Species diversity, habitat utilization and blood parasites of amphibians in and around Ndumo Game Reserve

EC Netherlands

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Supervisor: Prof LH du Preez

Co-supervisor: Prof NJ Smit

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Dedication

This dissertation is dedicated to my family and friends, thank you for all your support, especially to my Oupa Jannie Keeve and my father Ed Netherlands. Oupa baie dankie vir u liefde en ondersteuning, sowel as vir die lewenslesse wat Oupa my oor die jare geleer het. Dad, thank you for supporting me every step of the way, and motivating me to follow my passion and dreams. Both of you are true role models and I aspire to be like you.

"As I sat in the rain a little tree-frog, about half an inch long, leaped on to a grassy leaf, and began a tune as loud as that of many birds, and very sweet; it was surprising to hear so much music out of so small a musician."
David Livingstone

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"To fulfill a dream, to be allowed to sweat over lonely labor, to be given the chance to create, is the meat and potatoes of life." - Bette Davis.

ABSTRACT

Ndumo Game Reserve is the only officially protected area within the Phongolo Floodplain; an area in the northern parts of KwaZulu-Natal known to boast a rich diversity of amphibians, thus becoming one of the focal areas for this study. The study's aim was to monitor and record amphibian diversity, as well as associated blood parasite biodiversity. For the purpose of monitoring, a number of active and passive techniques were employed. Habitat preferences for the expected species were divided into five types, namely endorheic, lacustrine, palustrine, riverine and terrestrial. Endorheic habitats were found to harbour the highest diversity (70%) of frog species. A permanent song meter was used to passively record calling activity of frog species associated with endorheic systems. This call data indicated peak breeding season, preferred calling times and intensities of the different species. Historical records from the same area were used as a basis to which this study's data were compared. In the case of the polychromatic Argus Reed Frog *Hyperolius argus* Peters, 1854, questions were raised concerning the major colour changes during development of the apparent sub-adult to adult life stages, an observation which was has caused some confusion as to whether these forms represented a single species or multiple cryptic species. These issues were clarified using techniques such as DNA extraction and polymerase chain reaction (PCR). Furthermore, a blood parasite survey was conducted. Thin blood smears for morphometrics and whole blood for molecular work, were collected from 29 species and 436 individual frogs. For the majority of the recorded parasites, techniques such as light microscopy were utilized for the morphological description and classification of these parasites. Among the recorded frog blood parasites observed, 20% of the frog specimens were infected with at least one blood parasite group. *Hepatozoon* and *Trypanosoma* species accounted for most of the infections; the former demonstrated significant differences in intensity of infection across species, families and habitat types ($P = 0.028$; $P = 0.006$; $P = 0.007$ respectively). Methods, such as transmission electron microscopy, examining the ultrastructure, as well as parasite DNA extraction and 18S rDNA gene sequences for the molecular and phylogenetic characterization, were reserved for *Hepatozoon* species infecting common toad species (*Amietophrynus*). Parasite stages observed were measured and compared to each other, as well as to other described African bufonid haemogregarines. Resulting sequences were compared with each other and to comparative haemogregarine sequences selected from GenBank. In the current study a number of important aspects with regards to monitoring and assessment of amphibians in their natural environment were explored, including looking at and determining diversity and prevalence of blood parasites. Furthermore, important data on gaining a better understanding of amphibians and their behavioural activities were also gathered, which should be

able to assist in conservation actions to effectively protect South African anurans and their required habitat types.

Key words: Amphibian, haemoparasite, haematozoan, passive acoustic monitoring, PCR, polychromatic, song meter

OPSOMMING

Die Ndumo-wildreservaat is die enigste amptelik-beskermde gebied in die Phongola-Vloedvlakte. Hierdie gebied is geleë in die noordelike dele van KwaZulu-Natal, ‘n area wat spog met ‘n hoë amfibieërspesiediversiteit. Met die doel om amfibieër en bloedparasietbiodiversiteit te moniteer en te dokumenteer, is die Ndumo-wildreservaat as studiegebied geselekteer. 'n Aantal aktiewe en passiewe moniteringstegnieke is toegepas. Habitatvoorkeure vir die verwagte spesies is in vyf habitats verdeel, naamlik tydelike panne, mere, moerasse, rivier- en terrestriële gebiede. Daar is bevind dat tydelike habitattipes die hoogste diversiteit (70%) van paddaspesies bevat. 'n Permanente programmeerbare klankopnemer is gebruik om passiewe roepaktiwiteit van paddaspesies wat verband hou met tydelike mikrohabitatte op te neem. Hierdie opnames identifiseer piek-broeiseisoene, voorkeurroeptye en -roepintensiteite van die verskillende spesies. Historiese rekords van dieselfde gebied is gebruik as 'n basis waarteen hierdie studie se data vergelyk kon word. In die geval van die veelkleurige Argusrietpadda, *Hyperolius argus* Peters, 1854, is die kleurverandering tydens die ontwikkeling van die oënskynlike sub-volwasse en volwasse individue bestudeer. Die oogmerk was om te bevestig dat individue met verskillende kleurvariasie wel konspesifiek is. Hierdie navorsingsvraag is onderdoek deur gebruik te maak van onder andere DNA-ekstraksie en polimerase-kettingreaksie (PCR)- tegnieke. 'n Bloedparasietopname is ook gedeon deur dun bloedsmere vir morfologiese identifikasie te maak, sowel as om bloed te versamel vir molekulêre analises van 29 spesies en 436 individuele paddas. Vir die morfologiese beskrywing en klassifikasie van hierdie parasiete is gebruik gemaak van ligmikroskopietegnieke. Ten minste 20% van die paddas wat bestudeer is was besmet met ten minste een parasietgroep. *Hepatozoon* en *Trypanosoma* spesies verteenworrdig meeste van die infeksies. Eersgenoemde toon beduidende verskille in intensiteit van besmetting tussen spesies, families en habitattipes (P = 0,028; P = 0,006; P = 0.007 onderskeidelik). Metodes soos transmissie-elektronmikroskopie, die ondersoek van die ultrastruktuur, sowel as parasiet DNA-ekstraksie en 18s rDNA geen “sequences” vir die molekulêre en filogenetiese karakterisering, was gereserveer vir *Hepatozoon* spesies wat gewone skurwepaddaspesies (*Amietophrynus*) besmet. Parasietstadiums is gemeet en met mekaar vergelyk. Hulle is ook met ander bekende Afrika-bufonid-haemogregarine vergelyk. DNA “alingments” is vergelyk met haemogregarine belynings gekies uit GenBank. In die huidige studie is 'n aantal belangrike aspekte met betrekking tot die monitering en evaluering van amfibieë in hul natuurlike omgewing ondersoek. Dit het die bepaling van diversiteit en die voorkoms van bloedparasiete ingesluit. Verder is belangrike inligting ook verkry wat tot 'n beter begrip van amfibieërs en hul

gedragsaktiwiteite gelei het. Dit dra ook by tot die bewaring van Suid-Afrikaanse paddas en hul vereiste habitattipes.

Sleutelwoorde: Amfibieë, haemoparasite, “haematozoan”, passiewe akoestiese monitering, PCR, veelkleurigheid, klankopnemer

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CHAPTER 1

GENERAL INTRODUCTION

1.1

GENERAL INTRODUCTION

1.1.1 Introduction

Amphibians (class Amphibia) are divided into three orders and for the purposes of this study the focus will be on one of these orders, the Anura (frogs). The Anura is the most diverse of all the orders making up the Amphibia (Frost 2014), South Africa itself boasting a high diversity of frogs, but interestingly lacking in all the other orders of amphibians. According to du Preez & Carruthers (2009) such a rich diversity is as a result of southern Africa's diverse landscape, suitable climate and unique habitat types. One of South Africa's regions known for its high frog diversity is KwaZulu-Natal (KZN), however, very few protected natural areas remain in what is a fast evolving agricultural and urban landscape. One of the hardest hit areas in this regard is the Phongolo Floodplain, which is situated below the Pongolapoort Dam in northern KZN, a region recognised for its high aquatic biodiversity and unique ecosystem (Lankford *et al.* 2010). The only officially protected portion of the Phongolo Floodplain is the section of the Phongolo River and associated pans in the Ndumo Game Reserve (NGR). Very little is known about the amphibians of this area, specifically with regards to their habitat utilization and community structures, their parasitic infections, and in particular, the blood parasites that infect them.

To date, blood parasites have been recorded from a wide range of vertebrate and invertebrate hosts and vectors, stretching from aquatic to terrestrial habitats (see Barta *et al.* 2012). With very few frog blood parasites surveys carried out to date in sub-Saharan Africa, blood parasite diversity, particularly for the region being studied (KZN), remains largely unknown (Readel & Goldberg 2010; Netherlands *et al.* 2014). Yet, before being able to elucidate the effects that these parasites may have on their natural hosts, and the role these parasites may have in amphibian conservation, such diversity knowledge is vitally needed. Although South Africa boasts a high biodiversity of frog species, no multispecies blood parasite survey has ever been conducted within this area, resulting in very few records and descriptions of anuran blood parasites.

1.1.2 Aims of the study

The aims for this study were to:

1. document amphibian species diversity and abundance inside and outside Ndumo Game Reserve;
2. relate frog species diversity and abundance to the location and habitat type;
3. evaluate passive acoustic recording device "song meter" for long-term monitoring of amphibians;
4. compare all current data collected with historical records of the same area, and;
5. determine the amphibian blood parasite diversity and parasitaemia.

1.1.3 Objectives of the study

In order to achieve the aims of this study the following objectives were formulated:

1. Undertake a comprehensive survey and monitoring program of amphibian species diversity and richness, using both active and passive techniques over a two year period.
2. Document the micro-habitat in which each individual is collected and categorised it according to the appropriate systems (endorheic, lacustrine, palustrine, riverine or terrestrial).
3. Use a song meter to passively monitor amphibian activity at a selected locality for the duration of a year and compare recorded data to the other surveying methods in order to measure its effectiveness and efficiency.
4. Collect historical data from Lambiris (1989), Ezemvelo Wildlife as well as from the atlas and red data book (Minter *et al.* 2004), in order to gather information on the frog species previously recorded within the study area.
5. As a case study do morphological and molecular analysis of one of the species collected during the biodiversity survey.
6. Conduct a survey on the diversity, prevalence and parasitaemia of frog blood parasites, by means of blood smear preparation (fixing and staining with Giemsa stain), light microscopy screening and statistical analysis (on the prevalence and parasitaemia between frog species, families, habitat types and sampling periods). When possible classify parasite species to genus level based on their basic morphology.

7. As a case study, do a complete species description using morphological as well as molecular characteristics of one of the species of blood parasites found.

1.1.4 Outline of dissertation

Following the brief introduction (Chapter 1), the dissertation is divided in to two main sections, the first generally focusing on anurans (Chapters 2 & 3) and the second section focusing on blood parasites of anuran hosts (Chapters 4 & 5). These two sections are followed by a final summative discussion (Chapter 6), along with a thorough reference list (following the format of the journal, *African Zoology*), and additional appendices completes the dissertation. Chapters 2 to 5 consist of an introduction, materials and methods, a results section followed by a discussion. A summary of each chapter will follow below.

In **Chapter 2**, the results of the frog diversity within the study area are reported and compared to historical records, the habitat preferences and breeding activity of the encountered frog species as well as the effectiveness and efficiency of Passive Acoustic Monitoring (PAM). **Chapter 3**, is a case study on the polychromatic forms of the Argus Reed Frog *Hyperolius argus* Peters, 1854. Results on encountered sub-adult male, adult male and adult female frog forms, as well as on the unexplained phenomenon of sexually immature sub-adult males producing mating calls are reported on and discussed in this chapter. In **Chapter 4**, a detailed assessment on previous work of the intraerythrocytic blood parasites parasitemic to amphibian hosts throughout Africa and South Africa is provided, along with reports on the diversity, prevalence and parasitaemia of frog blood parasites observed in the current survey. **Chapter 5**, is a case study reporting on all the currently described African *Hepatozoon* species parasitising frogs from the family Bufonidae. This chapter also reports on the morphological and molecular description and characterisation of a *Hepatozoon* species from this family. In **Chapter 6**, the results of each of the pervious chapters in the dissertation are briefly discussed, along with recommendations for future research.

CHAPTER 2

DIVERSITY AND HABITAT UTILIZATION OF ANURAN COMMUNITIES WITHIN NORTHERN KWAZULU-NATAL

2.1

INTRODUCTION

2.1.1 General introduction to amphibians

Amphibians comprise a large component of the world's vertebrate fauna (Frost *et al.* 2006), and according to Frost (2014) there are currently 7,302 species that make up the class Amphibia. Along with the high diversity of species, amphibians are found in nearly all terrestrial and freshwater habitats globally, with the exception of the poles and some isolated oceanic islands (see Frost *et al.* 2006; du Preez & Carruthers 2009). In recent years there has been a huge scientific effort and focus on amphibians as being the most threatened vertebrate class, these threats are attributed to a number of factors ranging from habitat loss, pollution, climate change and disease (Stuart *et al*. 2004; Weldon & du Preez 2004; Beebee & Griffiths 2005). Ironically these efforts, with the use of DNA markers and increased scientific surveys to remote areas of the world, have led to the rapid biodiversity increase with new species being discovered and described on a frequent basis (see Frost *et al.* 2006; du Preez & Carruthers 2009).

2.1.2 Southern Africa's frog diversity and species richness

Globally the Anura is the most species-rich of the three amphibian Orders and currently consists of 6,418 species in 54 families (Frost 2014). In southern Africa, the anuran fauna currently comprises 13 families, 33 genera and 159 known species (see du Preez & Carruthers 2009; Channing & Baptista 2013; Channing *et al*. 2013a; Channing *et al*. 2013b; Conradie 2014). The diverse landscape ranging from desert to tropics contribute to the uneven distribution of frog species throughout southern Africa. Suitable breeding conditions are vitally important to frogs and thus, rainfall patterns and the numbers and diversity of frog species seem to parallel one another, increasing from the west (Namibia) to the east (Mozambique) (see du Preez & Carruthers 2009). At the latitude of the study site (western to eastern coast of southern Africa), anuran diversity varies from a single species along the Namibian coast to more than 40 along the KZN coast.

Another possible factor playing a role in the dispersal and diversity of frog species in southern Africa is the evolutionary origin and adaptation of frog species over time. Previous studies have argued

that, based on the climatic warming in the past, tropical frog species from northern Africa moved down to southern Africa, whereas species originally from the southern parts moved slightly north-eastwards. As the climate cooled down, this process was reversed and some populations became isolated evolving independently, whereas most of the tropical species established themselves on the more tropical north-eastern side of southern Africa (Poynton 1964). These events led to the possible increase of species diversity northwards, stretching along the coast from the southwestern Cape to KZN, with an increase of endemic species southwards towards the southwestern Cape. In other words, KZN contains high species richness and low numbers of endemics, compared to the southwestern Cape which is conspicuously rich in endemics, but average in species diversity. However, the distribution of frog species in the central and north-western parts, appear to have low diversity and very few endemic species (Alexander *et al*. 2004).

2.1.3 KwaZulu-Natal: A provincial haven for frog diversity

According to Alexander *et al.* (2004), KZN is one of the provinces with the greatest richness of endemic species in South Africa, second only to the Western Cape. The tropical conditions and moist savanna of KZN seems to be the preferred habitat for a vast diversity of frog species. This could be due to the relatively high rainfall and variety of rivers that arise on the escarpment and cross the coastal plain into the sea. As a result of the steep escarpment (towards the sea) numerous deeply incised river valleys facilitated the formation of a complex landscape with diverse habitats. With such a divers complexity of habitats it is possible that a greater number of amphibian refuges were available, increasing the potential for speciation over time (Alexander *et al*. 2004). KwaZulu-Natal is an important refuge for a number of endangered and endemic frog species, however, due to the drastic increase in anthropogenic influences on the natural environment, the stress on the survival of these species increases, and protected havens become more important for the survival of the amphibian species richness in KZN. Currently there are 71 different species (including subspecies) in KZN, these accounting for 43.3% of the total diversity of frog species that occur in southern Africa (see du Preez & Carruthers 2009; Channing & Baptista 2013; Channing *et al*. 2013a; Channing *et al*. 2013b; Conradie 2014).

2.1.4 Habitat and diversity of the Ndumo Game Reserve and surrounds

Ndumo Game Reserve (NGR) is situated in the West of the Maputaland bioregion, close to the borders of South Africa, Swaziland and Mozambique (Wesołowska & Haddad 2009). The Maputaland bioregion, located in northern KZN and crossing into southern Mozambique, is one of the most biologically rich areas in southern Africa (Haddad 2003). The climate of NGR and the surrounding areas can be described as subtropical. Ndumo is a large reserve at 10,117 ha, including habitats ranging from floodplains, subtropical bush, savannah and woodland, to riparian forest (Wesołowska & Haddad 2009). The area directly surrounding the NGR is not formally protected and covered in rural tribal villages, with the vegetation heavily impacted by the villagers' livestock and subsistence farming practices. Approximately 80 km to the south lies the Kwa Nyamazane Conservancy (KNC), a small conservation area running along the Phongolo River and surrounded by large sects of agricultural land, most of it utilized for sugar cane farming.

Officially, the only protected portion of the Phongolo Floodplain is the section consisting of the Phongolo River and associated pans in the NGR, an area recognised as a biodiversity hotspot. The NGR in particular is known for its magnificent bird life, large numbers of crocodiles and hippopotami. In addition, it is also a hotspot for amphibians (Alexander *et al*. 2004). According to the historical data provided by Lambiris (1989), Minter *et al.* (2004), as well as records from Ezemvelo Wildlife, a total of 40 different species have been documented (between 1929 and 2004) in the NGR and surrounds.

2.1.5 Importance of long term monitoring of frog communities

Amphibians play an intermediate role in the food web (Hirai & Matsui 1999). Both as predators and prey they play a key role in the stability of most ecomicrohabitat communities. According to Hirai & Matsui (1999) there is high correlation between the relative abundance of prey in the area, as well as the frequency found in the gut contents of frogs in that same area. Since amphibians contribute greatly to their surrounding ecohabitats, their decline may cause a snowball effect on other species as well as the malfunction of the affected ecomicrohabitat communities (Vonesh *et al*. 2009). Thus, it is important to increase the awareness of amphibian activity through conserving and protecting their diversity. The most effective way to achieve this is through the monitoring of amphibians and their communities as a whole and over a sufficient period of time.

The following chapter will thus provide an account of the frog diversity reported over a period of two years from the NGR and surrounds, comparing this data to the historical records, whilst also discussing the importance of the long term monitoring of these and similar communities.

2.2

MATERIALS & METHODS

2.2.1 Historical data

Historical data was obtained from Ezemvelo Wildlife, KZN for all frog species encountered in northern KZN. This data was recorded between 1929 and 2001. Additionally the atlas and red data book (Minter *et al.* 2004) as well as Lambiris (1989) were used to confirm, as well as add any missing species, from the Ezemvelo Wildlife KZN dataset. A total of 40 different frog species were recorded within the historical study, see results section (Section 2.5) for further details.

