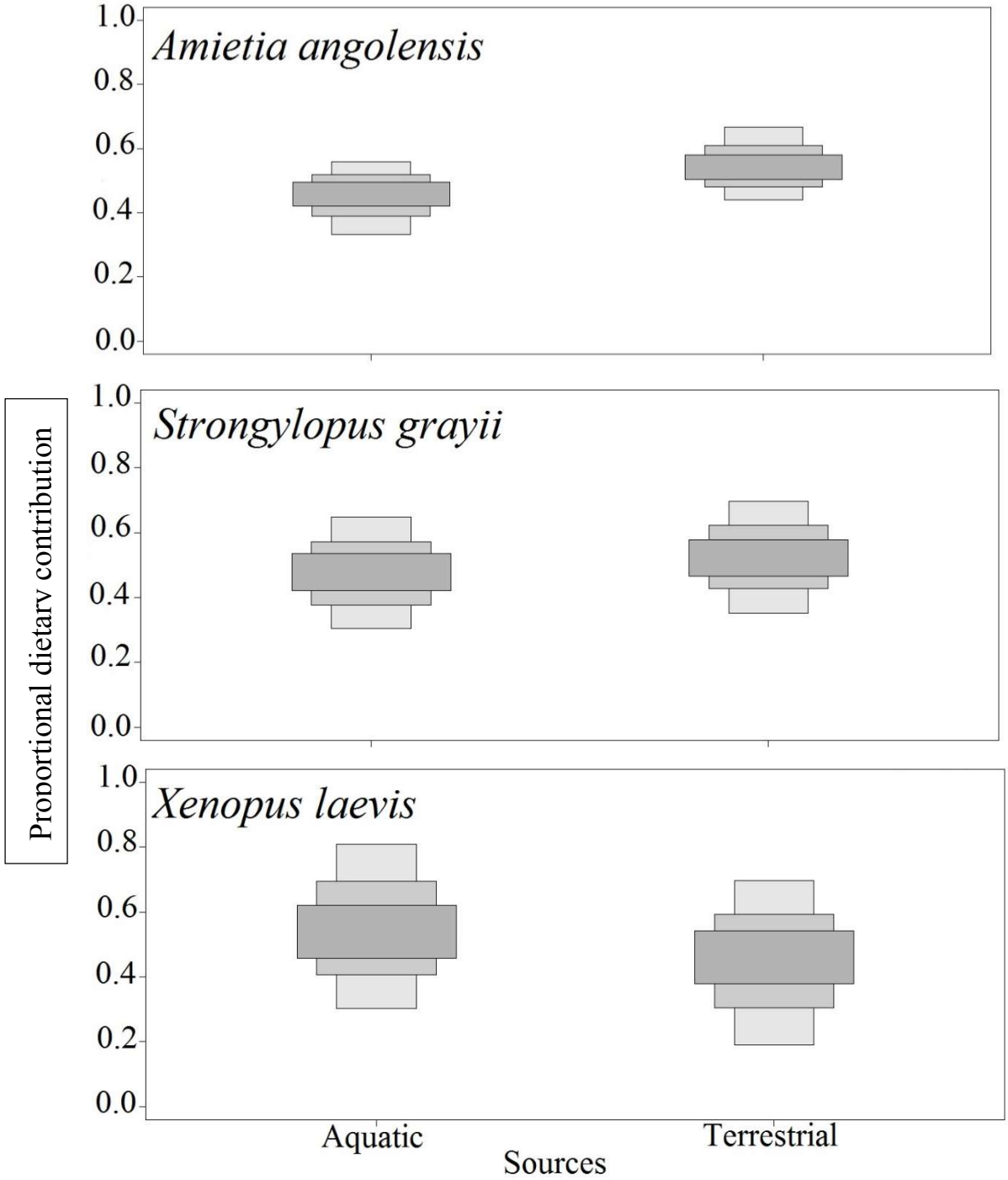


Figure 4.8 Boxplots from SIAR mixing models showing the dietary contributions of potential prey sources to the diets of the frog species collected from the upstream site. The dietary proportions indicate the credibility intervals at 95, 75 and 25%.

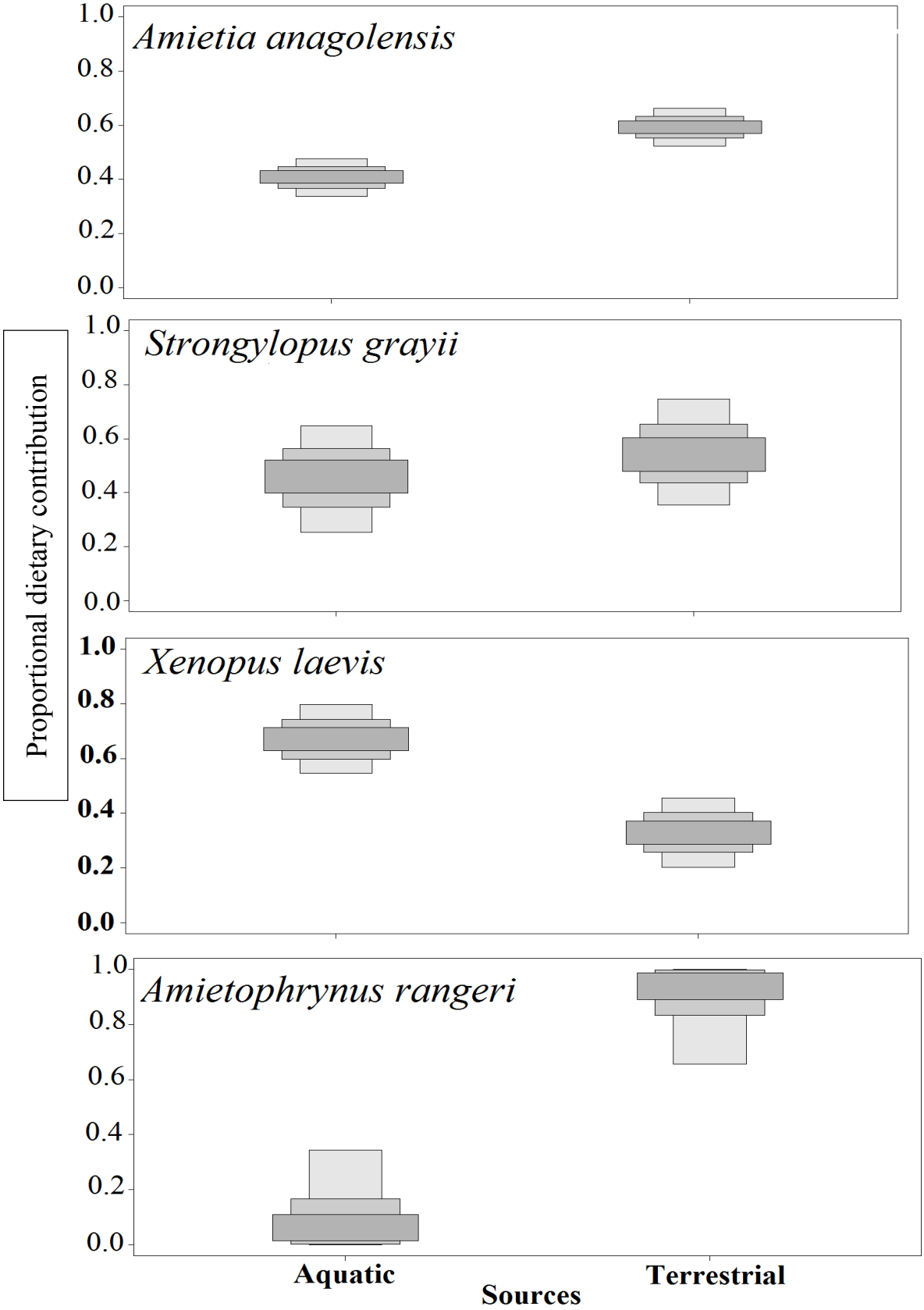


Figure 4.9 Boxplots from SIAR mixing models showing the dietary contributions of potential prey sources to the diets of the frog species collected from the downstream site. The dietary proportions indicate the credibility intervals at 95, 75 and 25%.