2.2.2 Site selection

The present study took place in northern KZN, at three different sites all directly or indirectly associated with the Phongolo River (see Figure 2.2.1). The NGR the largest (10,117 ha) of the three sites, contained the highest variety of microhabitats, ranging from endorheic, lacustrine, palustrine and riverine aquatic habitats, to subtropical bush, savannah, and riparian forest terrestrial habitats. The second site directly surrounding the NGR is not formally protected and thus is covered in rural tribal villages. The KNC was the third site, situated approximately 80 km to the south of the NGR, a small conservation area which runs along the Phongolo River and is surrounded by large sects of agricultural land, most of it utilized for sugar cane farming.

To cover the breeding season for all frog species expected to occur in the study area (as identified through historical data), surveys were conducted during the periods 15 – 21 February 2012 (summer), 17 – 18 August 2012 (winter), 15 – 23 November 2012 (spring), 15 – 26 April 2013 (autumn), 17 – 21 November 2013 (summer) and 3 – 7 February 2014 (summer). Specific localities were selected based on the different habitat preferences (see appendix 2) of the expected species in the different sites (see Table 2.2.1).

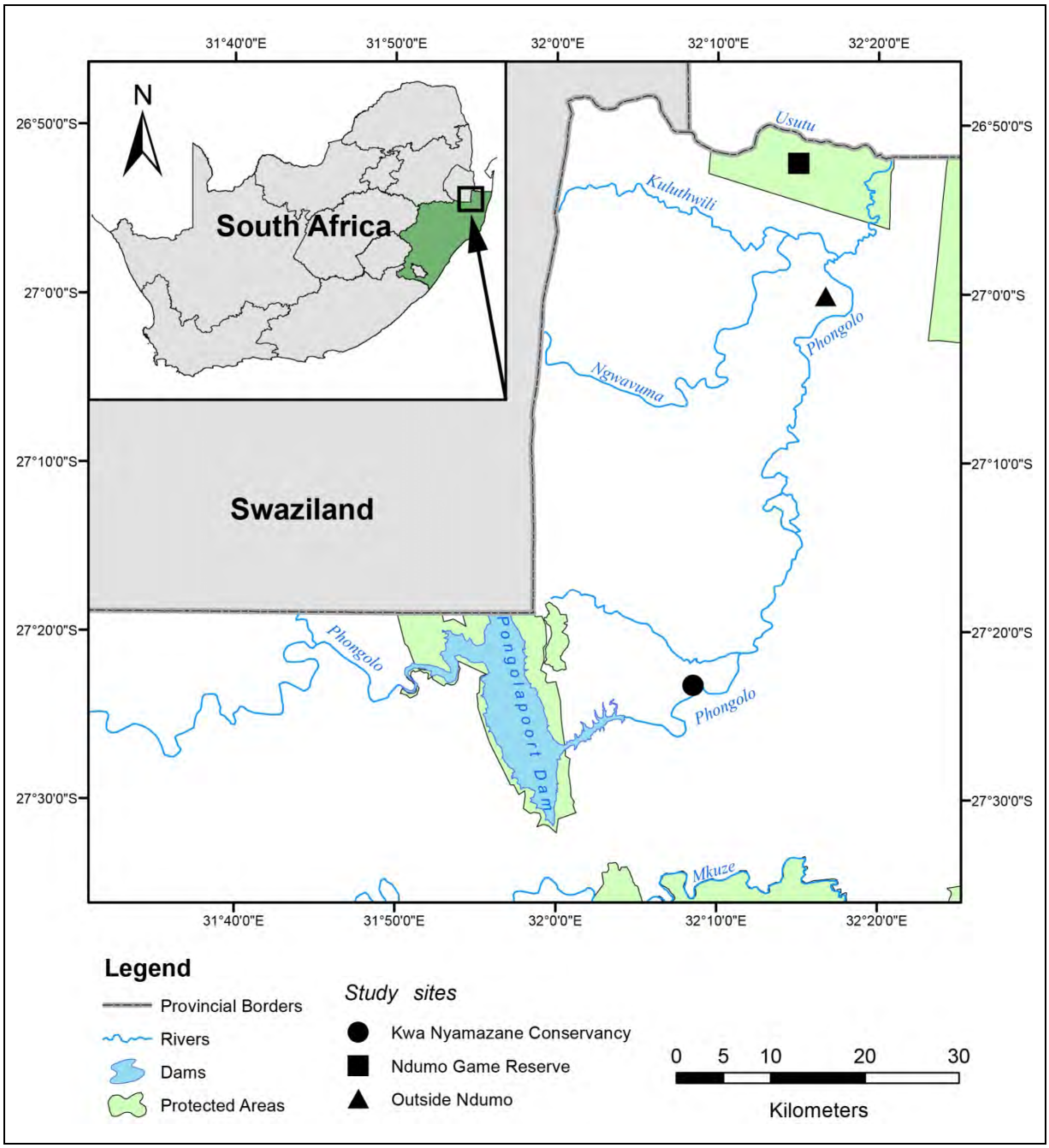


Figure 2.2.1: Map displaying the three different sampling sites in northern KZN.

Table 2.2.1: Different micro-habitats (within five habitats) identified through historical data to host all amphibian species expected to be recorded in the current study. Definitions according to du Preez & Carruthers (2009).

Endorheic habitats Depressions filled by rainwater; depleted by evaporation or absorption into the substrate; neither fed nor drained by a watercourse. These habitats are made up of:
Pan: These habitats are usually temporary, but may hold rainwater for an extended time. They fluctuate in size from many hectares to a few square metres. The banks may include open mud, inundated grass, reed beds or copses of overhanging trees, as well as certain hydrophytes - each of which attract different species of frogs.
Pool: Often a small depression such as a ditch or vehicle track filled with rainwater. Water is retained only for a short period, although continuous rain may keep a pool filled for several months. These habitats are usually exploited by opportunistic explosive breeders. Plants growing in, or associated with pools are generally not specialised hydrophytes.
Riverine habitats Watercourses contained within a channel except in times of flooding.
Permanent river: A continuous flow of water in a natural channel.
Dry river bed: Natural channel in which a river flows through on a seasonal basis.
Floodplain: A flat or depressed area along a riverbank that is periodically flooded and may retain this water once the river recedes.
Perennial stream: A slow yet continuous flow of water in a natural channel throughout all seasons.
Lacustrine habitats Open water bodies, with very little emergent vegetation, which are greater than 8 ha and situated in topographic depressions or dammed river channels.
Dam: A manmade barrier constructed to hold back water and raise its level, forming a reservoir which is mostly used for irrigation or watering of livestock. Frogs usually breed in the headwaters of a dam or along shallow parts of the bank.
Lake: A large, natural body of fresh water. Although lakes support relatively few breeding populations of frogs, due to the wave action and the presence of predators, in some areas that contain dense hydrophytes certain frog species may occur in great numbers.
Palustrine habitats Shallow wetland areas (less than 2 m deep) with more than 30% of the surface dominated by emergent hydrophytic vegetation.
Vlei: Forms part of a watercourse which spreads out over a flat valley forming a marshy wetland

Table 2.2.1. continued

with inundated grass, sedges, reeds and other specialised water-based vegetation. Vleis usually dry up partly or entirely during the dry season. They are the breeding grounds for many different species.
Inundated grass: Temporarily flooded open grassland.
Terrestrial habitats Ecological habitats with no conspicuous standing or flowing water bodies.
Forest floor: Ground below closed canopy woodland, usually comprising heavy deposits of humus and leaf litter.
Rock outcrop: An assembly of exposed rock deposits above the soil.
Sand dunes: Elevated deposits of loose sand.

Based on accessibility, safety, and habitat suitability a total of 23 official sampling sites were selected within the NGR. All selected sites were revisited and if possible (depending if certain sites still contained water or not) frogs were collected. Details of the localities, coordinates, and a brief description of each site is given below (see Table 2.2.2), followed by Figure 2.2.2 containing a photograph of each site.

Table 2.2.2: Official identified sampling localities in the Ndumo Game Reserve.

Locality	Coordinates	Description
1.1 Magongolwanini pan	26.87249 S 32.19878 E	Endorheic and riverine microhabitat: A small more permanent pan; well vegetated; areas with over hanging trees; muddy water clarity (Figure 2.2.2: A).
2.1 Phaphukhulu natural spring	26.87518 S 32.17032 E	Palustrine and riverine microhabitat: Small natural spring; various vegetation types (both terrestrial and aquatic); muddy to semi-clear water clarity (Figure 2.2.2: B).
3.1 Matendeni pan	26.87433 S 32.18638 E	Endorheic microhabitat: A small temporary pan; a few over hanging trees; muddy water clarity (Figure 2.2.2: C).
4.1 Ziposheni pan	26.89756 S 32.21565 E	Endorheic and riverine microhabitat: A small more permanent pan; well vegetated; areas with dense over hanging trees and shrubs; semi-clear water clarity (Figure 2.2.2: D).

Table 2.2.2. continued

Locality	Coordinates	Description
5.1 Fontane pan	26.86347 S 32.16112 E	Endorheic microhabitat: A large well vegetated (various hydrophytes) more permanent pan; semi-clear water clarity (Figure 2.2.2: E).
6.1 Matenini pan	26.86554 S 32.16415 E	Endorheic microhabitat: Medium sized shallow pan, with slight depressions (forming pools after rain) surrounding the main pan (Figure 2.2.2: F).
7.1 Riverbank at the inflow to Nyamiti	26.89984 S 32.26352 E	Riverine microhabitat: permanent flowing river (into Phongolo River); muddy water clarity (Figure 2.2.2: G).
7.2 Vlei area at the inflow to Nyamiti	26.90001 S 32.26378 E	Palustrine microhabitat: Small vlei area; various vegetation types (both terrestrial and aquatic); muddy to semi-clear water clarity (Figure 2.2.2: H).
7.3 Temporary pan at the inflow to Nyamiti	26.89980 S 32.26304 E	Endorheic microhabitat: A small temporary pan; a few over hanging trees; muddy water clarity (Figure 2.2.2: I).
7.4 Stream feeding into Nyamiti	26.90008 S 32.26323 E	Riverine microhabitat: Small temporary stream; various vegetation types (both terrestrial and aquatic); muddy to semi-clear water clarity (Figure 2.2.2: J).
8.1 Riverbank at pump station	26.90515 S 32.32352 E	Riverine microhabitat: permanent flowing river (Phongolo River); muddy water clarity (Figure 2.2.2: K).
8.2 Pan near pump station	26.90337 S 32.32266 E	Endorheic microhabitat: Medium sized pan – outflow from the Phongolo River; muddy water clarity (Figure 2.2.2: L).
8.3 Forest floor at pump station	26.90434 S 32.32353 E	Terrestrial microhabitat: Forest floor, with dense canopy cover and substantial leaf litter (Figure 2.2.2: M)
8.4 Stream feeding into the pan at pump station	26.90344 S 32.32219 E	Endorheic microhabitat: A small temporary pan; a few over hanging trees; muddy water clarity (Figure 2.2.2: N).
9.1 Riverbank at broken bridge	26.88265 S 32.31132 E	Riverine microhabitat: permanent flowing river (Phongolo River); muddy water clarity (Figure 2.2.2: O).
9.2 Vlei area at broken bridge	26.88135 S 32.31106 E	Palustrine microhabitat: Small vlei area; various vegetation types (both terrestrial and aquatic); muddy to semi-clear water clarity (Figure 2.2.2P).

Table 2.2.2. continued

Locality	Coordinates	Description
9.3 Medium sized pan at broken bridge	26.87782 S 32.30613 E	Endorheic microhabitat: Medium sized shallow pan; muddy water clarity (Figure 2.2.2: Q).
9.4 Stream at broken bridge	26.88055 S 32.31178 E	Endorheic microhabitat: A small temporary pan; a few over hanging trees; muddy water clarity (Figure 2.2.2: R).
10.1 Lukhondo pools	26.92345 S 32.31537 E	Endorheic microhabitat: Several medium to small sized temporary pools; moderate canopy cover; muddy water clarity (Figure 2.2.2: S).
11.1 Pan close to the Phongolo River	26.92831 S 32.32990 E	Endorheic microhabitat: Small temporary pool; muddy water clarity (Figure 2.2.2: T).
12.1 Wetland vlei area	26.90294 S 32.23714 E	Palustrine microhabitat: large vlei area; various vegetation types (both terrestrial and aquatic); semi-clear water clarity (Figure 2.2.2: U).
12.2 Wetland vlei area	26.89953 S 32.22217 E	Palustrine microhabitat: temporary vlei area; mostly terrestrial vegetation types; semi-clear water clarity (Figure 2.2.2: V).
13.1 Lake Nyamiti	26.89420 S 32.29607 E	Lacustrine microhabitat: A large lake; well vegetated (various hydrophytes) in certain areas; dangerous site to sample – due to crocodiles and hippopotami (Figure 2.2.2: W).
14.1 Camp site	26.90943 S 32.31321 E	Terrestrial microhabitat: Anthropogenically impacted site; small manmade pools (Figure 2.2.2: X).

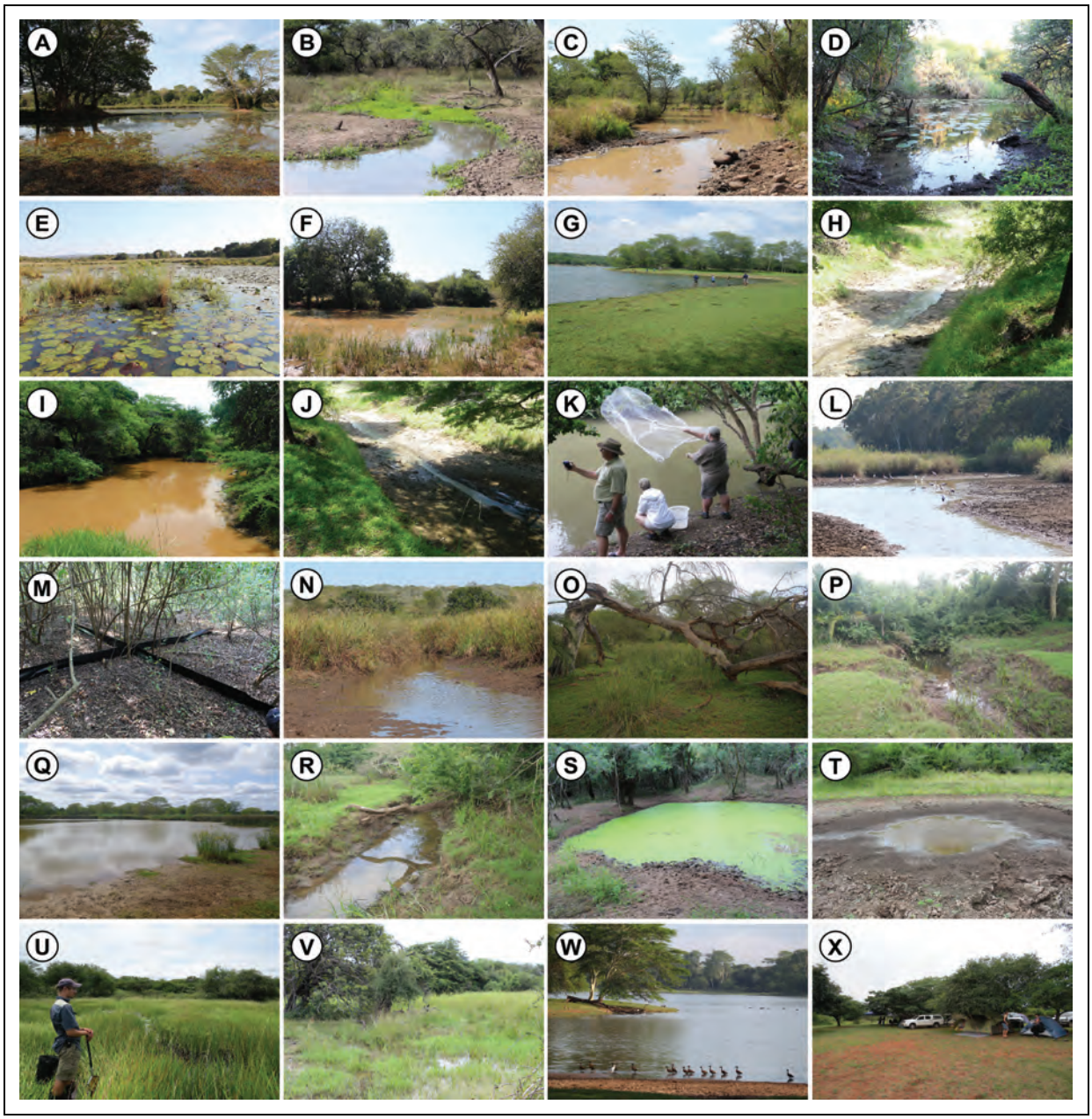


Figure 2.2.2: All sampling localities within the Ndumo Game Reserve. A: 1.1 Magongolwanini pan. **B:** 2.1 Phaphukhulu natural spring. **C:** 3.1 Matendeni pan. **D:** 4.1 Ziposheni pan. **E:** 5.1 Fontane pan. **F:** 6.1 Matenini pan. **G:** 7.1 Riverbank at the inflow to Nyamiti. **H:** 7.2 Vlei area at the inflow to Nyamiti. **I:** 7.3 Temporary pan at the inflow to Nyamiti. **J:** 7.4 Stream feeding into Nyamiti. **K:** 8.1 Riverbank at pump station. **L:** 8.2 Pan near pump station. **M:** 8.3 Forest floor at pump station. **N:** 8.4 Stream feeding into the. pan at pump station. **O:** 9.1 Riverbank at broken bridge. **P:** 9.2 Vlei area at broken bridge. **Q:** 9.3 Medium sized pan at broken bridge. **R:** 9.4 Stream at broken bridge. **S:** 10.1 Lukhondo pools. **T:** 11.1 Pan close to the Phongolo River. **U:** 12.1 Wetland vlei area. **V:** 12.2 Wetland vlei area. **W:** 13.1 Lake Nyamiti. **X:** 14.1 Camp site.

Based on accessibility, safety, and habitat suitability total of six official sampling sites were selected from the areas surrounding the NGR. Details of the localities, coordinates, and a brief description of each site is given below (see Table 2.2.3), followed by Figure 2.2.3 containing a photograph of each site.

Table 2.2.3: Official identified sampling localities surrounding the Ndumo Game Reserve.

Locality	Locality	Locality
M1 Road side burrow pit	27.05713 S 32.23254 E	Endorheic microhabitat: Small temporary pool; muddy water clarity (Figure 2.2.3: A).
M2 Temporary pan for rainy season	27.00393 S 32.28150 E	Endorheic microhabitat: A small temporary pan; a few over hanging trees and shrubs; muddy water clarity (Figure 2.2.3: B).
M3 Well vegetated pool	27.00785 S 32.28811 E	Endorheic microhabitat: A small pan; well vegetated (both terrestrial and aquatic); areas with dense over hanging trees and shrubs; semi-clear water clarity (Figure 2.2.3: C).
M4 Permanent pan used for livestock	27.00572 S 32.28936 E	Endorheic microhabitat: A medium more permanent pan; well vegetated (various hydrophytes); areas with dense over hanging trees and shrubs; semi-clear water clarity (Figure 2.2.3: D).
M5 Small natural spring	26.98893 S 32.26902 E	Palustrine microhabitat: Small natural spring; various vegetation types (both terrestrial and aquatic); muddy to semi-clear water clarity (Figure 2.2.3: E).
M6 Namaneni pan	26.98690 S 32.27142 E	Endorheic microhabitat: A large well vegetated (various hydrophytes) more permanent pan; various vegetation types (both terrestrial and aquatic); semi-clear water clarity (Figure 2.2.3: F).

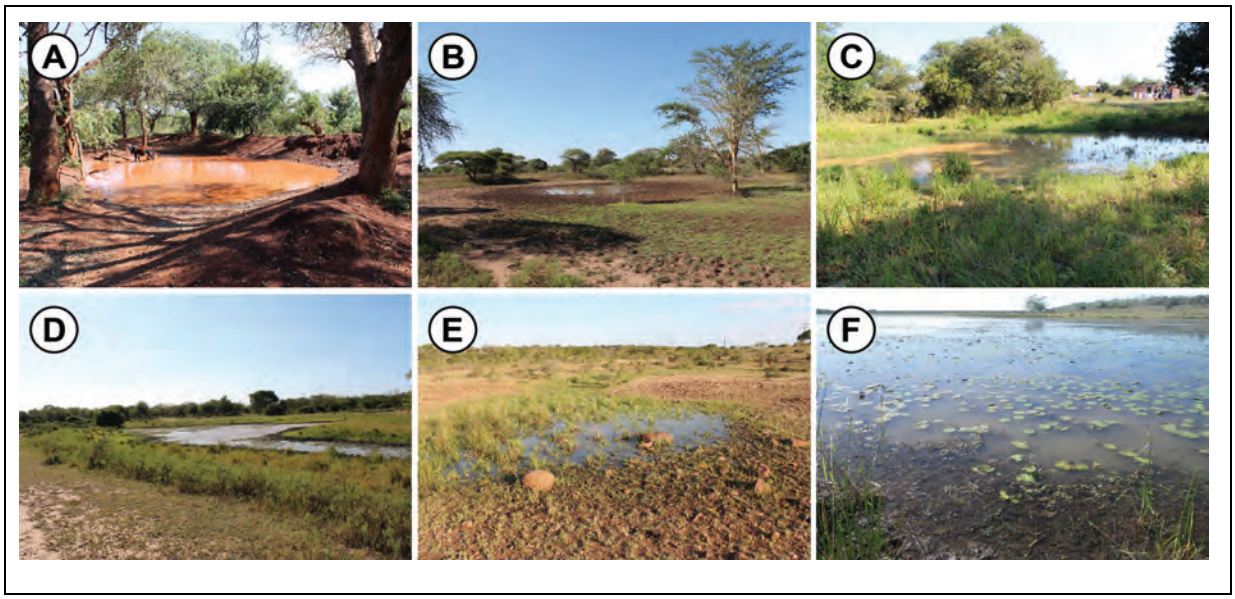


Figure 2.2.3: All sampling localities surrounding the Ndumo Game Reserve. A: M1 Road side burrow pit. **B:** M2 Temporary pan for rainy season. **C:** M3 Well vegetated pool. **D:** M4 Permanent pan used for livestock. **E:** M5 Small natural spring. **F:** M6 Namaneni pan.

From the KNC site a total of four official sampling sites were selected based on accessibility, safety, and habitat suitability. Details of the localities, coordinates, and a brief description of each site is given below (see Table 2.2.4), followed by Figure 2.2.4 containing a photograph of each site.

Table 2.2.4: Official identified sampling localities in the Kwa Nyamazane Conservancy.

Locality	Locality	Locality
P201 permanent catchment forming a shallow pan	27.40979 S 32.146578E	Endorheic microhabitat: A shallow permanent pan, formed and sustained through irrigation water; various vegetation types; semi-clear water clarity (Figure 2.2.4: A).
P202 permanent catchment from over irrigation	27.41809 S 32.14162 E	Palustrine microhabitat: large vlei area; well vegetated; semi-clear water clarity (Figure 2.2.4: B).
P203 Riverbank at campsite on the Phongolo River	27.39146 S 32.13994 E	Riverine microhabitat: permanent flowing river (Phongolo River); muddy water clarity (Figure 2.2.4: C).
P204 Stream feeding into the Phongolo River	27.42611 S 32.13601 E	Palustrine microhabitat: Small stream feeding into the Phongolo River; muddy to semi-clear water clarity (Figure 2.2.4: D).

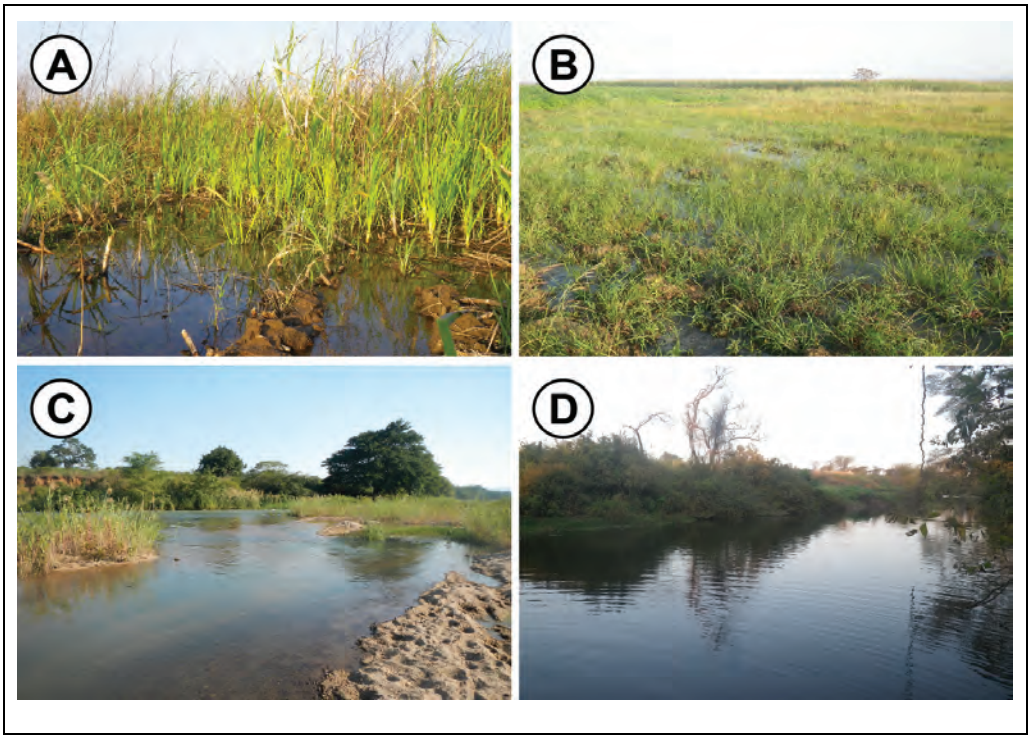


Figure 2.2.4: All sampling localities in the Kwa Nyamazane Conservancy. A: P201 permanent catchment forming a shallow pan. **B:** P202 permanent catchment from over irrigation. **C:** P203 Riverbank at campsite on the Phongolo River. **D:** P204 Stream feeding into the Phongolo River.

2.2.3 Frog collection

Active sampling

This method is done according to three types of sampling techniques. The first and most applied technique was site constrained sampling, defined as collecting as many specimens of different species as possible at a selected site (a selection of different microhabitats were sampled in this way). The second being time constraint sampling, defined by recording the numbers of observations in a specific time, this was conducted through visual or acoustic observations (receiving a score out of five for abundance and intensity) or specimens collected. The third technique was dip netting, defined by intensive sweeping for tadpoles using dip nets, at various possible breeding sites, over a set time period (approximately one minute) (see Figure 2.2.5: A-B).

Baited traps

Bucket traps were used to collect aquatic frog species. These consisted of a large 20 L bucket with a secure fitting lid, and an inward directed traffic cone for the frogs to enter. Traps were baited with chicken livers placed in gauze bags inside the buckets were used to bait the traps, which were then set and secured in the shallow waters of selected sites. Baited bottle traps were used to collect tadpoles. Traps consisted of a 2L bottles, which were subsequently cut in half and the top half was then inverted back into the bottle, creating a funnel trap effect. Traps were baited with trout pellets and checked and reset on a daily basis (see Figure 2.2.5: C).

Drift fence pitfall traps

Drift fences pitfall traps were set up in representative sites for forest floor dwelling amphibians (see Figure 2.2.5: D-E). The drift fences and pitfall traps (using four 5L buckets) were set up by channelling plastic sheets (approximately 300mm high) in the shape of a "+", these sheets were subsequently fixed to metal rods placed about 2m apart. The plastic sheeting was slightly submerged into the ground and directed across the centre of the bucket pitfall traps, preventing frogs from passing underneath and allowing frogs on either side of the sheet to fall into the traps (Carruthers & du Preez 2011) (see Figure 2.2.5: E). Some vegetation and water were placed into the buckets for trapped frogs to conceal themselves within and prevent desiccation. Traps were checked on a daily basis and covered if they are not to be used or checked the following day (Hill *et al.* 2005).

Handling of specimens

All specimens collected were placed in plastic bags or plastic containers that were marked according to the collection site and habitat, and transported back to a field laboratory in a coolbox where they were assigned unique field numbers, additional data such as the date of collection, locality coordinates, and type of species were also recorded. Specimens were identified to species level using field guides, and when necessary a dissection microscope was used to distinguish between cryptic species (looking for small and unique differences on various places such as the feet). Specimens were kept cool and moist in individual plastic containers with a small volume of water (see Figure 2.2.5: F).

Two voucher specimens per species and per locality were euthanised using MS222, a muscle tissue sample from one hind leg was preserved in 70% molecular grade ethanol and the carcass fixed in a natural position in 10% neutral buffered formalin (for future locality and genetic records). Preserved specimens and tissue samples were deposited in the African Amphibian Conservation Research

Group (AACRG) collection at the North-West University, Potchefstroom, South Africa. This collection forms part of the official South African Institute of Aquatic Biodiversity (SAIAB). Specimens not included in the above voucher collection were transported back to and released at their locality of collection.

Figure 2.2.5: Different sampling techniques used to collect and handle frog specimens. A-B: Active sampling. **C:** Checking of baited bucket traps. **D-E:** Drift fences pitfall traps. **F:** Handling and processing of collected specimens at the field laboratory.

2.2.4 Song meter monitoring

Passive acoustic monitoring (PAM) is an advanced, effective, and cost-efficient biodiversity-monitoring tool, producing huge quantities of data that are easily analysed. In order to enable long-term recordings and monitoring of as many frog species as possible a PAM recorder/song meter (see Figure 2.6) (Song Meter™ SM2; Wildlife Acoustics Inc., Concord, Massachusetts, USA) equipped with an ambient temperature sensor and a solar panel was deployed at a seasonal pan (locality 6.1 - Matenini pan, 26.86554; S 32.16415 E) in the NGR, recognised (through active sampling and other methods) to host a wide variety of different frog species. In order to record and monitor calling and breeding activity of as many frog species as possible, a song meter was set up for the duration of a year, recording at one night a week (51 weeks), for 10 minutes on the hour from 18h00 till 05h00 (first week started on the 20th April 2013 to week 51 on the 19th April 2014). The calls of the different frog species were analysed using Song Scope analysis software (Song Scope™ Wildlife Acoustics Inc., Concord, Massachusetts, USA). Calling males received a sore out of five for abundance and intensity (see Table 2.2.5).

Table 2.2.5: Male calling activity scores for abundance and intensity.

Score	Abundance	Intensity
1	One or two males calling	Males calling infrequently
2	Three to four males calling	Males calling repeatedly
3	More than four males calling	Males calling infrequently
4	More than four males calling	Males calling repeatedly
5	Difficult to count the number of males calling	Continues calling for long periods of time

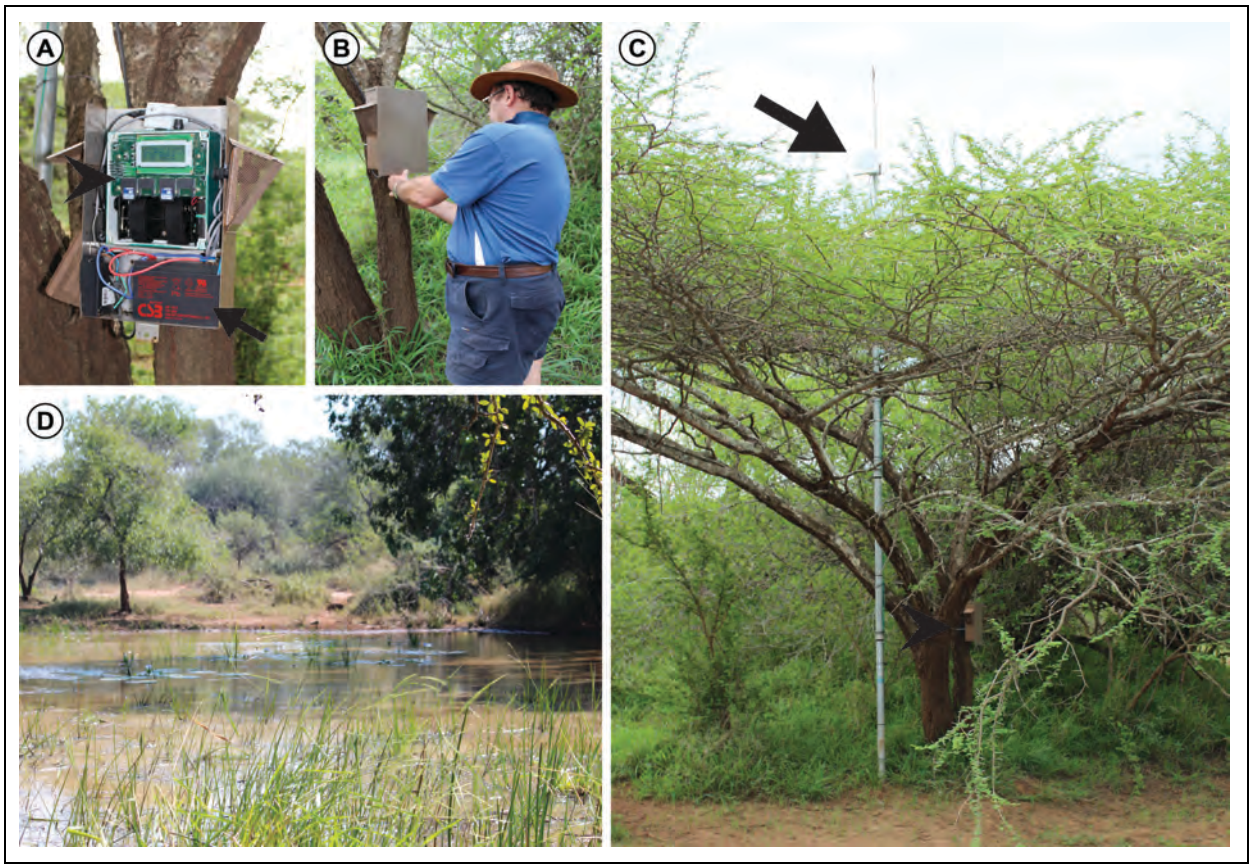


Figure 2.2.6: Solar powered song meter installed in protective housing and attached to a tree, at a seasonal pan in the Ndumo Game Reserve for long term monitoring. A: Song meter inside protective housing; black arrow head - showing the inside of the song meter; black arrow - showing the external battery pack connected to a solar panel. **B:** Song meter protective housing attached to a tree and locked to prevent unwanted tampering. **C:** Song meter attached to the tree; black arrow – showing solar panel above the tree line; black arrow head – showing the song meter in protective housing. **D:** Locality 6.1- Matenini pan, the long term monitoring site selected for the song meter.

RESULTS

2.3 Amphibian diversity in and around Ndumo Game Reserve

2.3.1 Frog diversity in Ndumo Game Reserve

The NGR being the only formally protected area in the Phongolo Floodplain was the most intensely sampled. This was to determine the true frog species diversity in this protected area, as well as if the NGR serves as a suitable conservation area for frog diversity and their required micro habitats. Within this area and over five sampling trips, a total of 11 families, 17 genera and 30 frog species were collected or encountered. In February 2014 (summer) and April 2013 (autumn) the highest diversity of frog species were collected, with a total of 19 and 18 species respectively. *Amietophrynus garmani* Meek, 1897*, Ptychadena anchietae* Bocage, 1867 and *Xenopus muelleri* Peters, 1844 were the only species encountered consistently during all the sampling trips (see Table 2.3.1).

2.3.2 Frog diversity outside Ndumo Game Reserve

Two sites outside the NGR were selected for sampling. In August 2012 (spring) sampling took place in the KNC, a site directly linked to the Phongolo River and situated approximately 80 km from the NGR. A total of four families, five genera and seven species, were collected in the KNC. In this study *Cacosternum boettgeri* (Boulenger, 1882) was the only species collected in the KNC that was not recorded in the NGR. In April 2013 another site outside the NGR was selected, this site was situated in the nearby villages surrounding the NGR. A total of five families, five genera and seven species were collected. The only species not recorded in the NGR in this study was *Hyperolius argus* Peters, 1854. These sites were selected in order to compare frog diversity inside the NGR to unprotected areas surrounding the NGR. A total number of six families, seven genera and 11 species were recorded from the two sites outside the NGR (see Table 2.3.2).

2.3.3 Habitat utilization

Frogs were collected in a variety of habitat types (see appendix 2), which were subsequently divided into the five systems, namely endorheic, lacustrine, palustrine, riverine and terrestrial (see Table 2.3.3). The vast majority of frog species and numbers were collected in endorheic systems, made up

of temporary pans or pools usually filled by rainwater. The smallest number of frogs and frog species were collected in lacustrine systems. Such a finding could be due to the challenging sampling conditions, in terms of the high numbers of crocodiles and hippopotami in these systems.

Table 2.3.1: All frog species collected in the Ndumo Game Reserve via active sampling, baited traps and pitfall traps, listed alphabetically, with family and sampling trip data.