4.4 DISCUSSION

In the present study, I investigated diets and ontogenetic diet shifts of four anurans in the Kowie River based on stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analysis. Stable isotopic mixing models showed that aquatic-derived sources had greater contributions to *Xenopus laevis* than terrestrial food sources, while for *Amietia angolensis* and *Strongylopus grayii* the models revealed equal nutritional contributions of aquatic and terrestrial food sources. In contrast, for *Amietophrynus rangeri* the model indicated that terrestrially-derived sources made up major portions of its diet. Higher trophic positions were measured in *A. angolensis* and *S. grayii* adults (2.5 and 3.0, respectively) compared to the tadpoles (1.6 and 1.6, respectively). Based on the isotopic compositions of the consumers, anurans from the upstream site were much more variable (intraspecific variability) in their diets as compared to those from the downstream site. Interestingly, potential food sources downstream were more enriched in nitrogen compared to upstream.

Isotopic data have demonstrated that the diet composition among and within frog species is more variable than previously thought based on stomach contents data (Trakimas et al. 2011). If different frogs from the same population were all feeding on similar proportions of the same prey, one would expect much less variation in $\delta^{13}C$ values within the population (Gillespie 2013). Interestingly, $\delta^{13}C$ values of frogs in the Kowie River showed some variation among individuals of the same species at both sampling sites (Figures 4.3 & 4.4). Extreme $\delta^{13}C$ values for some individual frogs and tadpoles indicated that these individuals may have been eating a very different combination of prey than much of the population (Figure 4.1 shows individuals with extremely low $\delta^{13}C$ values, Figure 4.2 shows some extremely high $\delta^{13}C$). This result of wide dietary variations within a population is similar that from a study by Araújo et al. (2009), who reported evidence of intraspecific diet variation in four frog species of Brazilian frogs. These authors also found evidence of a positive correlation between the population niche width and the degree of diet variation (Araújo et al. 2009). Furthermore, Schriever & Williams (2013) observed intraspecific dietary variation in amphibian larvae (wood frog tadpoles, *Lithobates sylvaticus,* and blue-spotted salamander larvae, *Ambystoma laterale*). *Lithobates sylvaticus* had a wide range of isotopic values ($\delta^{13}C$ = -37.6 - -27.2 ‰ and $\delta^{15}N$ = 3.8 – 5.0 ‰), suggesting generalist feeding (Schriever & Williams 2013). In the Kowie River populations, a similar pattern was also observed where *A. angolensis* and *S. grayii* showed wide ranges of isotopic values ($\delta^{13}C$ = -26.8 - -21.8 ‰ and $\delta^{15}N$ = 5.8 – 13.63 ‰; $\delta^{13}C$ = -30.3 - -20.5 ‰ and $\delta^{15}N$ ‰

= 7.3 - 14.9 ‰, respectively), confirming generalist feeding behaviors and in agreement with gut content findings (Chapter 3).

The present study also aimed at identifying any changes in the diet with frog development in the species analyzed. Throughout their life history cycles, anurans go through ontogenetic habitat (aquatic-terrestrial) and dietary (herbivory-carnivory) shifts. Associated with these changes during amphibian development are changes in their isotopic signatures, as primary aquatic and terrestrial production tends to vary in $\delta^{13}C$ (e.g. Rau 1980), while stepwise changes in trophic position are revealed by $\delta^{15}N$ (Minagawa & Wada 1984). I found that $\delta^{13}C$ values in *A. angolensis* adults were markedly enriched in comparison with tadpoles, suggesting differences in their diets. Ontogenetic diet shifts dictate the complex structure and function of food webs and are necessary to understanding community dynamics (Polis 1991); however, they are seldomly incorporated into food web studies. According to Post & Takimoto (2007), changes in resource consumption of intermediate level consumers can induce changes in higher trophic levels, resulting in shortening or lengthening effects on food chains. Discrete shifts in diet between larval and juvenile and adult life stages are abundant across animal groups (Werner et al. 1995). I found ontogenetic changes in the diets of frog populations of the Kowie River, as tadpoles switched to feeding on insects after metamorphosis. Changes in diet were reflected in increased trophic position of frogs, especially a marked shift between tadpoles that were ranked as primary consumers and post-metamorphic stages that were classified as tertiary consumers. Changes to a more terrestrial diet for *A. angolensis* and *S. grayii* through developmental stages (from tadpoles to adult form) at both sampled locations were reflected in their isotopic values and mixing models. Schriever & Williams (2013) also found ontogenetic diet shifts in amphibian larvae (*Lithobates sylvaticus*) based on both isotopic signature and gut content data. These researchers observed that *L. sylvaticus* switched from dependence on primarily non-filamentous algae and detritus as tadpoles, followed by an abrupt increase in the consumption of insects as adults (Schriever & Williams 2013). Furthermore, Trakimas et al. (2011) found ontogenetic dietary shifts in the European common frog (*Rana temporaria*). These authors found that $\delta^{13}C$ values in *R. temporaria* tadpoles were significantly depleted in comparison to adults, signifying a terrestrial to aquatic shift in energy sources.

Nitrogen signatures can provide insights into the trophic positions of consumers (Peterson & Fry 1987; Post 2002) and/or they may simply represent feeding on food types that differ in their $\delta^{15}N$ even though they are at the same trophic level (Bunn et al. 2003). Through the stable isotope approach, I observed an increase in the trophic positions of *A. angolensis* and *S. grayii*

from tadpole to adult, which confirmed that the tadpoles are primary consumers and adults are secondary consumers. *Amietia* frogs found downstream in the Kowie River were much higher in $\delta^{15}N$ values than their tadpoles, indicating a shift from aquatic diet to a terrestrial insect diet with the shift from herbivory (tadpoles) to carnivory (adults). Similarly, in *S. grayii* the nitrogen signatures increased after the transit from tadpoles in the aquatic habitat to adults in the terrestrial habitat, reflecting changes in diet to mostly terrestrial prey. Enrichments of $\delta^{15}N$ by $\geq$ 3‰ (one trophic level) were anticipated if adult frogs occupy a higher trophic level than tadpoles, and this pattern was observed as *Strongylopus* adults were 3.6 ‰ higher in $\delta^{15}N$ in comparison to their tadpoles at the downstream site.