			Ndumo Game Reserve				
SPECIES NAME		FAMILY	Feb 2012	Nov 2012	Apr 2013	Nov 2013	Feb 2014
1	*Afrixalus aureus*	Hyperoliidae			11	5	7
2	*Afrixalus delicatus*	Hyperoliidae	6				
3	*Afrixalus fornasinii*	Hyperoliidae	2				1
4	*Amietophrynus garmani*	Bufonidae	1	7	16	4	1
5	*Amietophrynus gutturalis*	Bufonidae		1		1	
6	*Amietophrynus maculatus*	Bufonidae			9		
7	*Breviceps adspersus*	Brevicipitidae				2	2
8	*Breviceps mossambicus*	Brevicipitidae	1	1			
9	*Chiromantis xerampelina*	Rhacophoridae	15		39	15	3
10	*Hemisus marmoratus*	Hemisotidae			14	7	7
11	*Hildebrandtia ornata*	Ptychadenidae	2		1	2	1
12	*Hyperolius marmoratus*	Hyperoliidae	15		20	5	13
13	*Hyperolius pusillus*	Hyperoliidae			1		15
14	*Hyperolius tuberilinguis*	Hyperoliidae	10	13	7		6
15	*Kassina maculata*	Hyperoliidae					10
16	*Kassina senegalensis*	Hyperoliidae			7		2
17	*Leptopelis mossambicus*	Arthroleptidae		6		2	
18	*Phrynobatrachus mababiensis*	Phrynobatrachidae	2		6	13	1
19	*Phrynomantis bifasciatus*	Microhylidae					4
20	*Ptychadena anchietae*	Ptychadenidae	11	14	26	18	9
21	*Ptychadena mascareniensis*	Ptychadenidae	4		1		
22	*Ptychadena mossambica*	Ptychadenidae	3		9	6	5
23	*Ptychadena oxyrhynchus*	Ptychadenidae		1			
24	*Pyxicephalus edulis*	Pyxicephalidae					15
25	*Schismaderma carens*	Bufonidae	4		1		1
26	*Tomopterna cryptotis*	Pyxicephalidae	1	2	2	3	
27	*Tomopterna krugerensis*	Pyxicephalidae	1				
28	*Tomopterna natalensis*	Pyxicephalidae			1		
29	*Xenopus laevis*	Pipidae		3			
30	*Xenopus muelleri*	Pipidae	5	1	206	3	5
Total number of frog species:			**16**	**10**	**18**	**14**	**19**

Table 2.3.2: All frog species collected via active sampling, baited traps and pitfall traps, listed alphabetically, with family and sampling trip data. Frogs collected in August 2012 were collected in Kwa Nyamazane Conservancy and those during April 2013 in the areas surrounding the NGR.

			Outside Ndumo	
SPECIES NAME		FAMILY	Aug 2012	Apr 2013
1	*Amietophrynus garmani*	Bufonidae	5	2
2	*Amietophrynus gutturalis*	Bufonidae	3	
3	*Cacosternum boettgeri*	Pyxicephalidae	10	30
4	*Chiromantis xerampelina*	Rhacophoridae		1
5	*Hyperolius argus*	Hyperoliidae		43
6	*Hyperolius marmoratus*	Hyperoliidae	6	12
7	*Hyperolius pusillus*	Hyperoliidae	3	1
8	*Kassina senegalensis*	Hyperoliidae	4	
9	*Ptychadena anchietae*	Ptychadenidae	1	
10	*Ptychadena mascareniensis*	Ptychadenidae		2
11	*Xenopus muelleri*	Pipidae		3
Total number of frog species:			**7**	**8**

Table 2.3.3: All frog species collected via active sampling, baited traps and pitfall traps, listed alphabetically, with family and habitat where specimens were collected.

			All frog collected across different habitat types				
SPECIES NAME		FAMILY	Endorheic habitats	Lacustrine habitats	Palustrine habitats	Riverine habitats	Terrestrial habitats
1	*Afrixalus aureus*	Hyperoliidae	21	0	2	0	0
2	*Afrixalus delicatus*	Hyperoliidae	6	0	0	0	0
3	*Afrixalus fornasinii*	Hyperoliidae	0	0	3	0	0
4	*Amietophrynus garmani*	Bufonidae	11	1	2	10	12
5	*Amietophrynus gutturalis*	Bufonidae	0	0	0	4	1
6	*Amietophrynus maculatus*	Bufonidae	0	0	0	9	0
7	*Breviceps adspersus*	Brevicipitidae	0	0	0	0	4
8	*Breviceps mossambicus*	Brevicipitidae	0	0	0	0	2
9	*Cacosternum boettgeri*	Pyxicephalidae	25	0	15	0	0
10	*Chiromantis xerampelina*	Rhacophoridae	54	0	1	18	0

Table 2.3.3. continued

11	*Hemisus marmoratus*	Hemisotidae	19	0	0	9	0
12	*Hildebrandtia ornata*	Ptychadenidae	5	0	0	0	1
13	*Hyperolius argus*	Hyperoliidae	43	0	0	0	0
14	*Hyperolius marmoratus*	Hyperoliidae	37	10	24	0	0
15	*Hyperolius pusillus*	Hyperoliidae	2	0	18	0	0
16	*Hyperolius tuberilinguis*	Hyperoliidae	8	10	8	10	0
17	*Kassina maculata*	Hyperoliidae	0	0	10	0	0
18	*Kassina senegalensis*	Hyperoliidae	9	0	4	0	0
19	*Leptopelis mossambicus*	Arthroleptidae	2	0	1	5	0
20	*Phrynobatrachus mababiensis*	Phrynobatrachidae	20	0	0	2	0
21	*Phrynomantis bifasciatus*	Microhylidae	3	0	0	0	1
22	*Ptychadena anchietae*	Ptychadenidae	42	0	1	35	1
23	*Ptychadena mascareniensis*	Ptychadenidae	7	0	0	0	0
24	*Ptychadena mossambica*	Ptychadenidae	21	0	0	0	2
25	*Ptychadena oxyrhynchus*	Ptychadenidae	0	0	1	0	0
26	*Pyxicephalus edulis*	Pyxicephalidae	8	0	7	0	0
27	*Schismaderma carens*	Bufonidae	5	0	0	0	1
28	*Tomopterna cryptotis*	Pyxicephalidae	2	0	0	0	6
29	*Tomopterna krugerensis*	Pyxicephalidae	0	0	0	0	1
30	*Tomopterna natalensis*	Pyxicephalidae	1	0	0	0	0
31	*Xenopus laevis laevis*	Pipidae	3	0	0	0	0
32	*Xenopus muelleri*	Pipidae	216	0	0	7	0
Total number of frog species:			**24**	**3**	**14**	**10**	**11**
Total number of frogs collected:			**570**	**21**	**97**	**109**	**32**

2.4 Monitoring of amphibian activity

2.4.1 Frog diversity from song meter data

A single site (Locality 6.1 - Matenini pan) in the NGR was selected for long-term passive acoustic monitoring (PAM) via song meter recordings. A total of 10 families, 13 genera and 19 species were recorded (see Table 2.4.1). An additional two species *Cacosternum nanum* Boulenger, 1887 and *Phrynobatrachus natalensis* Smith, 1849 were added to the total species recorded during this study through active sampling, drift fence pitfall traps, baited buck and bottle traps.

Table 2.4.1: All frog species recorded via passive acoustic monitoring listed alphabetically, with family. Song meter was set up from April 2013 till April 2014, at a temporary pan (Locality 6.1- Matenini pan) in the NGR.

	TAXON NAME	**FAMILY**
1	*Afrixalus aureus*	Hyperoliidae
2	*Afrixalus delicatus*	Hyperoliidae
3	*Amietophrynus garmani*	Bufonidae
4	*Amietophrynus gutturalis*	Bufonidae
5	*Amietophrynus maculatus*	Bufonidae
6	*Breviceps adspersus*	Brevicipitidae
7	*Cacosternum nanum*	Pyxicephalidae
8	*Chiromantis xerampelina*	Rhacophoridae
9	*Hemisus marmoratus*	Hemisotidae
10	*Hyperolius marmoratus*	Hyperoliidae
11	*Kassina maculata*	Hyperoliidae
12	*Kassina senegalensis*	Hyperoliidae
13	*Leptopelis mossambicus*	Arthroleptidae
14	*Phrynobatrachus mababiensis*	Phrynobatrachidae
15	*Phrynobatrachus natalensis*	Phrynobatrachidae
16	*Phrynomantis bifasciatus*	Microhylidae
17	*Ptychadena anchietae*	Ptychadenidae
18	*Ptychadena mossambica*	Ptychadenidae
19	*Tomopterna cryptotis*	Pyxicephalidae

2.4.2 Monitoring of amphibian breeding activity

Passive acoustic monitoring data was also used to monitor breeding activity of frog species associated with endorheic habitats. The first frog species recorded to call was *Phrynobatrachus mababiensis* FitzSimons, 1932 in week 17 on the 10th of August 2013 (spring). The call intensity and abundance was scored 2/5. After week 17 there was a gradual increase in frog species calling activity, with the exception of week 18, 23 and 32 of which no frogs were recorded. Week 37, recorded the most species in one evening, with a total of 11 species out of the total 19 species recorded at this site via the song meter. The peak breeding season of most species according to PAM is from week 34 to week 42 (1 December 2013 to 26 January 2014, with an average of 11 species calling per night (see Figure 2.4.1).

2.4.3 Average hourly calling activity and intensity

Six species of the 19 species recorded, namely *Chiromantis xerampelina* Peters, 1854*, Hemisus marmoratus* (Peters, 1854)*, Hyperolius marmoratus* Rapp, 1842*, Kassina maculata* Duméril, 1853*, Kassina senegalensis* Duméril & Bibron 1841 and *P. mababiensis* were recorded calling in all 12 hours. Only four species namely *Amietophrynus maculatus* Hallowell, 1854, *K. maculata, P. mababiensis* and *Phrynomantis bifasciatus* Smith, 1847 were recorded reaching an average call intensity of 5/5 (Figure 2.4.2)

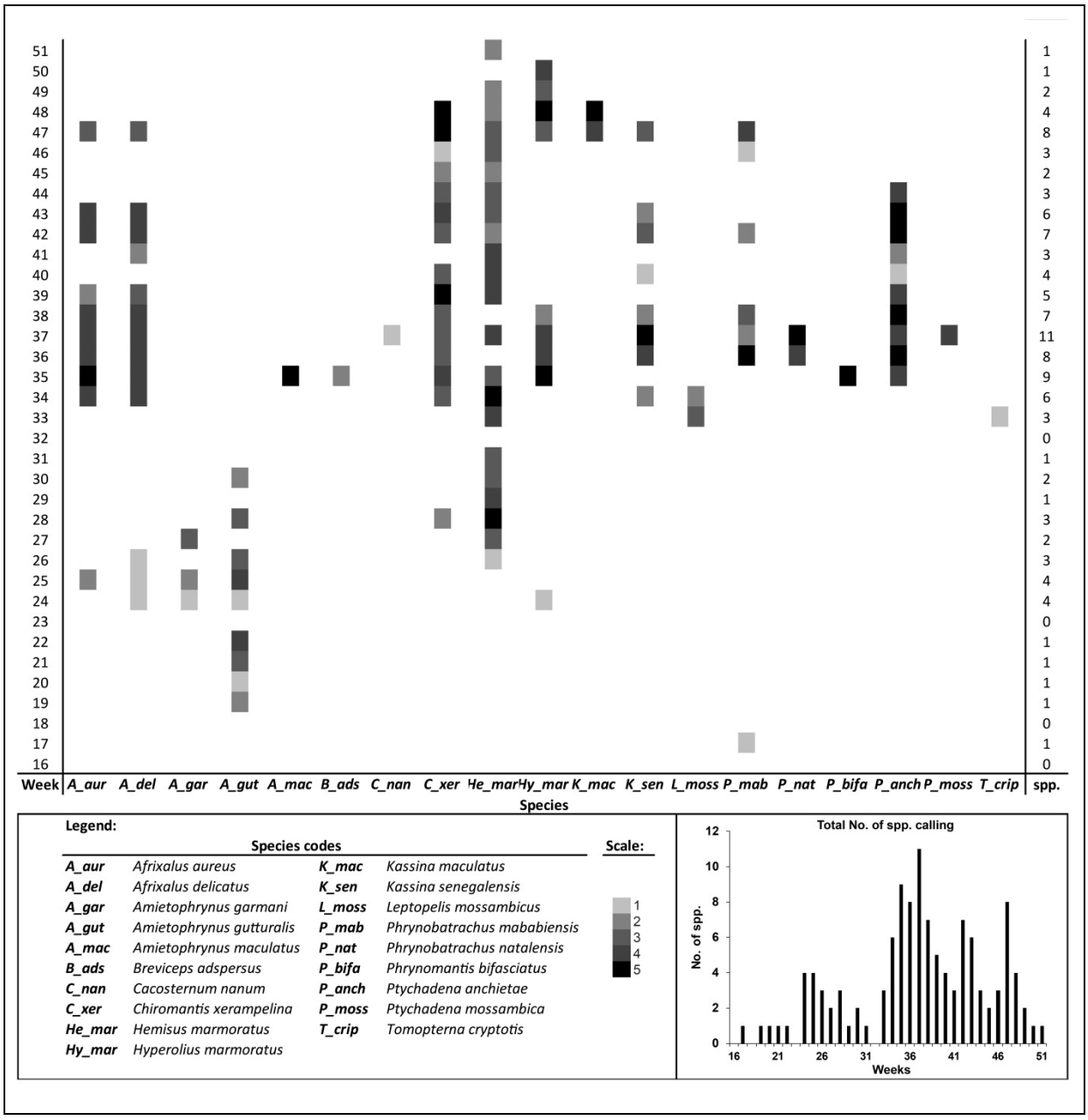


Figure 2.4.1: Song meter data on peak calling activity and intensity of male frog species.

Displayed is the maximum calling intensity and abundance of frog species recorded one night a week (18h00 to 05h00). Calling activity and intensity was recorded from April 2013 to April 2014 at a temporary pan (Locality 6.1- Matenini pan) in the NGR. The date, week, frog species and total number of species calling is represented. Only data is show from week 16 to 51 (August 2013 till April 2014), as no frogs were recorded calling in the preceding months. Calling intensity and abundance is rated according to the shaded scale bar (see Table 2.6).

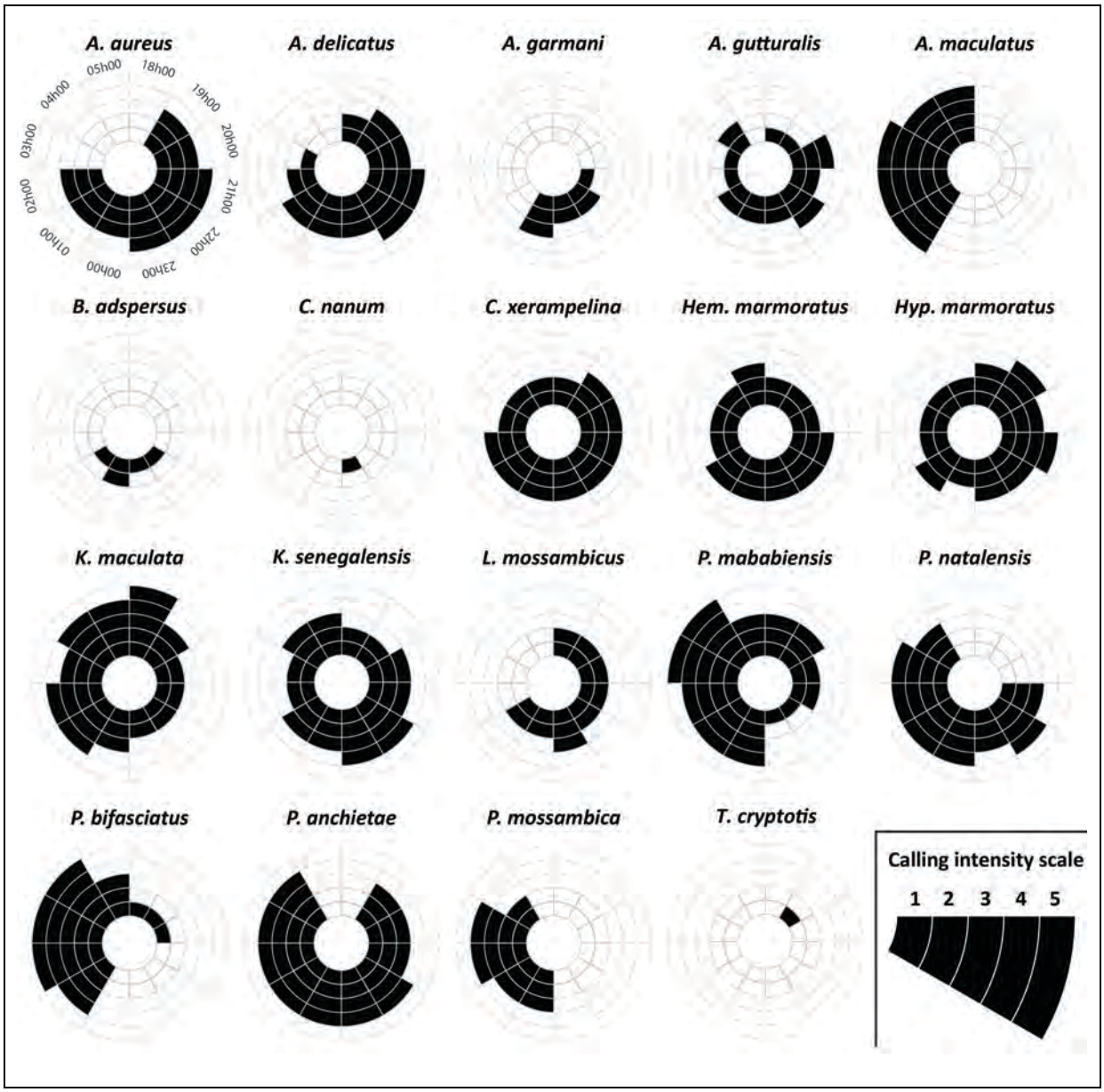


Figure 2.4.2: The average hourly activity and intensity of male frog species

Displayed are the data on the average calling activity and intensity of each frog species recorded by the song meter for the first 10min of each hour (18h00 to and including 05h00). The scale bar represents the hourly calling intensity.

2.5 Comparison of amphibian diversity to historical data

2.5.1 Historical frog diversity of the study area

As mentioned previously the historical data for all frog species encountered in northern KZN was obtained from Ezemvelo Wildlife data base, Lambiris (1989), as well as from the atlas and red data book (Minter *et al.* 2004). This data was obtained from 1929 till 2001, and 1996 till 2004 respectively. With the combined effort of both data sets a total of 11 families, 21 genera and 40 species were recorded in the past to occur in the study area (see Table 2.5.1).

Table 2.5.1: Historically identified frog species and families, occurring in the study area, listed alphabetically with the preferred habitats. Highlighted are the species that were not recorded in the current study.