The mixing models suggested that prey from aquatic sources made up the largest proportion of the potential *X. laevis* diet within the sampled community. The high contribution of aquatic sources to the diet of *Xenopus* was consistent with findings in previous studies (Measey 1998; Bwong & Measey 2010). On the other hand, terrestrial sources made up the largest proportions of the diets for the other species within the Kowie River community. Though stable isotope analysis does not give the taxonomic resolution of stomach content analysis, it did suggest that terrestrial prey constitute consistently significant portions of the diets of *Amietiophyrus* and *Amietia* sp. Other authors have noted that terrestrial material plays an important role in the aquatic environment. For instance, observations indicate that not only prey associated with aquatic vegetation, but insects that fall on the surface of water bodies make important contributions to the diet composition of some amphibian species (Dure and Kehr 2001; Texeira et al 2004). In contrast with these findings, my results suggested that some amphibians such as the *Xenopus* are strongly dependent on carbon sources originating in aquatic systems.

The stable isotope data showed that frogs in the Kowie River derived their nutrition from both terrestrial and aquatic sources. The $\delta^{15}N$ data provided an indication for arthropodivory by the adult amphibians and for herbivory by the tadpoles, thereby linking terrestrial and aquatic food webs. Substantial declines in amphibian abundance and species richness are occurring in many regions, but little is known about the ecological significance of these losses (Houlahan et al. 2000; Stuart et al. 2004). Field experiments have shown that invertebrate populations, primary production, nutrient cycling, and leaf litter decomposition are affected when tadpoles (Kiffeney & Richardson 2001; Ranvestel et al. 2004), frogs (Beard et al. 2002), and salamanders (Davic & Welsh 2004) are removed from systems. This study is a step towards a better understanding of the trophic ecology of anurans, their role in aquatic-terrestrial systems, and their dietary shifts within and between habitats.

5 ASSESSING THE DIETS OF FOUR FROG SPECIES IN A TEMPERATE RIVER USING FATTY ACID ANALYSES

5.1 INTRODUCTION

Central to ecology is understanding food web dynamics and trophic interactions, but this is often complicated because of the occurrence of organisms with wide diet regimes such as omnivores, particularly in systems with high consumer diversity and long food chains (Thompson et al. 2007; Whiles et al. 2010). The difference between what is consumed and what is assimilated creates difficulties in assessing trophic status and ecological roles (e.g. Altig et al. 2007). As a result, thorough datasets on prey ingestion and assimilation in omnivorous species are required for a precise assessment of trophic status (e.g. Benke & Wallace 1980; Evans-White et al. 2003).

Of the diversity of consumers in freshwater systems, anurans are some of the least understood in terms of trophic relations (Petranka & Kennedy 1999; Altig et al. 2007). Anurans are obvious consumers in a variety of freshwater and riparian systems, and are common components of freshwater mesocosm studies, nonetheless there is surprisingly a lack of information on their ecological roles. Even so, several studies have shown evidence that amphibians can affect ecosystem processes and functions in freshwater systems through their feeding activities (Davic & Welsh 2004; Whiles et al. 2006; Regester et al. 2008). Gaps in knowledge concerning the ecological roles of amphibians are becoming more and more evident as there is an increasing loss of amphibians worldwide (Stuart et al. 2004; Whiles et al. 2006). Considering the current biodiversity crisis (Connelly et al. 2008), the necessity for quantitative information on trophic interactions and the ecological roles of these consumers is growing. These losses in biodiversity may in turn have negative effects (such as reduced energy transfers between and within habitants) on ecosystem function and processes in freshwater habitats (e.g. Whiles et al. 2006; Verburg et al. 2007).

In freshwater systems, analysing diets of consumers and omnivores is ecologically significant, but difficult methodologically. Customary methods that have been used for many years include direct field observation and visual examination of stomach contents, though these are time-consuming processes that are logistically difficult and prone to bias (e.g. Evans-White et al. 2003; Ranvestel et al. 2004; Alfaro et al. 2006). Any soft-bodied or highly digestible food items are underestimated, and the importance of food items that were recently eaten is often over-

estimated (Kelly & Scheibling 2012). These shortcomings of traditional techniques have led to the advancement of biochemical methods such as fatty acid analysis (e.g. Gladyshev et al. 2000; Torres-Ruiz et al. 2007). Fatty acid (FA) analysis has the potential advantage of being less biased, and may reveal information on the quality and type of resources assimilated by consumers over a longer period of time (Dalsgaard & John 2004; Iverson et al. 2004). Because some FAs are specific to certain dietary sources and can be transferred to higher trophic levels without significant change, they can be suitable for use as dietary indicators (Dalsgaard & John 2004; Koussoroplis et al. 2010). Furthermore, FA compositions in consumers are useful for detecting differences in trophic niches between co-occurring species (Goedkoop et al. 1998; Richoux et al. 2014).

Given the general lack of information on the amphibians in South Africa, and the continued development of biochemical techniques for assessing diet and trophic interactions, the present study used FA analysis to investigate the trophic dynamics of four anurans found in the Kowie River. Specifically, FA profiles of frogs were used to assess diets within and among species at two locations along the river.

5.2 MATERIALS AND METHODS

5.2.1 Study area, sample collection and treatment

Full descriptions of the study sites (Section 2.1) and processes followed for sampling methods (Section 2.22), sample treatment (Section 2.3.1) and FA analyses (Section 2.3.4) are provided in Chapter 2.

5.2.2 Data analysis

Non-metric multidimensional scaling (n-MDS), analysis of similarity (ANOSIM) and similarity percentages (SIMPER) of the qualitative FA data were performed to determine the differences in FA signatures within and among species and identify the FAs responsible for any differences. The data were also processed using principal components analysis (PCA) to assist in visually representation of the influential FA in the n-MDS plots (factor loadings with > 0.2 % contributions to the variability were considered influential and were superimposed in the n-MDS graphics). ANOSIM outputs include R values (with 1 showing complete dissimilarity and zero showing complete similarity), mean ranks of variability within and between groups, and a related p-value for each factor. All statistical analyses were performed using PAST 3.0 (Hammer et al. 2001).

5.3 RESULTS

Thirty-six fatty acids were detected at concentrations > 1 % of total fatty acids (TFA) in at least three individuals of the sampled amphibians. Fatty acid profiles were significantly different among frogs collected from different sites ($R = 0.07$; $p < 0.0261$) (Table 5.3). Major FA in the individuals from upstream included the monounsaturated fatty acids (MUFAs) 18:1ω9 and 16:1ω7; the saturated fatty acids (SFAs) 16:0 and 18:0, and the polyunsaturated fatty acids (PUFAs) 18:3ω3, 18:3ω6, 20:4ω6, 20:5ω3, 22:5ω3 and 22:6ω3. Downstream, the major FA included the MUFAs 18:1ω9, 18:1ω7 and 16:1ω7, the SFAs 16:0 and 18:0, and the PUFAs 18:3ω3, 18:3ω6, 18:2ω6, 20:3ω6 and 20:4ω6.

Proportions of SFAs ranged from 32.2 to 50.0 % downstream, with the highest proportions found in *Strongylopus* tadpoles, *Am. rangeri* and *A. angolensis*, and the lowest levels found in *A. angolensis* tadpoles (Table 5.2). Upstream there was relatively little variation in SFA proportions (ranged between 30.6 and 36.0%), with *S. grayii* tadpoles having the highest proportions (36.0 %) and *S. grayii* having the lowest. Palmitic acid (16:0) was the most common SFA identified in all species at both sites, followed by 18:0, 17:0, and 14:0, respectively. Of all the MUFAs identified, oleic acid 18:1ω9 was the most dominant in all species, followed by 16:1ω7 and 18:1ω7 at both sites (Tables 5.1 and 5.2). The highest levels of long-chained MUFAs (i.e. 17:1ω7, 20:1ω8 and 20:1ω9) occurred in *X. laevis* at the downstream site. The diatom-associated FA 16:1ω7 occurred in substantial proportions in tadpoles at both the upstream and downstream sites.