	Species	Family	Habitat preferences
1	*Afrixalus aureus*	Hyperoliidae	Endorheic habitats
2	*Afrixalus delicatus*	Hyperoliidae	Endorheic habitats
3	*Afrixalus fornasinii*	Hyperoliidae	Endorheic habitats
4	*Amietophrynus garmani*	Bufonidae	Endorheic, palustrine habitats
5	*Amietophrynus gutturalis*	Bufonidae	Endorheic & palustrine habitats
6	*Amietophrynus maculatus*	Bufonidae	Palustrine & riverine habitats
7	*Amietophrynus rangeri*	Bufonidae	Palustrine & riverine habitats
8	*Arthroleptis stenodactylus*	Arthroleptidae	Terrestrial habitats
9	*Breviceps adspersus*	Brevicipitidae	Terrestrial habitats
10	*Breviceps mossambicus*	Brevicipitidae	Terrestrial habitats
11	*Cacosternum boettgeri*	Pyxicephalidae	Endorheic habitats
12	*Cacosternum nanum*	Pyxicephalidae	Endorheic habitats
13	*Chiromantis xerampelina*	Rhacophoridae	Endorheic, palustrine, riverine habitats
14	*Hemisus marmoratus*	Hemisotidae	Endorheic, terrestrial habitats
15	*Hildebrandtia ornata*	Ptychadenidae	Endorheic habitats
16	*Hyperolius argus*	Hyperoliidae	Endorheic, lacustrine & palustrine habitats
17	*Hyperolius marmoratus*	Hyperoliidae	Endorheic, lacustrine & palustrine habitats
18	*Hyperolius poweri*	Hyperoliidae	Palustrine habitats
19	*Hyperolius pusillus*	Hyperoliidae	Endorheic, lacustrine & palustrine habitats
20	*Hyperolius tuberilinguis*	Hyperoliidae	Endorheic, lacustrine & palustrine habitats
21	*Kassina maculata*	Hyperoliidae	Lacustrine, palustrine habitats

Table 2.5.1. continued

	Species	Family	Habitat preferences
22	*Kassina senegalensis*	Hyperoliidae	Endorheic, palustrine habitats
23	*Leptopelis mossambicus*	Arthroleptidae	Endorheic, riverine, terrestrial habitats
24	*Phrynobatrachus acridoides*	Phrynobatrachidae	Endorheic habitats
25	*Phrynobatrachus mababiensis*	Phrynobatrachidae	Endorheic habitats
26	*Phrynobatrachus natalensis*	Phrynobatrachidae	Endorheic habitats
27	*Poyntonophrynus fenoulheti*	Bufonidae	Endorheic habitats
28	*Phrynomantis bifasciatus*	Microhylidae	Endorheic habitats
29	*Ptychadena anchietae*	Ptychadenidae	Endorheic & palustrine habitats
30	*Ptychadena mascareniensis*	Ptychadenidae	Endorheic & palustrine habitats
31	*Ptychadena mossambica*	Ptychadenidae	Endorheic & palustrine habitats
32	*Ptychadena oxyrhynchus*	Ptychadenidae	Endorheic & palustrine habitats
33	*Pyxicephalus edulis*	Pyxicephalidae	Endorheic & palustrine habitats
34	*Schismaderma carens*	Bufonidae	Endorheic, lacustrine & terrestrial habitats
35	*Strongylopus fasciatus*	Pyxicephalidae	Endorheic, palustrine & riverine habitats
36	*Tomopterna cryptotis*	Pyxicephalidae	Endorheic, lacustrine & terrestrial habitats
37	*Tomopterna krugerensis*	Pyxicephalidae	Endorheic, lacustrine & terrestrial habitats
38	*Tomopterna natalensis*	Pyxicephalidae	Endorheic, lacustrine & terrestrial habitats
39	*Xenopus laevis*	Pipidae	Endorheic, lacustrine, palustrine & riverine habitats
40	*Xenopus muelleri*	Pipidae	Endorheic, lacustrine, palustrine & riverine habitats

In the current study, data was collected and recorded from three different sites, namely NGR, outside NGR and KNC. Within each of the sites a total of 34 different localities were sampled. 18 were endorheic habitats, eight palustrine habitats, five riverine habitats, two terrestrial habitats and one was a lacustrine microhabitat. The combination of all the above methods resulted in the recording of a total of 11 families, 18 genera and 34 species (see Table 2.5.1 and Figure 2.5.1).

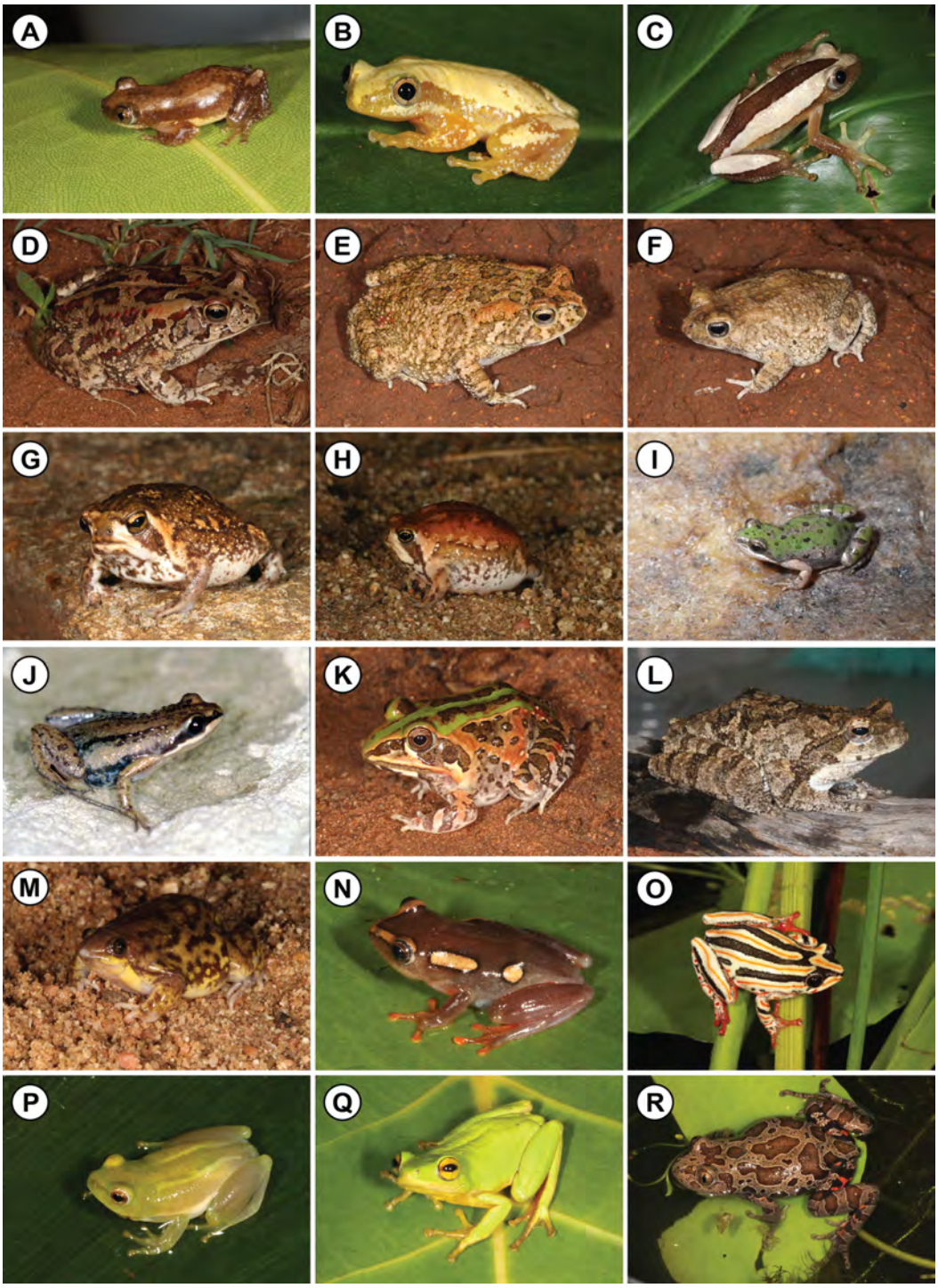


Figure 2.5.1: Photo plate of the frog species recorded during the current study. A: *Afrixalus aureus.* **B:** *Afrixalus delicatus.* **C:** *Afrixalus fornasinii.* **D:** *Amietophrynus garmani.* **E:** *Amietophrynus gutturalis.* **F:** *Amietophrynus maculatus.* **G:** *Breviceps adspersus.* **H:** *Breviceps mossambicus.* **I:** *Cacosternum boettgeri.* **J:** *Cacosternum nanum.* **K:** *Chiromantis xerampelina.* **L:** *Hemisus marmoratus.* **M:** *Hildebrandtia ornata.* **N:** *Hyperolius argus.* **O:** *Hyperolius marmoratus.* **P:** *Hyperolius pusillus.* **Q:** *Hyperolius tuberilinguis.* **R:** *Kassina maculata.*

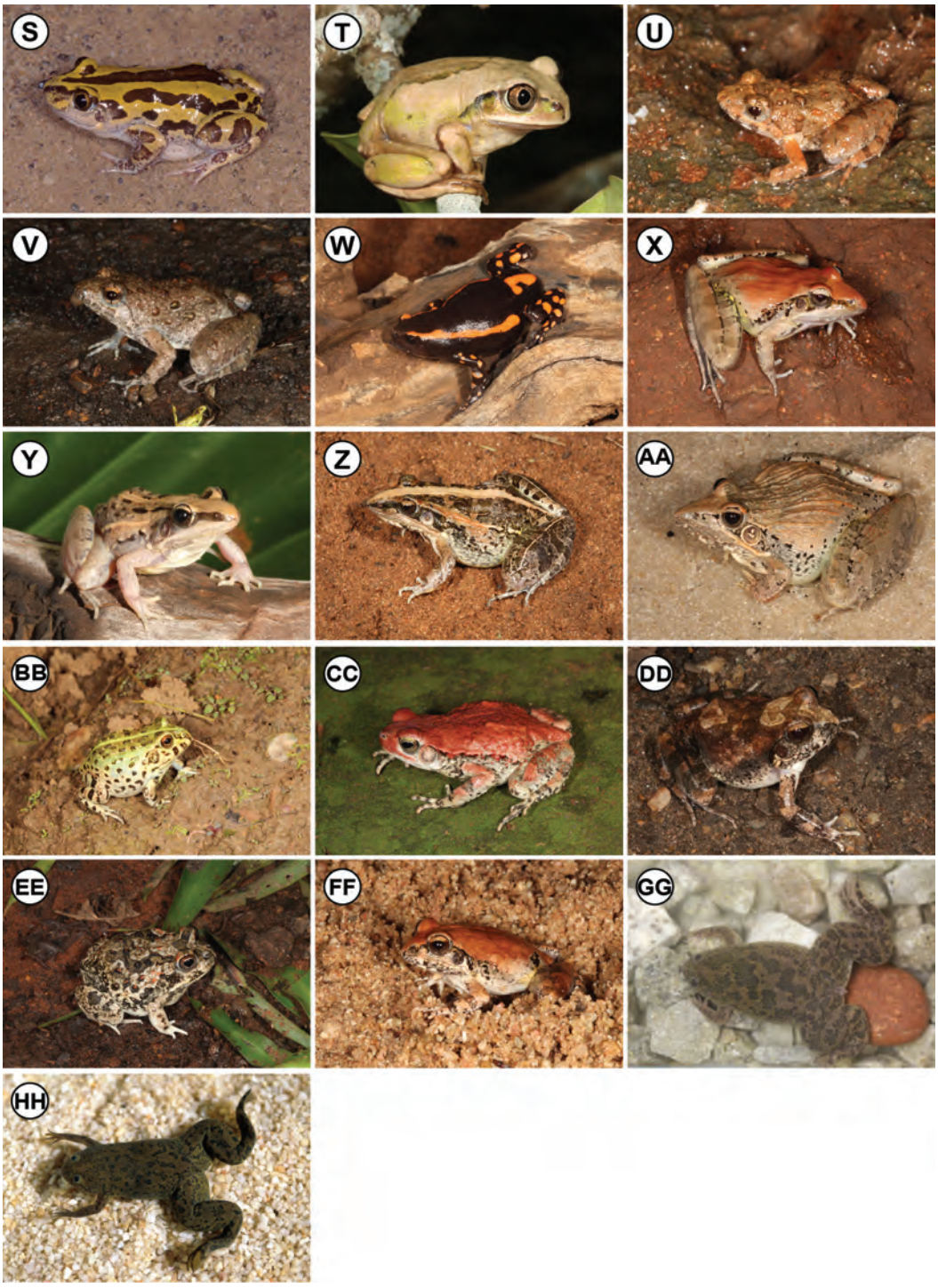


Figure 2.5.1 continued: S: *Kassina senegalensis.* **T:** *Leptopelis mossambicus.* **U:** *Phrynobatrachus mababiensis.* **V:** *Phrynobatrachus natalensis.* **W:** *Phrynomantis bifasciatus.* **X:** *Ptychadena anchietae.* **Y:** *Ptychadena mascareniensis.* **Z:** *Ptychadena mossambica.* **AA:** *Ptychadena oxyrhynchus.* **BB:** *Pyxicephalus edulis.* **CC:** *Schismaderma carens.* **DD:** *Tomopterna cryptotis.* **EE:** *Tomopterna krugerensis.* **FF:** *Tomopterna natalensis.* **GG:** *Xenopus laevis.* **HH:** *Xenopus muelleri.*

2.6

DISCUSSION

KwaZulu-Natal is known for its tropical environment and vast diversity of habitats, and due to these optimal conditions a high biodiversity of frog species are known to occur throughout this area (see Measey 2011; Alexander *et al*. 2004). Amphibians, a class known to serve an intermediate role in the food web, are considered to be a keystone group in most microhabitat communities. This means that, not only do they control and consume large quantities of invertebrates, but they also serve as a primary source of food to a large number of vertebrates, such as snakes, birds and even other frogs (see Hirai & Matsui 1999; du Preez & Carruthers 2009; Vonesh *et al*. 2009). Since KZN is such a biodiversity hotspot for amphibians, it is important to locate sufficient and conservable areas within KZN to protect this diversity of frog species and their communities as a whole. On the other hand NGR is regarded as the top birding hotspot in South Africa and a large number of these birds prey on amphibians.

The NGR is the only officially protected area within the Phongolo Floodplain. Within this area a total of 11 families, 17 genera, and 32 frog species were recorded during this study. In comparison to areas surveyed outside the NGR, a total of six families, seven genera and 11 frog species were recorded. Although NGR was found to contain the highest frog diversity compared to the other sampled areas outside the reserve, it does not necessarily mean that these additional areas do not contain the same diversity as inside the NGR. This could be due to the fact areas were simply not as thoroughly surveyed for various reasons as discussed below. However, two species, namely *C. boettgeri* and *H. argus* were only recorded outside the NGR. Both these species prefer specific habitat types, *C. boettgeri* occurs in temporary habitats, with short folded tufts of grass serving as male call sites. This species lays its eggs in shallow pans or puddles, while it prefers foraging in open grassland areas (see appendix 2; du Preez & Carruthers 2009). *Hyperolius argus* breeds in a number of different habitats, particularly those with floating vegetation being the favoured call site for males. This species lays its eggs on submerged vegetation in temporary habitats (see appendix 2; du Preez & Carruthers 2009). Reasons why these species were possibly missed in the NGR, could be attributed to the inability of sampling these habitat types, due to unfavourable sampling conditions, such as surveys taking place at the wrong time of the year or dangerous sampling conditions as mentioned above.

Focusing on the preferred microhabitats for frogs in this area, five main habitat types, namely endorheic, lacustrine, palustrine, riverine and terrestrial were identified. From the 34 species

collected or recorded, 20 species were recorded from more than one microhabitat, and 14 were found in only one specific microhabitat. Endorheic habitats accounted for the highest diversity of species with 76% (26/34) found to occur in these temporary habitats (see Table 2.2.2; Table 2.3.3). This means that most of the frog species in this area would be expected to be opportunistic and explosive breeders, taking advantage of the suitable habitat supporting these activities and species in the NGR. Lacustrine habitats accounted for the least frog diversity with only 8.7% (3/34) found in these habitats (see Table 2.2.2; Table 2.3.3). This low diversity could be attributed to the challenging sampling conditions, as mentioned above.

Temporary endorheic habitats accounted for the highest diversity, thus a long-term passive acoustic monitoring tool (song meter) was set up at a selected seasonal pan in the NGR (Figure 2.2.2: F). The purpose of this long-term monitoring was to document the breeding and calling activity of frog species associated with this microhabitat for the duration of a year (April 2013 to April 2014). This data recorded a total of 10 families, 13 genera and 19 frog species. Additionally two frog species were added to the species list from the current study namely *C. nanum* and *P. natalensis*. Three of the 19 species recorded namely *A. maculatus*, *Breviceps adspersus* Peters, 1882, and *K. maculata* were not expected to be recorded in this microhabitat, as their preferred habitat types are palustrine and riverine habitats, terrestrial habitats, as well as lacustrine and palustrine habitats respectively. However it is possible that these species were recorded a fair distance away (approximately 1 km), most likely from locality 5.1 - Fontane pan (26.86347 S 32.16112 E) (Figure 2.2.2: E). Although this microhabitat is classified as an endorheic habitat, it is a large well vegetated (various hydrophytes) pan, which is fed by the Usutu River, thus possibly containing suitable conditions supporting the breeding habits of these species.

According to the PAM data the earliest calling activity of male frogs was in August 2013 (week 17). Thereafter, there was a gradual increase in frog species calling activity, with the exception of week 18 (18 August 2013), 23 (19 September 2013) and 32 (15 November 2013) during which no frogs were recorded. This could be due to a number of environmental factors, such as heavy wind or rain, or a sudden increase or decrease in temperature. The peak breeding season for the majority of the species, with 79% (15/19) of calling males recorded, was in summer between December 2013 and January 2014 (week 34 to 42). Of the 19 frog species recorded, the hourly calling activity and intensity (18h00 to and including 05h00) differed among species, with only four species reaching an average call intensity of 5. It may be argued that these species, namely *A. maculatus*, *K. maculata, P. mababiensis* and *P. bifasciatus*, could be explosive breeders, with high calling intensity for only a few weeks a year depending on the weather conditions.

Over the course of two years of surveying, using various active and passive techniques for sampling and recording data, a total number to 11 families, 18 genera and 34 frog species were recorded. As mentioned previously according to historical data obtained from Lambiris (1989) , Ezemvelo Wildlife as well as from the atlas and red data book (Minter *et al.* 2004), there are 11 families, 21 genera and 40 frog species expected to occur in the study area. Thus, in the current study three genera and six frog species previously recorded were not recorded in the present study. These species were *Amietophrynus rangeri* Hewitt, 1935 and *Poyntonophrynus fenoulheti* (Hewitt & Methuen 1913) from the family Bufonidae, *Arthroleptis stenodactylus* Pfeffer, 1893 from the family Arthroleptidae, *Hyperolius poweri* Loveridge, 1938 from the family Hyperoliidae, *Phrynobatrachus acridoides* Cope, 1867 from the family Phrynobatrachidae and *Strongylopus fasciatus* Smith, 1849 from the family Pyxicephalidae.