The proportions of PUFAs were high at both sites in different species (upstream: *A. angolensis* tadpole, *S. grayii* tadpole and *X. laevis*, and downstream: *S. grayii* tadpole and *Am. rangeri*) having low MUFA and/or SFA content. Levels ranged from 39.1 % in *X. laevis* at the upstream site to 50.0 % in *S. grayii* tadpoles at the downstream site. The most prominent PUFAs were 20:5ω3, 22:5ω3 and 22:6ω3 in all species at both locations. However, the dinoflagellate-associated FA 22:6ω3 was found in small proportions in *Am. rangeri* (2.4 ± 0.4%) and *X. laevis* (3.5 ± 1.3%) at the downstream site. While the proportions of ω3 PUFAs in frogs from the downstream site showed relatively little variation among species (14.3 – 18.9 %), proportions in frogs at the upstream site were highly variable, with values ranging from 14.7 % in *S. grayii* to 23.7 and 23.0 % in *S. grayii* tadpoles and *A. angolensis* tadpoles, respectively. The proportions of essential fatty acids (EFAs) in specimens from the upstream site were considerably high (ranged from 13.8 to 20.0 %), whereas at the downstream site EFAs in frogs

were found in moderate proportions that showed more variability. There were also moderate proportions of bacterial fatty acids (BAFAs; *i*15:0, *ai*15:0, *i*17:0 and 17:0), with *A. angolensis* tadpoles and *S. grayii* tadpoles having high proportions of these FA followed by *X. laevis* at upstream. Downstream, *A. angolensis* tadpoles and the adult form had similar proportions of BAFAs, and the remaining species showed high levels of these FA. The BAFA 17:0 occurred consistently in substantial concentrations in all the different species at both sampled regions. Fatty acid profiles of all species are detailed in Tables 5.1 and 5.2.

Table 5.1 Fatty acid composition (mean % TFA ± SD) of three amphibian species from the upstream site of the Kowie River

FA	*Amietia* tadpole	*A. angolensis*	*Strongylopus* tadpole	*S. grayii*	*X. laevis*
14:0	1.00 ± 0.39	0.45 ± 0.27	1.30 ± 0.57	0.46 ± 0.40	0.79 ± 0.38
i-15:0	0.00 ± 0.00	0.03 ± 0.10	0.07 ± 0.20	0.11 ± 0.21	0.11± 0.20
ai-15:0	0.05 ± 0.12	0.05 ± 0.13	0.13 ± 0.27	0.07 ± 0.13	0.11 ± 0.18
15:0	0.68 ± 0.24	0.26 ± 0.20	0.64 ± 0.19	0.38± 0.23	0.38 ± 0.21
16:0	20.51 ± 1.97	17.97 ± 2.60	21.43 ± 1.18	19.10 ± 1.82	19.18 ± 2.74
i-17:0	0.93 ± 0.45	0.01 ± 0.04	0.15 ± 0.45	0.01 ± 0.03	0.32 ± 0.23
ai-17:0	1.00 ± 0.56	0.20 ± 0.49	0.46 ± 1.00	0.18 ± 0.37	0.37 ± 0.30
17:0	1.81 ± 0.94	0.71 ± 0.44	1.94 ± 0.57	1.14 ± 0.71	1.00 ± 0.59
18:0	8.91 ± 1.84	10.03 ± 2.36	10.00 ± 0.90	9.68 ± 2.71	9.67 ± 2.52
20:0	0.43 ± 0.22	0.60 ± 0.52	0.38 ± 0.31	0.54 ± 0.81	0.33 ± 0.28
22:0	0.42 ± 0.40	0.66 ± 0.61	0.31 ± 0.46	0.36 ± 0.36	0.34 ± 0.54
23:0	0.17 ± 0.46	0.12 ± 0.49	0.38 ± 1.14	0.04 ± 0.10	0.00 ± 0.00
24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:1ω7	7.31 ± 1.94	1.78 ± 1.60	7.05 ± 1.84	4.36 ± 2.64	4.26 ± 1.33
17:1ω7	0.21 ± 0.24	0.04 ± 0.10	0.00 ± 0.00	0.08 ± 0.26	0.22 ± 0.22
18:1ω7	6.79 ± 1.53	3.82 ± 1.93	5.78 ± 1.75	3.71 ± 1.61	7.11 ± 1.78
18:1ω9	11.78 ± 3.58	24.38 ± 7.23	12.85 ± 6.11	25.33 ± 8.66	16.29 ± 5.11
18:2ω6	3.38 ± 1.46	16.74 ± 6.52	4.10 ± 2.60	12.56 ± 4.65	8.48 ± 1.74

18:3ω3	0.00 ± 0.00	3.09 ± 1.32	0.00 ± 0.00	2.64 ± 1.05	3.80 ± 1.67
18:3ω6	3.80 ± 1.14	0.00 ± 0.00	2.41 ± 1.87	0.00 ± 0.00	0.00 ± 0.00
18:4ω3	0.00 ± 0.00	0.02 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20.2ω7	0.27 ± 0.73	0.00 ± 0.00	0.00 ± 0.00	0.37 ± 0.25	0.00 ± 0.00
20:1ω8	0.00 ± 0.00	0.02 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.76
20:1ω9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:2ω9	0.00 ± 0.00	0.65 ± 1.24	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.35
20:3ω6	0.22 ± 0.59	0.41 ± 0.53	0.00 ± 0.00	0.62 ± 0.33	0.45 ± 0.32
20:3ω7	1.28 ± 1.80	0.66 ± 1.99	1.09 ± 0.80	0.47 ± 0.903	0.59 ± 1.57
20:3ω9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4ω3	1.29 ± 1.76	0.39 ± 0.97	1.10± 1.49	0.02 ± 0.07	0.93 ± 1.70
20:4ω6	5.27 ± 2.34	4.74 ± 2.48	6.34 ± 1.01	5.15 ± 2.08	7.06 ± 3.09
20:5ω3	8.44 ± 2.33	2.61 ± 2.17	9.39 ± 1.42	3.41 ± 1.67	5.10 ± 2.77
22:4ω6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.14
22:5ω3	6.58 ± 1.17	2.80 ± 2.32	4.87 ± 3.03	3.15 ± 1.67	4.23 ± 1.20
22:5ω6	1.01 ± 1.47	0.34 ± 0.89	0.19 ± 0.58	1.03 ± 0.93	0.58 ± 0.36
22:6ω3	6.32 ± 1.55	6.408 ± 3.24	7.656 ± 2.46	5.01 ± 2.80	7.64 ± 2.38
ΣPUFA	**38.00 ± 5.46**	**38.34 ± 8.20**	**37.22 ± 2.98**	**34.61 ± 11.45**	**39.14 ± 5.32**
ΣEFA	**20.02 ± 3.51**	**13.75 ± 5.83**	**22.57 ± 4.11**	**13.59 ± 7.26**	**19.80 ± 5.21**
ΣSFA	**33.93 ± 4.29**	**30.84 ± 4.36**	**35.95 ± 2.09**	**30.62 ± 4.25**	**31.79 ± 4.28**
ΣMUFA	**26.10 ± 2.84**	**30.41 ± 7.72**	**25.68 ± 4.09**	**34.55 ± 12.39**	**27.88 ± 3.82**
ΣBAFA	**24.98 ± 3.26**	**19.33 ± 2.79**	**24.34 ± 1.67**	**20.59 ± 2.31**	**21.47 ± 2.80**
ΣHPFA	**3.61 ± 1.86**	**18.85 ± 8.23**	**4.78 ± 4.46**	**14.65± 5.72**	**12.28 ± 1.70**
Σω3	**23.01 ± 3.87**	**15.07 ± 6.01**	**23.69 ± 4.77**	**14.77 ± 6.33**	**21.74 ± 5.01**
ω3/ω6	**1.76 ± 0.45**	**0.79 ± 0.51**	**2.273 ± 1.62**	**0.82 ± 0.37**	**1.34 ± 0.39**

**ΣSFA*: total sum of all saturated fatty acids, Σ*MUFA*: monounsaturated fatty acids, sum of fatty acids with one double bond; Σ*PUFA*: polyunsaturated fatty acids, sum of fatty acids with two or more double bonds; Σ*EFA*: essential fatty acids (20:4ω6+20:5ω3+22:6ω6); Σ*BAFA*: bacterial fatty acids (*i*-14:0+*i*-15:0+*ai*-15:0+15:0+*i*-16:0+*i*-17:0+*ai*-17:0+17:0); *ΣHPFA*: higher plant fatty acids (18:2ω6+18:3ω3+24:0+25:0+26:0+28:0).

Table 5.2 Fatty acid composition (mean % TFA ± SD) of the four amphibian species from the downstream site

FA	*Amietia* tadpole	*A. angolensis*	*Strongylopus* tadpole	*S. grayii*	*Am. rangeri*	*X. laevis*
14:0	0.86 ± 0.523	0.89 ± 0.92	1.15 ± 0.74	1.18 ± 1.05	1.35 ± 0.81	2.51 ± 1.97
i-15:0	0.12 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.77	0.00 ± 0.00	0.55 ± 0.64
a-15:0	0.13 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.55	0.2 ± 0.28	0.32 ± 0.47
15:0	0.84 ± 0.39	0.62 ± 0.85	0.48 ± 0.33	0.52 ± 0.38	0.66 ± 0.38	0.90 ± 0.83
16:0	17.42 ± 4.78	17.74 ± 1.41	20.06 ± 3.10	21.79 ± 3.69	13.62 ± 0.81	21.30 ± 1.32
i-17:0	0.31 ± 0.54	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.99 ± 0.72
ai-17:0	0.65 ± 0.15	0.00 ± 0.00	0.49 ± 0.85	0.25 ± 0.61	0.00 ± 0.00	1.04 ± 0.71
17:0	1.51 ± 0.54	1.45 ± 0.69	0.37 ± 0.28	1.39 ± 0.43	1.14± 0.42	1.92 ± 0.56
18:0	8.79 ± 1.11	15.57± 2.05	11.31 ± 1.18	10.73 ± 1.02	10.03 ± 2.81	7.85 ± 1.16
20:0	0.56 ± 0.54	0.98 ± 0.61	0.42 ± 0.24	0.26 ± 0.16	0.59 ± 0.45	0.26 ± 0.31
22:0	1.00 ± 0.28	1.78± 0.65	0.79 ± 0.63	0.20 ± 0.19	0.74 ± 0.49	0.54 ± 0.85
23:0	0.00 ± 0.00	0.38 ± 0.65	0.00 ± 0.00	0.29 ± 0.28	0.25 ± 0.35	0.12 ± 0.31
24:0	0.17 ± 0.29	0.39 ± 0.68	0.00 ± 0.00	0.00 ± 0.00	0.39 ± 0.55	0.13 ± 0.35
16:1ω7	7.02 ± 0.88	1.99 ± 2.21	5.15 ± 2.44	3.95 ± 3.01	3.95 ± 1.82	6.85 ± 3.05
17:1ω7	0.54 ± 0.70	0.44 ± 0.75	0.00 ± 0.00	0.00 ± 0.00	0.46 ± 0.09	1.22 ± 0.42
18:1ω7	6.42 ± 0.29	3.39 ± 0.95	3.40± 0.39	3.96 ± 0.45	2.72 ± 0.16	7.79 ± 1.97
18:1ω9	11.13 ± 3.42	23.79 ± 6.24	14.87 ± 0.62	24.08 ± 5.71	21.61 ± 3.13	12.96 ± 2.46
18:2ω6	3.97 ± 0.83	11.98 ± 4.23	11.72 ± 1.02	8.14 ± 4.37	19.82 ± 3.75	5.79 ± 1.38

18:3ω3	1.02 ± 1.77	3.64 ± 0.65	3.71 ± 0.51	0.44 ± 1.07	2.51 ± 1.03	0.59 ± 1.05
18:3ω6	4.10 ± 3.44	0.00 ± 0.00	0.00 ± 0.00	2.85 ± 1.60	0.31 ± 0.44	6.39 ± 0.98
18:4ω3	0.14 ± 0.25	0 .00± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20.2ω7	0.15 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.19
20:1ω8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.77
20:1ω9	0.18 ± 0.32	0.11 ± 0.30	0.00 ± 0.00	0.00 ± 0.00	0.49 ± 0.08	0.06 ± 0.16
20:2ω9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.32 ± 0.45	0.16 ± 0.31
20:3ω6	4.12 ± 2.92	0.19 ± 0.33	6.95 ± 0.51	0.00 ± 0.00	0.74 ± 0.27	3.19 ± 1.24
20:3ω7	0.18 ± 0.31	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.18	0.00 ± 0.00
20:3ω9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4ω3	0.89 ± 0.38	0.10 ± 0.26	2.89 ± 0.69	1.38 ± 0.93	0.00 ± 0.00	6.03 ± 1.53
20:4ω6	2.45 ± 3.37	3.97 ± 0.90	0.64 ± 0.35	1.75 ± 1.34	9.91 ± 1.52	0.72 ± 0.24
20:5ω3	9.13 ± 1.73	2.69 ± 1.95	0.14 ± 0.24	6.11 ± 1.88	2.69 ± 0.35	1.27 ± 0.75
22:4ω6	0.12 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.55 ± 0.18	0.38 ± 0.38
22:5ω3	6.83 ± 1.00	2.73 ± 1.28	5.89 ± 0.66	2.79 ± 0.81	1.72 ± 0.45	3.18 ± 1.83
22:5ω6	1.22 ± 0.38	0.00 ± 0.00	1.29 ± 0.19	0.00 ± 0.00	0.39 ± 0.11	0.61 ± 0.46
22:6ω3	6.66 ± 4.44	4.61 ± 1.47	8.28 ± 0.073	7.06 ± 2.13	2.39 ± 0.35	3.46 ± 1.31
ΣPUFA	**32.61 ± 5.22**	**37.51 ± 18.64**	**50.01± 16.46**	**33.25 ± 5.81**	**42.21 ± 0.53**	**33.29 ± 8.45**
ΣEFA	**14.16 ± 6.40**	**3.64 ± 2.39**	**5.03 ± 3.12**	**5.63 ± 3.73**	**8.77 ± 2.78**	**5.91 ± 3.28**
ΣSFA	**37.05 ± 3.83**	**30.77 ± 7.51**	**22.83 ± 12.74**	**33.24 ± 11.69**	**31.16 ± 1.60**	**31.70 ± 6.89**
ΣMUFA	**30.34 ± 5.83**	**30.42 ± 18.39**	**24.18 ± 5.69**	**32.55 ± 7.55**	**25.92 ± 1.12**	**33.74 ± 11.72**