The favoured microhabitats of *A. rangeri* for breeding necessitate slow flowing streams or rivers (Cunningham 2004; du Preez & Carruthers 2009). Sites typical for this species could not be intensely surveyed due to dangerous sampling conditions in these habitats, resulting in the species, if present, not being recorded. *Poyntonophrynus fenoulheti* occurs is a variety of bushveld vegetation types, usually occurring in rocky outcrops, where breeding takes place in shallow rain filled pools (du Preez & Carruthers 2009). Not many microhabitats suitable for this species specialised breeding habits were surveyed, drastically decreasing the chance of encountering this species in the current study. *Arthroleptis stenodactylus* is associated with forest floor terrestrial habitats occurring within dense leaf litter. This genus demonstrates no free living tadpole stage and metamorphosis is completed within the egg capsule (du Preez & Carruthers 2009). Sites typical for this species were once again not surveyed, due to the high numbers of hippopotami active in these habitats at night, resulting in the species, if present, not being recorded. *Hyperolius poweri* occurs in coastal bushveld or grassland, breeding in shallow pans, vleis or inundated grasslands (Bishop 2004a). Very little is known about the behaviour of this small frog species, and it is possible that due to specialised breeding habits and the periods of breeding, that this species was overlooked during the study. *Phrynobatrachus acridoides* is found in a variety of different habitat types. This opportunistic species breeds in pans, puddles, pools and vleis (du Preez & Carruthers 2009). Owing to the opportunistic habits of this species it is difficult to estimate the time of year or exact habitat at which this species could be found. According to Minter *et al.* (2004) the last time this species was recorded in this area was pre-1996. *Strongylopus fasciatus* occurs in a variety of different grassy areas close to water sources, such as dams, pans and streams. This species was not recorded during this study, possibly due to the fact that it is a winter breeder and only the April survey was completed in the winter

months. According to Minter *et al.* (2004) the last time this species was recorded in this area was pre-1996.

As mentioned above a number of factors played a role in not recording all the species accounted for by historical data. Additionally, a limiting number of surveying field trips, as well as the unpredictable weather conditions and dangerous sampling conditions all contributed to not being able to collect or record all the expected species. An alternative method to recording all the expected species for future studies could be to exploit the use of song meters in the various available microhabitats within the study area. As this was proved to be a rather effective and efficient tool, but was unfortunately only utilized at one particular locality, its efficiency should be tested at additional, possibly less accessible, sites.

In the current study 85% (34/40) of the expected frog species were recorded in as little time as two years, compared to the 75 years (1929 to 2004) of historical data recorded from the same area. In the NGR alone, a total of 32 frog species were recorded, stressing the importance and value of naturally protected areas and how they support not only specific species, but also whole communities. These results indicate that even though there are major global amphibian declines, areas such as the NGR still provide a safe haven for frog species to flourish in, and should remain protected at all costs. Especially in terms of anthropogenic influences and the dispersal of various diseases (such as amphibian chytrid), which could have detrimental effects in such confined and highly populated areas.

CHAPTER 3

HYPEROLIUS ARGUS: CASE STUDY

3.1

INTRODUCTION

In most animals some form of dimorphism is present. Sexual dimorphism, in which males and females differ in body size or colour, is one of the most commonly observed forms of dimorphism among anurans (Han & Fu 2013). According to Willink *et al.* (2013), one of the fundamental questions in evolutionary biology is the origin and complexity of colour variation, among different sexes, individuals, species, and populations. This variation which places a species under selective pressure may be subject to multiple evolutionary forces.

Differences in the body size of male and female frogs are attributed to either, fecundity in which larger females are naturally selected, due to the fact that they are able to produce more offspring; or sexual selection in which larger and more dominant males are selected, based on aspects related to their size, such as vocal comparison or territorial combat (Channing *et al.* 1994; Bell & Zamudio 2012; Han & Fu 2013). Sexual dimorphism in which males and females differ in colour is known as sexual dichromatism, and has been reported to occur in a vast range of vertebrates and invertebrates (see Kodric-Brown 1998; Allen *et al.* 2011; Badyaev & Hill 2003; Bell & Zamudio 2012). Sexual dichromatism appears to be driven by sexual selection depending on which sex is influenced. For instance, if males have conspicuous colours, then male to male competition probably promotes female selection, if females have conspicuous colours then the roles are possibly turned around, and finally if both sexes have conspicuous colours, mate selection may be a mutual choice (see Bell & Zamudio 2012).

In a recent review on anuran sexual dichromatism by Bell & Zamudio (2012), two forms were referred to. The first is dynamic dichromatism, which promotes males of certain species to undergo a temporary change in colour over the breeding season. The second is the more commonly encountered form referred to as ontogenetic dichromatism, in which both males and females permanently differ in colour.

The reed frog *Hyperolius argus* Peters, 1854 is a well distributed species throughout the eastern side of Africa, stretching from as far north as Somalia and south to South Africa, where it seems to be restricted to the coastal plains of KZN (see Bishop 2004b; Poynton 1986). Although little else is known about the true breeding habits of this species, adult males are known to form large calling

choruses at night (see Bishop 2004b). *Hyperolius argus* is also known to contain ontogenetic dichromatic forms across both male and female individuals (see Stewart 1967; Poynton 1986; Noriega & Hayes 2000; du Preez & Carruthers 2009). Parker (1930) stated that adults retain juvenile colour patterns throughout their development. An experimental study by Hayes & Menendez (1999) also showed the expected and gradual ontogenetic colour changes of male and female tadpoles through to adulthood. However, in the current study *H. argus*, collected from one locality in sub-tropical northern KZN, is reported to contain polychromatic forms between sub-adult male, adult male and female frogs. The aim of this section was to clarify some of the taxonomic confusion and speculation from in the literature surrounding this species, reporting on the high degree of colour variation or possible hybridisation in *H. argus* with *Hyperolius semidiscus* Hewitt, 1927 (see Loveridge 1941; Poynton 1986). Additionally, the author will be reporting on the unexplained phenomenon of sexually immature sub-adult males producing mating calls, questioning what the possible advantages or evolutionary implications of this sort of behaviour may be. To the author's knowledge this is the first report on this unusual behaviour.

3.2

MATERIALS & METHODS

3.2.1 Frog collection and processing

Sites were selected based on mating calls of male *Hyperolius argus*, numbers estimated by scoring of individual frog calls. On 21 and 22 April 2013 specimens were collected from Nomaneni pan, a large and well vegetated site situated in a heavily populated part of the Makatinhi floodplains in northern KZN province, South Africa (26°59'12.8"S, 32°16'17.1"E). Frogs were collected by hand and were temporarily kept in plastic bags, separating adults from sub-adults, until further analysis at the field laboratory. Analysis included photography of external characteristics using a Canon 650D camera fitted with a Canon100mm macro lens and Canon twin macro flash; acoustic recording (see below); as well as the processing of voucher specimens. Voucher specimens were euthanised using MS222, a muscle tissue sample from one thigh was fixed in 70% molecular grade ethanol and the carcass fixed in a natural position in 10% neutral buffered formalin.

3.2.2 Advertisement call recordings and analysis

Calls were recorded several times in a laboratory setup, the setup assembled to simulate their natural environment, using an automated recorder (SongMeter SM2, Wildlife Acoustics), as well as a Nagra Ares-M digital recorder equipped with a senheizer M6 rifle microphone. Recordings were done at a temperature of about 20°C over a period of 24 hours spanning over two nights, adult and sub-adults recorded separately. Calls were subsequently analysed using SongScope (version 4.1.2A, Wildlife Acoustics) comparing calls of adult and sub-adult males.

3.2.3 Histology preparation and sectioning

From the euthanised voucher specimens, gonads of five sub-adult and two adult *H. agrus* were dissected out and fixed in Bouin's fixative and stored in 70 % ethanol for histological sectioning. Samples were dehydrated in an ethanol series of 70 %, 80 %, 96 % and twice in 100 % for 10 - 15 min each. Submersion of the tissue for ten minutes in a xylene-ethanol mixture, followed by two

replacements of pure xylene for 20 minutes respectively, this was used to clear the material. The tissue was then impregnated with paraffin wax at 60 °C for 24 h. Following this, the tissue was embedded in paraffin wax with a melting point of 65 °C using a Slee MPS/P2 histocene embedding machine. Sections (5 µm thickness) were cut using a Reichert Yung motorised microtome. Wax sections were subsequently placed on glass slides covered with an albumin adhesive solution and stretched on a stretching plate. Excess adhesive solution was drained off and slides dried overnight in an oven at 40 °C. Sections were routinely stained using Harris' Haematoxylin and Eosin. Cover slips were permanently mounted using Entellan mounting medium.

3.2.4 DNA extraction and phylogenetic analysis of *Hyperolius argus* vouchers

Muscle tissue obtained from one of the hind legs of each specimen (n=6) (two sub-adults, two adult males and two adult females), was transferred to sterile 1.5 ml cryo vials containing 70% molecular grade ethanol. DNA was extracted from the samples using the standard protocol for human or animal tissue and cultured cells as detailed in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Düren, Germany). The tissue samples were digested using standard Proteinase-K protocol according to the manufacturer's instructions. Following extraction, the concentration of the DNA was determined using the NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, US).

To amplify the extracted DNA, polymerase chain reaction (PCR) sequence runs were undertaken in a Bio-Rad C1000 Touch™ 155 Thermal Cycler (Bio-Rad, Hemel Hempstead, UK). The primer set, 16SaR-F (5'-CGCCTGTTTAYCAAAAACAT-3') and 16SbR-R (5'-CCGGTYTGAACTCAGATCAYGT-3') sourced from Kocher et al. (1989), was used to amplify a region of the 16S mitochondrial (mtDNA) gene. The PCR reaction mixture contained 12.5 µl double strength PCR master mix [(0.05 U/µl Taq DNA Polymerase in reaction buffer, 0.4 mM of each dNTP; 4 mM MgCl2 (Fermentas Life Sciences, US)]; 0.75 ml of each primer (Applied Biosystems, UK); 10 ml (approximately 50 ng) template DNA; and PCR-grade nuclease-free water (Fermentas Life Science, US) to adjust the volume to 25µl. The reaction took place in an ICycler thermal cycler (BioRad, UK) under the following conditions: initial denaturation at 95°C for 90 seconds, followed by 34 cycles entailing a 95°C denaturation for 45 seconds, annealing at 51°C for 45 seconds, with an end extension of 72°C for 90 seconds respectively, and a final extension at 72°C for 5min. Gel electrophoresis was used to visualise the DNA, using Ultraviolet (UV) light and Gel Red (Biotium, US) to illuminate a 1 % agarose gel (see Figure 3.2.1). A BioRad GelDoc Imaging System (BioRad, UK) was used to view the contents (see Kruger et al. 2011).

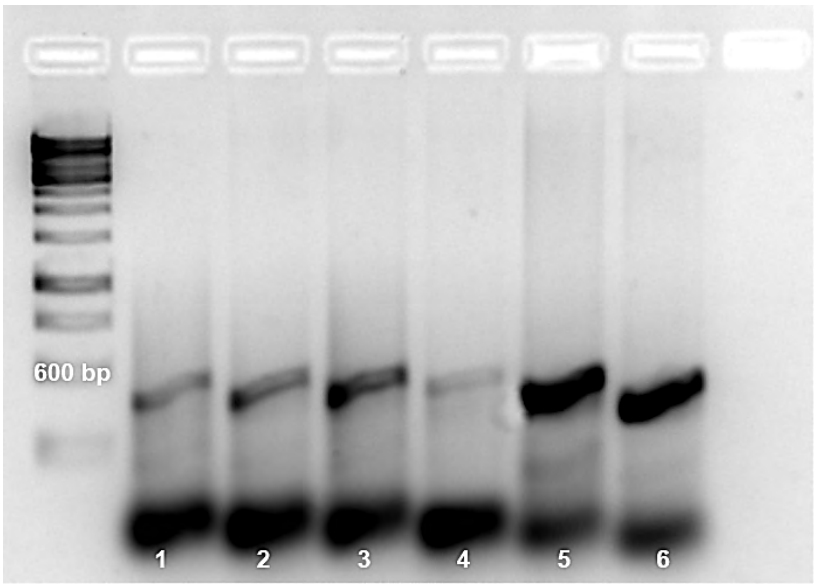


Figure 3.2.1: Amplified partial 16S rDNA fragments displayed on a 1 % agarose gel stained with Gel Red. Fragments of the 16S mt gene were amplified from the DNA extracted tissue samples of six *H. argus* (two sub-adults, two adult males and females) using the 16SaR-F and 16SbR-R primer set. Lane 1-6 displays the ± 500 bp fragment that was amplified for each of the samples.

Samples which yielded positive results were selected for sequencing. The NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) was used to purify the amplicon DNA and to remove traces of PCR reagents and primer-dimers. The concentration and 260:280 ratio of the purified amplicon DNA were determined using the NanoDropTM 1000 Spectrophotometer (Thermo Fischer Scientific, US). This DNA then served as the template for sequencing PCR.

The BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, UK) was used for the sequencing PCR. The reaction mixture contained 2.5 X Ready Reaction Premix (Applied Biosystems, UK), 5 X BigDye Sequencing Buffer (Applied Biosystems, UK), 3.2 pmol of either the forward or reverse primer. The same as was used in the original PCR reaction and PCR-grade nuclease-free water (Fermentas Life Science, US) to make up a final volume of 20 µl. The reaction took place in an ICycler thermal cycler (BioRad, UK) under the following conditions: initial denaturation at 96°C for 60 seconds, followed by 25 cycles entailing a 96°C for 10 seconds denaturation, annealing at 50°C for 5 seconds, and extension at 60°C for 4 min followed by holding at 4°C until purification as per the manufacturer's instructions.

The products of this reaction were purified again using the Zymo Research DNA Sequencing Clean-up Kit™ (Zymo Research, US) as per the manufacturer's instructions. The eluent was loaded into a 96-

well plate and placed in the 3130 Applied Biosystems Genetic Analyser, where sequencing electrophoresis took place using a 36 cm capillary array and POP-7™polymer (all Applied Biosystems, UK). Analysis of the data was done by the 3130 Genetic Analyser Data Collection software (Applied Biosystems, UK). A 500 bp fragment (approximately) of the mt 16S gene was amplified for each the samples.

Resulting sequences were edited by generating chromatogram-based contigs using the Geneious (Ver. 7.1) bioinformatics software package (created by Biomatters, available from http://www.geneious.com). Sequences were matched to existing Genbank sequences and were entered into the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) to confirm their identities. In addition, 19 comparative *Hyperolius* species sequences were obtained from GenBank, along with a single *Kassina senegalensis* [GenBank: AF215445] used as an outgroug (since it is from the same family, but a different genus). Sequences were aligned and analysed using the MUSCLE tool and Maximum Likelihood (ML) analysis respectively, and were all implemented in the MEGA6 bioinformatics software program (http://www.megasoftware.net) (Tamura *et al*. 2013). A ML phylogenetic tree was constructed based on the General Time Reversible + Gamma model (GTR+G) also identified in MEGA6 (Nei and Kumar 2000; Tamura et al. 2013), based on having the lowest Bayesian information criteria relative to other models. The initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.4915]). The analysis involved 23 nucleotide sequences, with a total of 468 positions in the final dataset. The performed phylogenetic analysis and nodal support was based on 1000 bootstrap replicates, with only bootstrap values of >50% shown.

3.3

RESULTS

3.3.1 Sampling

A total of 22 adult *H. argus* were collected and used for advertisement call recordings. Eleven of these were subsequently processed as voucher specimens (total adults: n=11), consisting of males (n=5) and females (n=6). Additionally, sub-adult males (n=9) were collected for vocal and morphological comparison and thereafter processed as voucher specimens.

3.3.2 Morphological comparison

Hyperolius argus is known to be a sexually dichromatic species in which adult females typically display white to yellow spots or stripes on a maroon-brown dorsum (Figure 3.3.1: A,B), whereas males vary from light to bright green with a fair amount of brown staining spots or flecks (Figure 3.3.1: C,D) (Noriega & Hayes 2000). Males also usually have a lateral line, which starts at the anterior tip of the nose and follows through to the posterior section of the body (Figure 3.3.1: C) in some cases the line is more prominent. All sub-adult males collected in this study were bright green or brown in colour with a very prominent yet slim yellow lateral line/stripe, extending, very similarly to the adult males, from the anterior tip of the nose and following through to the posterior section of the body (Figure 3.3.1: E,F).

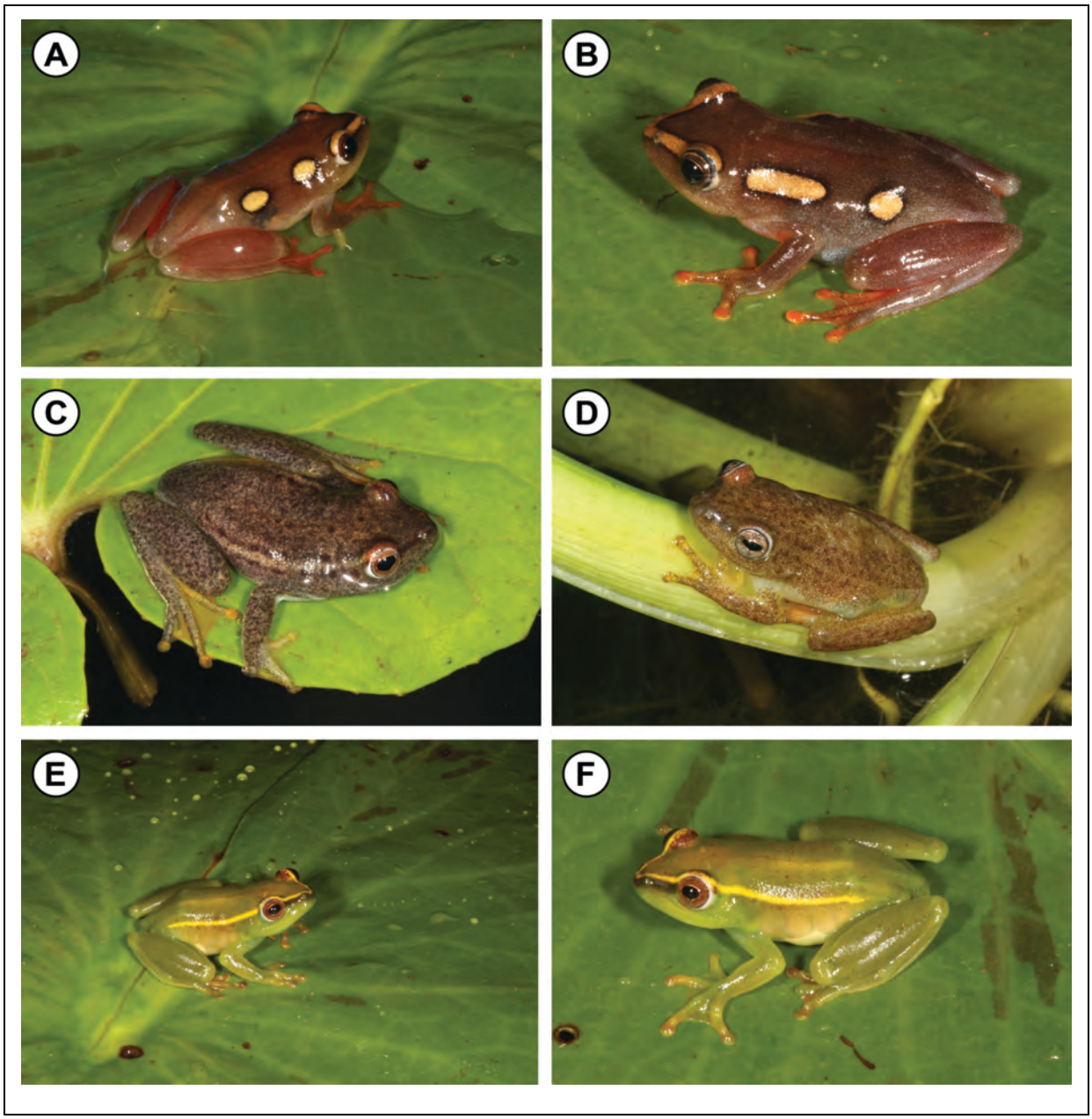


Figure 3.3.1: Various colour dimorphisms across different individuals of males and females as well as adult and sub-adult *H. argus*. A-B: Typical colouring for adult *H. argus* females from KwaZulu-Natal. **C-D:** Typical colour pattern for adult male *H. argus*. **C-D:** Light brown with very faint lateral stripes extending from the anterior tip of the nose across to the posterior section of the body. **E-F:** Unfamiliar colour pattern for sub-adult males.