ΣBAFA	**16.02 ± 7.49**	**16.96± 13.46**	**10.97 ± 11.79**	**19.76 ± 11.55**	**19.66 ± 1.44**	**19.79 ± 7.13**
ΣHPFA	**15.09 ± 8.00**	**7.76± 9.12**	**0.76 ± 0.69**	**5.76 ± 6.15**	**3.57 ± 0.54**	**2.92 ± 2.45**
Σω3	**16.22 ± 6.78**	**14.34 ± 8.80**	**15.39 ± 12.09**	**14.37 ± 4.04**	**18.92 ± 8.42**	**15.71 ± 8.27**
ω3/ω6	**1.26 ± 1.06**	**2.47 ± 1.99**	**2.99 ± 2.54**	**1.50 ± 1.81**	**0.97 ± 0.73**	**0.90 ± 0.45**

**Σ*SFA*: total sum of all saturated fatty acids, Σ*MUFA*: monounsaturated fatty acids, sum of fatty acids with one double bond; Σ*PUFA*: polyunsaturated fatty acids, sum of fatty acids with two or more double bonds; Σ*EFA*: essential fatty acids (20:4ω6+20:5ω3+22:6ω6); Σ*BAFA*: bacterial fatty acids (*i*-14:0+*i*-15:0+*ai*-15:0+15:0+*i*-16:0+*i*-17:0+*ai*-17:0+17:0); *ΣHPFA*: higher plant fatty acids (18:2ω6+18:3ω3+24:0+25:0+26:0+28:0).

Table 5.3 One-way ANOSIM outputs for the significant differences in the fatty acid profiles among frogs collected from the upstream and downstream regions.

		ANOSIM output		
FACTOR	R	mean rank within	mean rank between	p
species	0.37	1079	1655	< 0.0001
site	0.07	1488	1602	< 0.0261

Table 5.4 Follow up pair-wise comparison from the ANOSIM to determine how fatty acid profiles of the different sampled species differ among each other.

	A. angolensis tadpole	*A. angolensis*	*S. grayii tadpole*	*S. grayii*	*X. laevis*	*Am. rangeri*
A. angolensis tadpole		**<0.0001**	0.3150	**<0.0001**	**<0.0043**	**<0.01**
A. angolensis	**0.70**		**<0.0001**	**<0.0138**	**<0.0001**	0.43
S. grayii tadpole	0.01	**0.55**		**<0.0001**	**<0.0482**	**<0.02**
S. grayii tadpole	**0.58**	0.10	**0.39**		**<0.0001**	0.12
X. laevis	0.26	**0.51**	0.11	**0.35**		**<0.01**
Am. rangeri	**1.00**	0.04	**0.77**	0.27	**0.73**	

**Lower left: R values, upper right p-values.*

SIMPER analysis (>50% cumulative percent contribution) and PCA revealed the FA that greatly influenced the differences among the four species sampled (superimposed in Figure 5.1). n-MDS ordination suggested differentiation among the species at each site (Figure 5.1). Pairwise comparisons using the FA dataset showed that *S. grayii* tadpoles were completely similar to *A. angolensis* tadpoles at the upstream site, and the remaining species were statistically dissimilar (Table 5.5). Downstream, the pairwise comparisons using FA profiles indicated complete dissimilarities of *Am. rangeri* and *X. laevis* to the other species (Table 5.6).

Table 5.5 Follow up pair-wise ANOSIM comparisons to determine how fatty acid signatures differed among the frogs sampled at the upstream site of the Kowie River.

	Amietia tadpoles	*A. angolensis*	*Strongylopus* tadpoles	*S. grayii*	*X. laevis*
Amietia tadpoles		**<0.05**	0.35	**<0.05**	**<0.05**
A. angolensis	0.69		**<0.05**	0.24	**<0.05**
Strongylopus tadpoles	**0.00**	**0.65**		**<0.05**	**<0.05**
S. grayii	0.59	0.03	**0.55**		0.06
X. laevis	0.49	**0.29**	**0.34**	0.18	

**Lower left: R values, upper right p-values.*

Table 5.6 Follow up pair-wise ANOSIM comparison to determine how fatty acid signatures differed among the frogs sampled at the downstream site of the Kowie River.

	Amietia tadpoles	*A. angolensis*	*Strongylopus* tadpoles	*S. grayii*	*Am. rangeri*	*X. laevis*
Amietia tadpoles		**<0.05**	0.10	**<0.05**	0.10	**<0.05**
A. angolensis	0.81		**<0.05**	**<0.05**	0.06	**<0.05**
Strongylopus tadpoles	0.70	0.64		**<0.05**	0.10	**<0.05**
S. grayii	0.65	0.43	0.57		0.03	**<0.05**
Am. rangeri	**1.00**	0.51	**1.00**	0.82		**<0.05**
X. laevis	0.75	**0.91**	**0.99**	0.84	**1.00**	

**Lower left: R values, upper right p-values.*

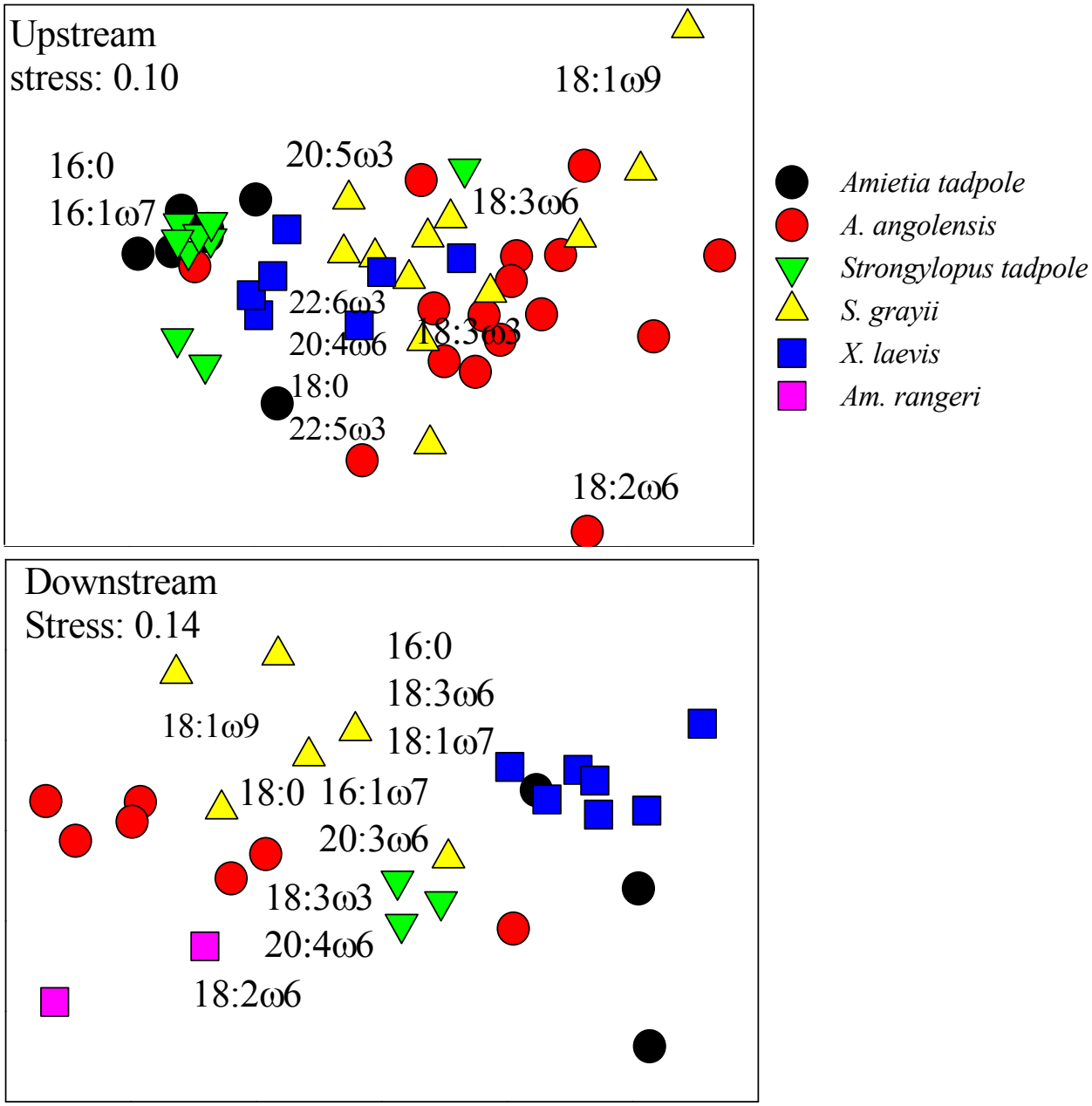


Figure 5.1 Non-metric multidimensional scaling output using fatty acid signatures of *A. angolensis, Am. rangeri, S. grayii and X. laevis* from both sampling sites (A= upstream and B= downstream). Fatty acids responsible for separating the four species (derived from SIMPER and PCA) are overlaid in the plots.

5.4 DISCUSSION

Trophic relationships in omnivores are complex because their lipid profiles originate from a variety of dietary sources (Dalsgaard & John 2004), and it is not often possible to determine whether FA were transferred to consumers directly or via lower-order consumers (Iverson 2009; Pitt et al. 2009). Nevertheless, FA composition techniques have been used previously to assess diets of aquatic consumers (e.g. Desvilettes et al. 1997; Koussoroplis et al. 2008). I used FA analysis to elucidate the diets of four anurans in the Kowie River, South Africa. The anurans analysed were characterized by different FA compositions which varied with location and among species. Fatty acid profiles were more similar among species from the upstream region, while downstream specimens were much more spread out from each other. All FA showed no distinct ontogenetic patterns. The FA profiles for *A. angolensis* and *S. grayii* indicated no obvious pattern, and the adult forms and tadpoles and were similar regardless of life stage. The FA 16:0, 20:5ω3 and 22:6ω3 were always present in high proportions in both the adults and tadpoles (Tables 5.1 and 5.2).

5.4.1 Fatty acids of consumers

The use of different biomarkers can potentially provide a plethora of information over a wide range of food targets. High levels of SFAs 16:0 and 18:0 were expected in the tissue muscles of the analysed samples as these components represent the most common FA in nature and they form an important part of cellular membrane components (Garret & Grisham 1999). According to Freites et al. (2002), detritus is recognized as a source of SFAs with 14 to 18 carbons. High levels of these FA could indicate significant inputs of detritus and bacterial material in the diets of consumers. All four anuran species in the Kowie River had substantial levels of BAFAs (upstream; 19.3 – 24.9 % and downstream; 10.9 – 19.8 %, reflecting detrital contributions to the diets of these consumers. The tadpoles (*A. angolensis* tadpoles and *S. grayii* tadpoles) had greater fractions of BAFAs (i.e. 15:0, *ai*-17:0 and 17:0; *A. angolensis* = 0.7, 1.0, 1.8 % and *S. grayii* tadpole = 0.6, 0.5, 1.9 %, respectively) at both sites. Similarly, Whiles et al. (2010) used FA to examine tadpole diets from ponds in southern Illinois and found that tadpoles consumed substantial amounts of detritus.

High levels of oleic acid (18:1ω9) are often used as the general indicator of carnivorous feeding (Graeve et al. 1997, Falk-Petersen et al. 2002; Drazen et al. 2008). Many animals can produce 18:1ω9 from the desaturation of 18:0, the modification of 18:1ω7 (Mayzaud et al 1989; Nichols et al 1991), or the elongation of 16:1ω7, which is algal or bacterial in origin (Graeve et al.

1997). As a result, proportionally high levels of the FA 18:1ω9 can be used as an indicator of carnivorous feeding in some cases. The levels of 18:1ω9 were elevated in the anurans from the Kowie River (11.8 – 25.3 % upstream and 11.1 – 24.1 % downstream), supporting high levels of carnivory and/or omnivory in the species' diets. Whiles et al. (2010) reported high degrees of omnivory and dietary plasticity by analysing FA compositions in pond dwelling tadpoles in southern Illinois, USA. Furthermore, Gillespie (2013), who used stable isotope analysis (which also accounts for prey assimilation) to study the foraging ecology of a highly endangered amphibian, *Eurycea sosorum*, in Eliza Spring Texas, USA, found that this amphibian demonstrated high levels of carnivory. Several researchers have shown that anurans feed on a variety of food sources in general (Altig et al. 2007; Whiles et al. 2010; Caut et al. 2012; Schriever & Williams 2013). The summation of FA 18:2ω6 and 18:3ω3 represents higher plant fatty acids (HPFAs) and these FA can be found in plant and macroalgae species, and represent a terrestrial input in diets of consumers(Dalsgaard et al. 2003). These HPFAs were found in low concentrations in *X. laevis, A. angolensis* tadpole and *S. grayii* tadpole, suggesting that terrestrial organic matter does not constitute an important part of these consumer's diets.

In higher trophic level consumers, the PUFAs 20:5ω3 and 22:6ω3 are very important for growth and survival and these components must be acquired mainly from feeding (González-Félix et al. 2003). Generally, high levels of PUFAs correspond with high quantity and quality food (i.e. good nutritional condition in the field) (Pazos et al. 2003; Narváez et al. 2008). Based on the FA in anurans from the Kowie River, the proportions of PUFAs were high at both upstream and downstream locations (upstream; 34.1 – 39.1 % and downstream; 32.6 – 50.0 %) and relatively similar among some of the species (*A. angolensis* tadpole, *S. grayii* tadpole, *A. angolensis, S. grayii* and *X. laevis*). The frogs and tadpoles at the upstream site showed large proportions of PUFAs and EFAs (PUFAs; 34.6 – 39.1 and EFAs; 13.6 – 22.6 %), reflecting a good quality of food as consumers were acquiring sufficient lipids to meet their dietary needs. Interestingly, variations in EFAs levels were reflected amongst species in the downstream (3.6 – 14.2 %) suggesting a large variation in the food sources available.