3.3.3 Acoustic recording

The recorded calls of both the adult and sub-adult *H. argus* were analysed using SongScope (version 4.1.2A, Wildlife Acoustics) (Figure 3.3.2). When compared, the advertisement calls for both the adult and sub-adult males were very similar (frequency and call similarity), with the exception of a few minor differences in the intensity and amplitude of the calls. This could be due to the size difference in the adults and sub-adults. The recorded calls were similar/identical to the recording of *H. argus* provided on the compact disk that accompanied the book by du Preez & Carruthers (2009).

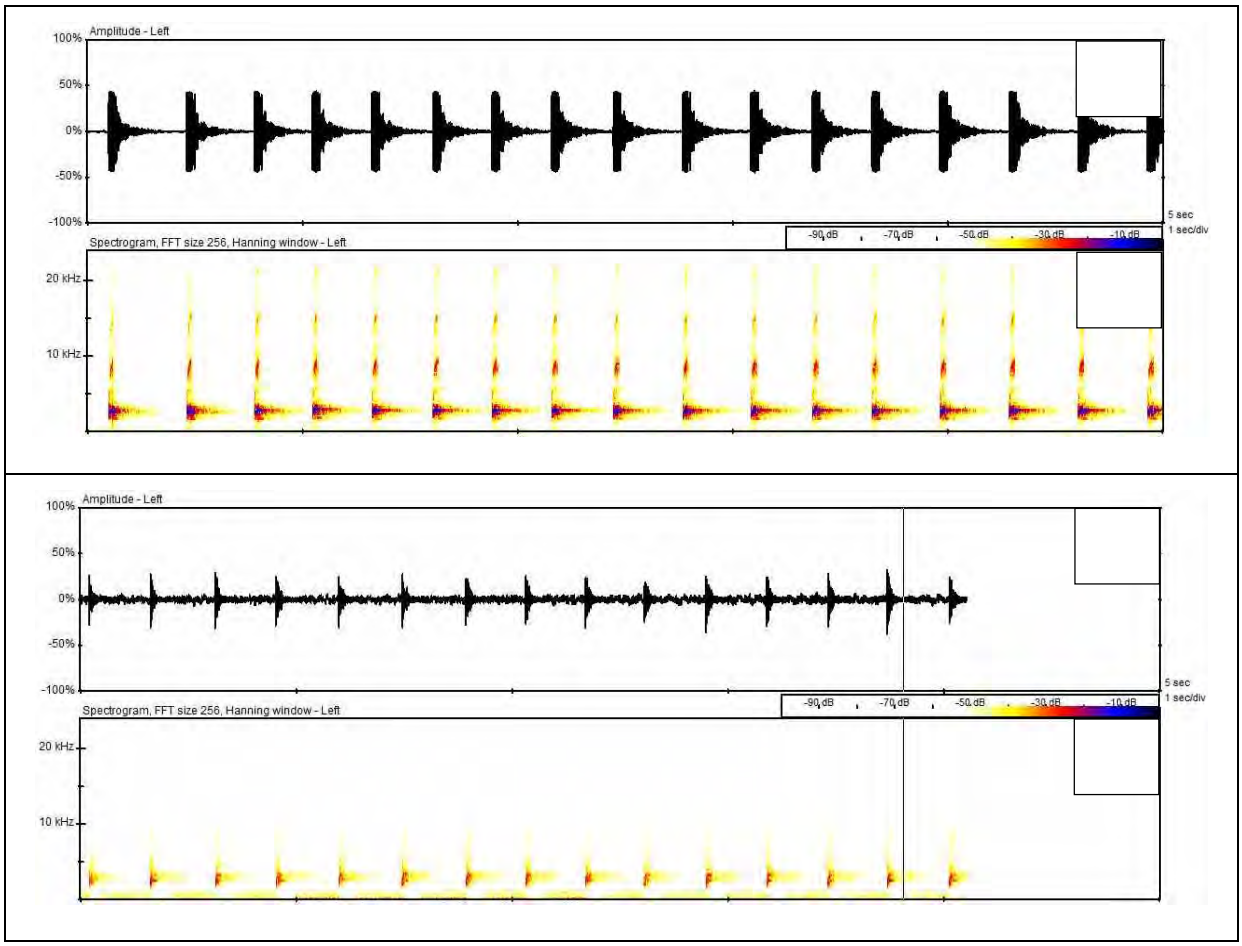


Figure 3.3.2: Representation of male *H. argus* adult and sub-adult advertisement calls. A-B: Representation (amplitude and spectrogram) of *H. argus* adult male advertisement call. **C-D:** Representation (amplitude and spectrogram) of *H. argus* sub-adult male advertisement call. **A,C:** Representing the amplitude as well as the rate at which the specimens were calling. **B,D:** Presenting the intensity (in decibels dB) the advertisement calls of the different males (adult/sub-adult). Calls were analysed and represented using SongScope (version 4.1.2A, Wildlife Acoustics).

3.3.4 Molecular analysis of *Hyperolius argus*

Sequences generated in the current study were edited and compared with each other, and three representatives of each were selected and aligned with 19 comparative sequences of various *Hyperolius* species, and one *Kassina senegalensis* (used as an outgroup), downloaded from GenBank. Ten species of *Hyperolius*, including *H. argus* were used in the phylogenetic analysis (see Figure 3.3.3). The three representative sequences of *H. argus*, consisting of a sub-adult, an adult male and an adult female, formed a monophyletic clade (Clade 1) along with previously published *H. agrus* sequences downloaded from GenBank (see Figure 3.3.3). It is also apparent that the various individuals are one and the same species, despite the fact that they vary morphologically (in terms of colouration).

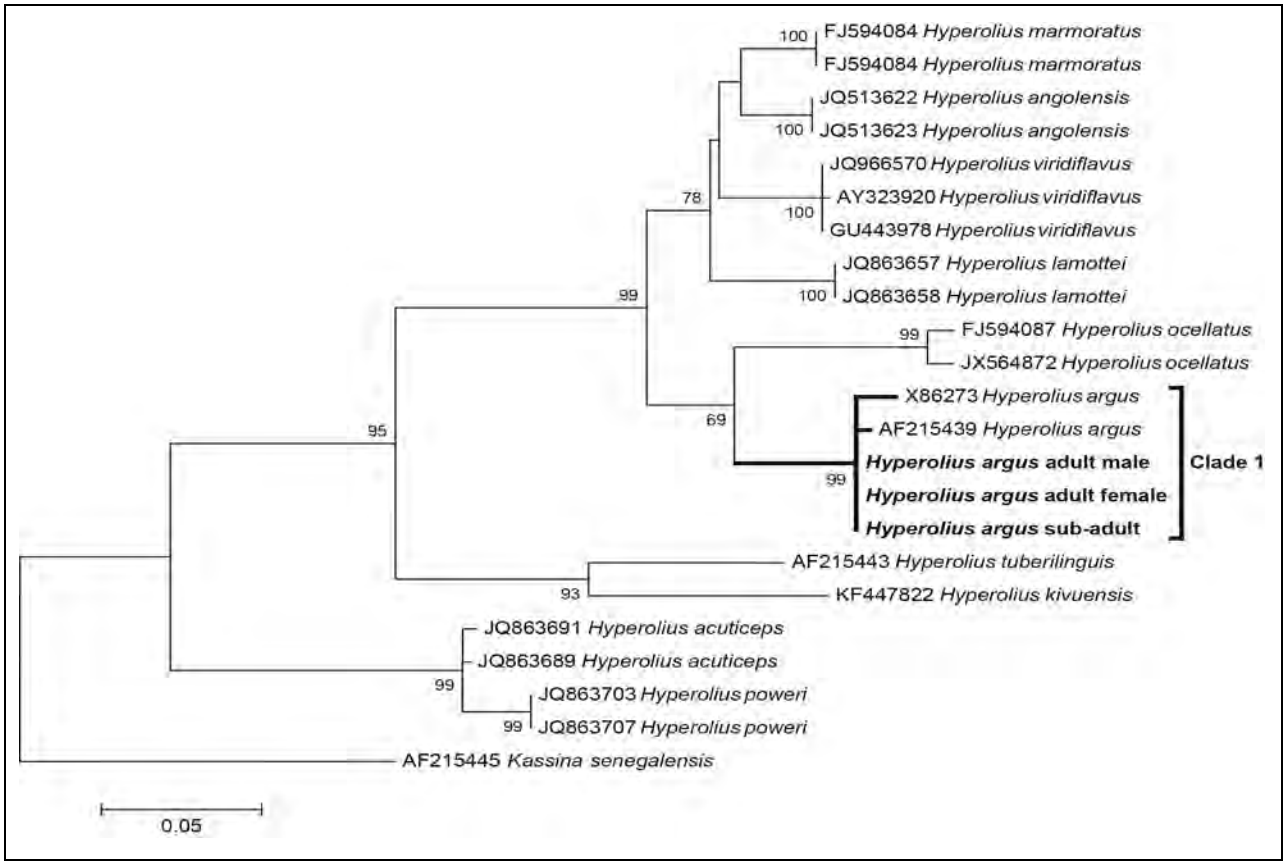


Figure 3.3.3: Molecular phylogenetic analysis of *Hyperolius* species by Maximum Likelihood (ML) method. Maximum Likelihood phylogenetic analysis highlighting the position of *H. argus* sub-adult and adult individuals, forming a monophyletic clade (Clade 1) with previously published sequences of *H. argus.* Tree was constructed under the conditions of the GTR+G model as implemented in MEGA6. The tree with the highest log likelihood (-2072.6319) is shown. The analysis involved 23 nucleotide sequences, and a total of 468 positions in the final dataset. *Kassina senegalensis* [GenBank: AF215445] was used as an outgroug.

3.3.5 Histological sectioning of *Hyperolius argus* adult and sub-adult gonads

Histology on the gonads of both adult and sub-adult individuals confirmed the difference in sexual maturity between the two (see Figure 3.3.4: A-B). High concentrations of fully developed sperm were found in the seminiferous tubules of the septa within the gonads of the adult males (Figure 3.3.4: A). However, the seminiferous tubules of the septa within the gonads of the sub-adult males did not contain any fully developed sperm (Figure 3.3.4: B).

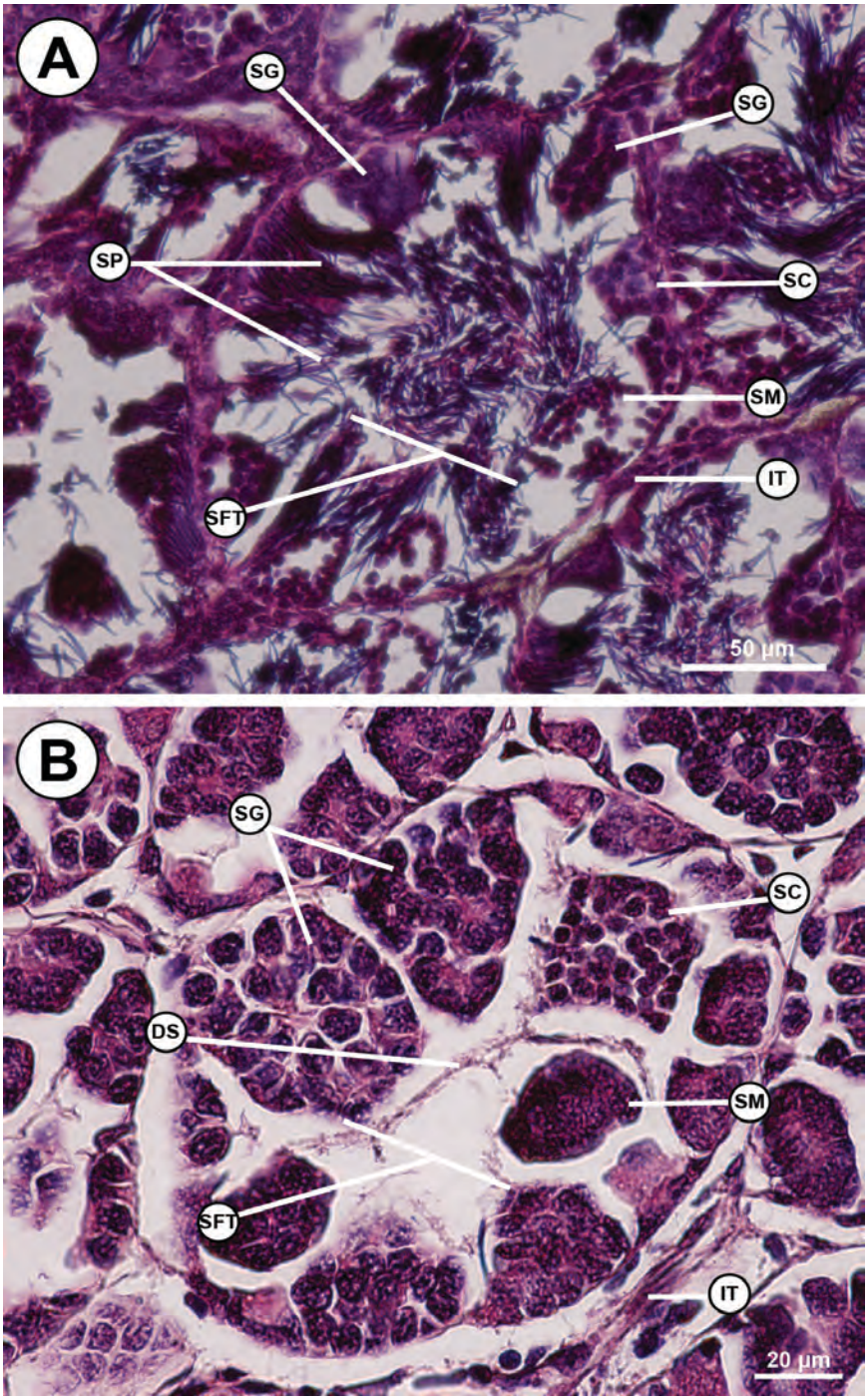


Figure 3.3.4: Histological sections of gonads displaying a mature and immature septum of two *H. argus*, one adult and one sub-adult respectively. A-B: Lables: Developing sperm (DS), Interstitial tissue (IT), Sperm (SP), Spermatocytes (SC), Spermatogonia (SG), Spermatids (SM), Seminiferous tubule (SFT). **A:** Showing the cross section of a mature septum in the gonad of an adult male *H. argus*. Note the large amount of sperm (SP) visable. **B:** Showing the cross section of an immature septum in the gonad of a sub-adult male *H. argus*. Note there are no fully developed sperm (DS) visable. Sections were routinely stained using Harris' Haematoxylin and Eosin.

3.4

DISCUSSION

Hyperolius argus is a commonly encountered frog species throughout eastern Africa, and is known to have ontogenetic dichromatism (permanent colour differentiation between sexes) (see Poynton 1986; Bishop 2004b; Bell & Zamudio 2012). On average females are slightly larger and demonstrate more conspicuous colours as compared to males (see du Preez & Carruthers 2009). Although there are several reports on small changes or variations in the colour patterns of different populations in Africa, based on other morphological characteristics, all are regarded as *H. argus* (see Poynton 1986; Hayes & Menendez 1999; Bishop 2004b; du Preez & Carruthers 2009). Previous developmental studies on *H. argus* have documented that adult species retain their colour patterns from metamorphosis (see Parker 1930; Hayes & Menendez 1999). In addition to the above, few reports comment on strange or unexplained colour variation within the species (see Passmor & Curruthers 1979; Poynton 1986). A possible theory that arose is the hybridisation between *H. argus* and *H. semidiscus* (see Loveridge 1941). Until now no further studies, in terms of molecular or experimental work have been conducted to challenge this theory and to sort out some of the taxonomic confusion associated with this species.

In the current study, three different forms in terms of colour variation were observed, the most obvious being the traditionally recognised ontogenetic dichromatism between the adult sexes. Even though *H. argus* females are larger, with more conspicuous colour patterns (characteristic purple-brown body, with striking yellow markings) (see Figure 3.3.1: A-B), there is no proof, as of yet, to support the theory of sexually dichromatic vertebrates making use of sexual selection as the major driving force in maintaining these traits seen in *H. argus* (see Bell & Zamudio 2012). In other words no previous literature mentions any observations of female to female rivalry over a possible mate. Males on the other hand seem to form large calling choruses competing amongst each other for mating rights.

The sub-adults collected (n=9) in the current study, occurring in the same locality as the above mentioned adult forms, were morphologically similar to the adult forms, but have completely different colours and patterns (more closely resembling *H. semidiscus*) as compared to adult forms (see Figure 3.3.1: C-F). This was in contrast to previous literature stating that adults keep juvenile colours and patterns throughout their development, as stated above. Thus, it should not be assumed

that all populations contain similar unusual differences in developing sub-adults, and it would seem to be an uncommon occurrence within this species.