The FA compositions of aquatic animals mostly depend on the diets consumed in their habitats and the specific requirements related to physiological adaptations to the environment (Ackman 1995). Fatty acid profiles differed between upstream and downstream, with a greater overall similarity in profiles among species in the upstream region, while downstream the species were overall much more distinct from each other. These results suggested that there may be more opportunity for diet differentiations (perhaps a larger food spectrum available) in the

downstream site. Additionally, the intraspecific dietary differences across sites may have been linked to the differences in the relative availabilities and nutritional values of the food sources, which in turn may be a function of physical differences between the sites. Of the two sites sampled, upstream was narrow making it to have a less complex habitat for the different species to take advantage of, and as a result their diets were more similar to each other.

Fatty acid analysis proved to be a useful tool in assessing trophic relationships within the Kowie River. In most cases, information derived from interpreting the FA compositions was in agreement with the results obtained using gut content analysis (Chapter 3) and stable isotope analysis (Chapter 4). While stable isotopes were able to provide information on the contributions of potential food sources within each location, FA profiles could also identify the degree of contribution of potential food sources to the diet of the consumers. The SIAR results illustrated that aquatic-derived food sources constituted most of the diet of *X. laevis, A. angolensis* tadpoles and *S. grayii* tadpoles, indirectly agreeing with FA results that highlighted that terrestrial-derived sources did not constitute an important part of the diet of these species. Similar to FA results, which illustrated a variation in FA profiles of consumers downstream (Figure 5.1), stable isotopes showed variation among individuals at both sites (Figures 4.3 & 4.4). Gut content analysis and stable isotopes were not able to provide any evidence on the bacterial sources consumed. However, FA biomarkers indicated that the four species had consistent BAFAs levels at both, and high proportions of SFAs with 18 carbons, suggesting that bacterial and detritus food sources played a significant role in their diets.

6 SUMMARY AND CONCLUSIONS

Investigating the diets of amphibians is important for understanding their basic ecology, trophic interactions, and community-level responses to abiotic and biotic changes. This study aimed to use a combination of gut content, stable isotope and fatty acid analyses to provide a time-integrated view of the assimilated feeding history of four co-occurring anurans in the Kowie River, South Africa. Gut contents (e.g. Guidali et al. 2000; França et al. 2004; Santos et al. 2004), stable isotopes (Najera□Hillman et al. 2009; Caut et al. 2012; Gillespie 2013) and fatty acids (Alfaro et al. 2006; Whiles et al. 2010; Lança et al. 2013) have been used in food web ecology to better understand links between resources and consumers. Gut content analysis provides a quantitative measurements of what organisms have ingested and stable isotopes and fatty acids in organisms' tissues provide a useful tool to derive long period dietary information (Kelly & Scheibling 2012). In this thesis, I provided quantitative measurements and discussed the relative importance of what co-occurring anurans in the Kowie River consumed, and my results provided evidence that species utilized a large prey range of both aquatic and terrestrial insects. My study provides the first report on the feeding patterns of anurans in the Kowie River, Eastern Cape, South Africa.

I have demonstrated that the different anuran species in the Kowie River showed differences in their gut contents, stable isotope composition, and fatty acid composition, which all confirmed the prediction that anurans derive their nutrition to different degrees from aquatic and terrestrial sources. Distinct shifts in diet among larval or juvenile and adult life stages are abundant across animal groups (Werner & Hall 1988). I found ontogenetic changes in diet in the anurans; for example, I found that $\delta^{13}C$ and $\delta^{15}N$ values in adult stages were markedly higher in comparison with tadpole stages. Furthermore, FA data illustrated that aquatic-derived resources constitutes an important part of the diet especially in tadpoles. Gut content analysis confirmed the prediction that frogs are generalist consumers feeding on a variety of prey items. The agreement between diet and isotopic composition has been described for anurans (Schriever & Williams 2013). Changes in diet were reflected in increases in trophic position. The adult life stages occupied a higher trophic position in comparison with the tadpoles. Using stable isotope analyses allowed me to differentiate and describe consumer diets, assign trophic positions within among the consumers, and determine which resources were assimilated by

consumers over a long period of time. Results from mixing models for *A. angolensis* and *S. grayii* suggested that aquatic- and terrestrial-derived source contributed relatively equal proportions to their diets, and gut content analysis also illustrated that these two species used a large range of food sources represented by both aquatic and terrestrial sources.

Distinct differences between upstream and downstream regions in Kowie River were observed in fatty acid signatures in adult frogs and tadpoles (Table 5.2 and Figures 5.1). Interestingly, all frogs contained large proportions of PUFAs and EFAs, indicating that food quality was similar among the different species and regions. In addition, all specimens contained microalgal-associated fatty acids (20:5ω3 and 22:6ω3) and 16:0 in large proportions, indirectly highlighting the importance of microalgae at the base of their food chains. Fatty acid analysis identified low levels of 18:2ω6 and 18:3ω3 in the tissues of *X. laevis, A. angolensis* tadpoles and *S. grayii* tadpoles, suggesting that terrestrial organic matter (TOM) is not an important food source for these organisms. Isotopic analyses also confirmed the limited assimilation of terrestrial-derived sources in these species due to their enriched carbon values.

A combination of gut content analysis with biochemical techniques (stable isotope and fatty acid analyses) have been successfully applied to determine the trophic interactions of four co-occurring anurans in two regions of the Kowie River in the Eastern Cape of South Africa. Gut content data use a snapshot of information accounting for recent feeding activity, while tracer analysis integrated diet information over longer time periods (Vander Zanden et al. 1997). Using all three techniques allowed me to differentiate diet items, assign trophic positions, and determine which resources were assimilated by consumers over a relatively long time period.

Future research

Aquatic-terrestrial connections allow for resource subsidies to flow between different habitats. Transfers are mediated by organisms that cross between habitats, and such transfers are important for conserving the structure and function of ecosystems. Various studies have demonstrated that oceans subsidize terrestrial primary production via the transportation of nutrients through terrestrial consumers feeding on marine organisms (Polis & Hurd 1996; Anderson & Polis 1998). Freshwater systems and adjacent terrestrial systems are linked by the emergence of freshwater insects that can increase abundances of the riparian arthropod assemblage (Hoekman et al. 2011). Habitats are considered to have permeable boundaries that make them open to the transfer of nutrients, detritus, and/or organisms (Ballinger & Lake 2006). Freshwater habitats are reciprocally connected to the adjacent riparian habitat through

the flow of energy out of the river (Bartels et al. 2012) via insects and amphibians and input from terrestrial carbon sources (Schriever et al. 2014). There is growing concern for conservation and management of the relationships between biodiversity and ecosystem functioning due to the declines in species diversity on earth (Rugenski et al. 2012). Researchers have suggested that declines in biodiversity will result in the loss of ecosystem functions and services (Greig et al. 2012; Reinhardt et al. 2013). Given the worldwide decline of amphibians organisms and the importance of conserving biodiversity, it is vital to assess the ecological roles amphibians fill to better understand how ecosystems may be affected by their loss (Whiles et al. 2006; Halliday 2008; Gillespie 2013). There are only a few studies that have quantified the movements of amphibians from the freshwater system to the adjacent terrestrial system, and their role in the aquatic-terrestrial food web connections. My thesis provides a foundation for future research into amphibian trophic ecology and the possible role of aquatic-terrestrial connections via amphibians.

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