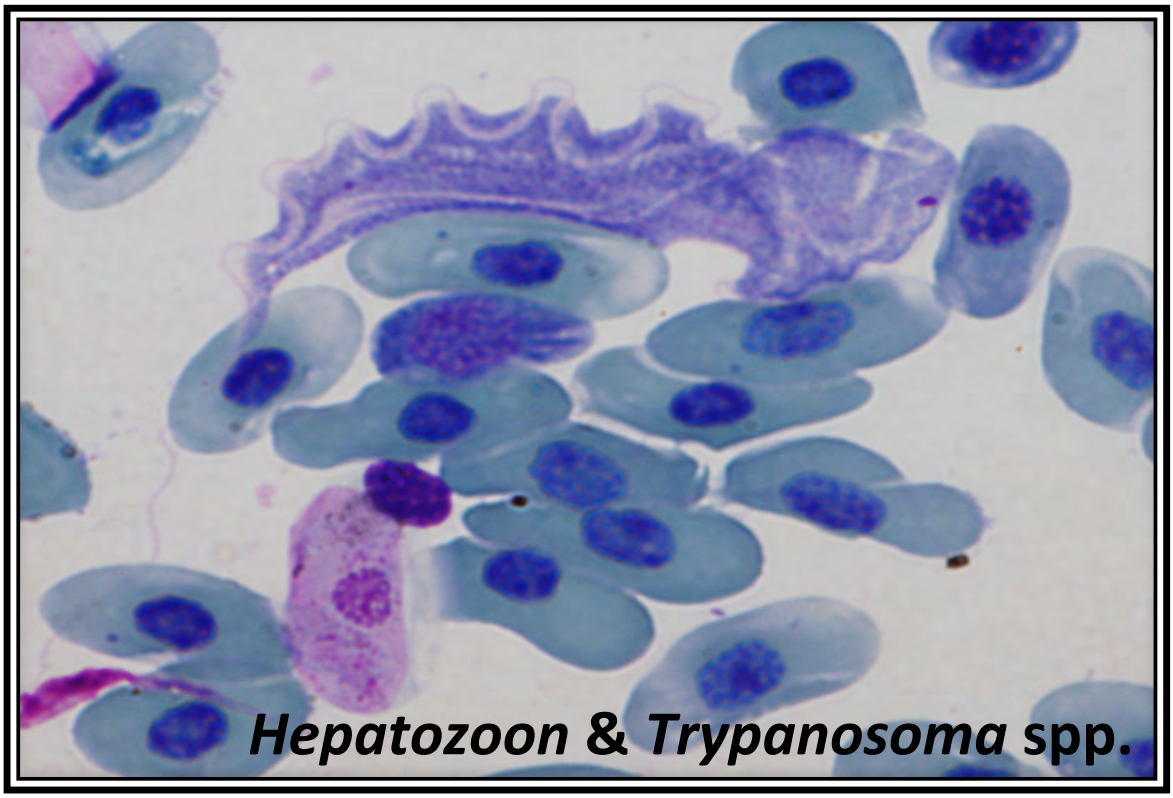
In order to prove that all the collected individuals were in fact one and the same species, fragments of the mt 16S gene were amplified from the tissue samples of six specimens, namely two sub-adults, two adult males and two adult females. Based on phylogenetic analysis these individuals all formed a monophyletic clade with other *H. argus* [GenBank: AF215439; X86273], confirming their taxonomic classification. An interesting question for future study will be to determine when and how fast transformation takes place from this conspicuous sub-adult form to the dull adult form (see Figure 3.3.1: C-F).

In conjunction with the unique appearance of the sub-adults from this study, an unexplained phenomenon was encountered as sub-adult frogs were located and collected listening for their distinctive (slightly softer and faster) calls, this in the midst of the overpowering chorus of adult male advertisement calls (see Figure 3.3.2: A-D).

At first it was assumed that the sub-adult males were mature and able to mate, but not developed enough to call properly. However, on examination of histological sections of sub-adult gonads in comparison to adult male gonads, it was revealed that the sub-adults were in fact sexually immature, without fully developed sperm cells (see Figure 3.3.4: A-B). Further experimental work is needed to determine or even propose a possible hypothesis on this behaviour. This leaves us with the question of what are the advantages for a frog to advertise itself if it does not lead to the successful reproduction of offspring?

CHAPTER 4

BLOOD PARASITE PREVALENCE, DIVERSITY AND PARASITEMIA OF AMPHIBIANS



4.1

INTRODUCTION

Amphibians are the most threatened vertebrate group, suffering large-scale declines in species diversity since at least, according to historical data, the 1970s (Stuart *et al.* 2004). A decade ago the IUCN's Global Amphibian Assessment indicated that a third of the estimated amphibian species had either declined or become extinct (Stuart *et al.* 2004; Beebee & Griffiths 2005). Such declines may be attributed to a number of factors ranging from habitat destruction, pollution and exploitation, to climate change and disease (Beebee & Griffiths 2005). The disease known as chytridiomycosis (amphibian chytrid), caused by the fungal pathogen *Batrachochytrium dendrobatidis*, has been responsible for major global amphibian declines (Readel & Goldberg 2010). Along with chytrid, amphibians are host to a wide variety of parasites (du Preez & Carruthers 2009; Netherlands *et al.* 2014), including intraerythrocytic and extracellular haemoparasites ranging from protozoans, comprising both intracellular apicomplexans (Davies & Johnston 2000) and extracellular flagellates (Acosta *et al.* 2013), to extracellular nematode microfilariae (Baker 2008) as well as those intracellular parasites of uncertain identity such as the rickettsia and viral-like infections (Davies & Johnston 2000; Davis *et al.* 2009). The most attention, however, has been given to those parasites of the first three groups mentioned, most likely due to the frequent findings and thus greater basis of knowledge of these organisms in anuran hosts. Furthermore, of these three groups, those of the protozoa, particularly the apicomplexans, would appear to be the most studied of all (see Davies & Johnston 2000; Netherlands *et al.* 2014).

However, since few parasite surveys on frogs have been carried out in sub-Saharan Africa, the degree of this haemoparasite diversity remains unknown (Readel & Goldberg 2010; Netherlands *et al.* 2014). Yet, such diversity of knowledge regarding these parasites is necessary before further studies can be done on elucidating the effects that these parasites may have on their natural hosts, and the role these parasites may have in amphibian conservation.

Southern Africa currently boasts 159 known species of frogs in 33 genera and 13 families (du Preez & Carruthers 2009; Channing & Baptista 2013; Channing *et al.* 2013a; Channing *et al.* 2013b; Conradie 2014). This section of the current study presents the results of a haemoparasite survey of frogs from three localities in KwaZulu-Natal, South Africa (see Figure 2.2.1). Localities including the formally protected Ndumo Game Reserve (NGR) and the reserve's anthropogenically impacted surrounds, as

well as Kwa Nyamazane Conservancy (KNC). All three sampling areas fall within a sub-tropical region known for its biological richness and as such the province, KZN, boasts the highest diversity of frog species in South Africa (see du Preez & Carruthers 2009). This section of the present study thus aimed to determine and record, through a multispecies haemoparasite survey on frogs, if this parasite diversity paralleled that of its rich frog diversity.

4.1.1 General introduction to blood parasites

To date, blood parasites have been recorded from a wide range of vertebrate and invertebrate hosts and vectors, stretching from aquatic to terrestrial habitats (see Barta *et al.* 2012). Throughout the 19th century a reasonable amount (over 100 publication) of work was done on the blood parasites of many different mammalian, reptilian and amphibian hosts. Among the most studied blood parasites, however, are those found infecting mammals, particularly those of humans such as *Plasmodium* Marchiafava Celli, 1885 and *Trypanosoma* Gruby, 1843 species, causing the diseases malaria and sleeping sickness, respectively (see Davies & Johnston 2000; Hamilton *et al.* 2007; Smith 1996). This bias could be due largely to the fact that only a few blood infections of amphibians, fish, and reptiles are observed to harm their hosts (Davies & Johnston 2000). According to Telford (2009), before the 1960's almost all haemogregarines were inadequately described, and Ball (1967) attempted to discourage any further descriptions of blood parasites especially from host peripheral blood stages alone. This fortunately contributed to the decline in inadequate descriptions since that time. With the improvement of both technology and different scientific techniques, such as microscopy and molecular work respectively, enhanced methods of characterizing, describing and redescribing new and already known blood parasites has encouraged recent and further work on blood parasites. Since in the past it was thought that blood parasites were solely host-specific, describing new species from new hosts, the above has led to many described species being subsequently synonymised (see Cook *et al.* 2010). In recent years, apart from the improved taxonomic and molecular work of blood parasites, there has been a progressive focus on experimental work determining the life cycles as well as host-parasite relationships, which will continue to be an important step in learning and understanding more about these parasites.

4.1.2 Intraerythrocytic and extracellular blood parasites of anurans

A wide variety of intraerythrocytic and extracellular blood parasites have been recorded infecting anuran hosts. These range from protozoans, including both intracellular apicomplexans and extracellular flagellates, to extracellular nematode microfilariae as well as those intracellular parasites of uncertain identity such as the rickettsia and viral-like infections. The most attention, however, has been given to those parasites of the above first three groups, most likely due to the frequent findings and thus greater basis of knowledge of these organisms in anuran hosts. Furthermore, of these three groups, those of the protozoa, particularly the apicomplexans, would appear to be the most studied of all.

Most of the currently recorded apicomplexan parasites from anurans are of the genera *Babesiosoma* Jakowska and Nigrelli, 1956; *Dactylosoma* Labbé, 1894; *Haemogregarina* Danilewsky, 1885; *Hemolivia* Petit, Landau, Baccam and Lainson, 1990; and *Hepatozoon* Miller, 1908 within the suborder Adeleorina Léger, 1911. Genera recorded from the suborder Eimeriorina Léger, 1911 are *Lankesterella* Labbé, 1899 and *Schellackia* Reichenow, 1919. From within the order Haemospororida Danilewsky, 1885 the genus *Haemoproteus* Kruse, 1890 is the only one currently recorded to parasitise anurans. From the order Rickettsiales Gieszczkiewicz, 1939 numerous rickettsia and rickettsia-like infections such as *Aegyptianella* Carpano, 1929 *Bertarellia* Carini, 1930; *Cytamoeba* Labbé, 1894; and *Haemobaronella* Tyzzer and Weinman, 1939 have been reported. Several uncertain mycoplasma-like infections such as *Pirhemocyton* Chatton and Blanc, 1914 and *Toddia* França, 1910 have also been reported in anurans across the globe (see Barta 1991; Davies & Johnston 2000).

Within the Kingdom Protozoa Goldfuss, 1818, haemogregarines are among the most commonly recorded apicomplexans to parasitise frogs. These parasites belong within the phylum Apicomplexa Levine, 1970, which comprises approximately 5000 described species, most of which have been poorly described in the past (Escalante & Ayala 1995). All species within this phylum are unicellular parasites characterised by the apical complex allowing the infective stages of the parasite to effortlessly enter into the host cell (Morrison 2009). Haemogregarines comprise a large group of apicomplexan blood parasites recorded from a wide range of tetrapod vertebrates and haematophagous invertebrates (Smith 1996; Davies & Johnston 2000). Haemogregarines are heteroxenous parasites and the group presently includes three families, namely the Haemogregarinidae Léger, 1911, Hepatozoidae Wenyon, 1926, and Karyolysidae Wenyon, 1926. Within these families there are six genera of blood parasites, differentiated on the sporogonic development in their invertebrate hosts (Telford 2009; Barta *et al.* 2012). Prior to the clarification of

the haemogregarine life cycles in anuran hosts by Desser *et al.* (1995), most were placed in the genus *Haemogregarina*. The genus *Haemogregarina* is characterised by merogony in the peripheral blood, transmission occurring via the bite of an infected, most often an aquatic vector, such as a leech. Conversely, the genus *Hepatozoon* is characterised by merogony in the vascular endothelial cells of the final vertebrate host and typically no merogony takes place in the peripheral blood erythrocytes, with only intraerythrocytic or rare intraleucocytic gamont stages being present. Transmission occurs via the ingestion of parasitised intermediate vertebrate hosts such as frogs or lizards, or definitive invertebrate vectors including haematophagous insects, mites, ticks, and possibly, but doubtfully, leeches, in which, sporogony typically occurs in the haemocoel (see Smith *et al.* 1994; Smith 1996; Davies & Johnston 2000; Van As *et al.* 2013; Netherlands *et al.* 2014). However, with further insight into the above, Smith (1996), suggested that these haemogregarines were better suited to the genus *Hepatozoon* and thus transferred them accordingly. As a result, *Hemolivia* and *Hepatozoon* are the only two haemogregarine genera currently known to parasitise anuran hosts (Davies & Johnston 2000), with species of the latter currently representing the most common intraerythrocytic protozoan parasites of anurans worldwide (Smith 1996).

The Dactylosomatidae is a small family comprising the genus *Babesiosoma* and *Dactylosoma*. Species of this family have only been found in amphibians or fish, the common factor of all these species being the prolonged contact with water. Thus the definitive vector of all *Babesiosoma* and *Dactylosoma* species is suggested to be haematophagous leeches (see Barta 1991; Smit *et al.* 2003). *Babesiosoma stableri* Schmittner & McGhee, 1961 is currently the only recorded species to parasitise frogs and the genus is characterised by the unique merogonic and sporogonic duplication within the vertebrate host and invertebrate vector (see Levine 1985; Barta 1991). Primary merogony takes place when a merozoite enters the vertebrate host's erythrocyte. Within the erythrocyte the merozoite undergoes nuclear division, transforming into a binucleate meront. This meront then undergoes a second nuclear division, forming the characteristic and tetranucleate cruciform meront (four merozoites arranged in a cross shape) typical of the genus *Babesiosoma* (see Barta & Desser, 1986, 1989; Barta 1991). In contrast, *Dactylosoma* species undergo two morphologically distinct merogonic cycles, during the primary merogonic development in the erythrocytes, large trophozoites or meronts are observed producing up to sixteen merozoites arranged in the characteristic fan or hand-like nature for which the genus was named. This is followed by secondary merogonic division of the meronts, which subsequently produce six merozoites by peripheral budding. These smaller merozoites will then from into gamonts (see Barta 1991; Lainson 2007). Because of the above mentioned development, the family Dactylosomatidae exhibits characteristics intermediate between some adeleid blood parasites such as *Haemogregarina*, and that of the

piroplasms such as *Theileria* Bettencourt, França & Borges, 1907 (Barta 1991). This is well supported by phylogenetic analysis shown by Barta *et al.* (2012).

Within the family Lankesterellidae, the genera *Lankesterella* and *Schellackia* are known to parasitise anuran hosts (see Davies & Johnston 2000). There are similar life cycle aspects to those of the haemogregarines, such as having stages infecting erythrocytes and leucocytes, as well as having members with heteroxenous life cycles. However, representatives of the family can be differentiated from those of the haemogregarines in having sexual reproduction take place in the tissues (gut, connective tissue and/or viscera) of the vertebrate host where most of the life cycle development takes place. In the genus *Lankesterella,* merogony, gamogony and sporogony takes place within the vertebrate host, in the cells of the reticuloendothelial system, and is known to contain oocysts with 32 or more sporozoites. Although dormant sporozoites have been reported to exist in some tissues, most sporozoites seem to exist in the erythrocytes (see Davies & Johnston 2000). In contrast to the latter, the genus *Schellackia* is characterised by merogony, gamogony and sporogony, which takes place in the intestinal epithelium or lamina propria, with possible development in the spleen or liver. The octonucleate oocysts produce only eight sporozoites, which then enter the erythrocytes and lymphocytes. Dormant sporozoites can also be found in tissues. For both the above genera transmission occurs through the ingestion of a sporozoite-bearing invertebrate or predation of a parasitised vertebrate (see Davies & Johnston 2000). Although the 18S rRNA phylogenetic analysis of *Schellackia* parasites by Megía-Palma *et al.* (2013), shows that species of the genus *Schellackia* are clustered with *Eimeria* species compared to *Lankesterella* species, further morphological and molecular work will have to be done on these species to sort out the uncertainty of their classification.

Along with the more commonly found and studied protozoan parasites of amphibian erythrocytes (see above), are other intraerythrocytic organisms, too small to be characterised by light microscopy (Desser & Barta 1984). After ultrastructural evidence presented by Bernard *et al.* (1969), it was recognised that these organisms were of a viral nature. Further ultrastructural studies of the same or similar organisms have revealed their viral-like and rickettsial natures. Based on ultrastructural morphology these were since then referred to, among other designations, as species of *Aegyptianella*, *Pirhemocyton* and *Toddia* (see Johnston 1975; Desser & Barta 1984). Although the original description of *Aegyptianella ranarum* (Desser, 1987) is ultrastructurally similar to other rickettsiae (Moulder, 1974), particularly the intraerythrocytic stages of *Aegyptianella pullorum* (Carpano, 1928), recent molecular studies have indicated that *A. ranarum* is more closely related to *Chryseobacterium meningosepticum* (King, 1959), a bacterium in the family Flavobacteriaceae

(Reichenbach, 1992) as compared to *A. pullorum* (see Rikihisa *et al.* 2003; Zhang & Rikihisa 2004). It was then proposed that *A. ranarum* be transferred to the family Flavobacteriaceae, in the newly erected genus *Hemobacterium* Zhang & Rikihisa, 2004 and was renamed Candidatus *Hemobacterium ranarum*. This proves that morphological characteristics alone are not enough to correctly classify these small intraerythrocytic organisms and molecular tools are definitely needed (see Davis *et al.* 2009).

According to Gruia-Gray *et al.* (1989), all intraerythrocytic viruses in the family Iridoviridae are icosahedral, contain double stranded DNA and are confined to the cell cytoplasm; some of these are even reported to be enveloped. Because of the confusion and uncertain taxonomic classification between icosahedral iridovirus-like organisms, such as *Pirhemocyton*, *Toddia* and other similar species recorded to occur in amphibian and reptilian host erythrocytes, it has been suggested that all iridovirus-like organisms be referred to as either Lizard Erythrocytic Virus (LEV), Snake Erythrocytic Virus (SEV) and/or Frog Erythrocytic Virus (FEV) according to the host species (see Gruia-Gray *et al.* 1989; Telford & Jacobson 1993; Smith *et al.* 1994). Apart from their ultrastructural characteristics very little is known about these viruses (see Gruia-Gray *et al.* 1989; Alves de Matos & Paperna 1993), and as stated above, attempts to classify these organisms should only be attempted using both morphological and molecular characterisation.

Extracellular blood parasites, such as the genus *Trypanosoma* (Euglenozoa: Kinetoplastea: Trypanosomatidae) comprise a large group of flagellate extracellular blood parasites (see Su *et al.* 2013). Trypanosomes have been recorded or described infecting an extensive range of vertebrate hosts namely mammalians, reptilians, fishes and amphibians, and have been reported to occur on all continents, as reviewed by Bardsley & Harmsen (1973). The first anuran trypanosome was discovered in 1842, in the European frog, *Pelophylax esculentus* (Linnaeus, 1758) (syn. *Rana esculenta*). It was at first classified as *Amoeba rotatoria* (Mayer, 1843). After the re-examination and a fairly complete description of a similar species by Gruby (1843), it was designated the name *Trypanosoma sanguinis* Gruby, 1843, who subsequently created the genus *Trypanosoma* (see Gruby 1843; Laveran & Mesnil 1907; Ferreira *et al.* 2007). The life cycles of these parasites alter between vertebrates and invertebrates. Although most trypanosome species, particularly those of mammals, develop in haematophagous arthropod vectors, those of fish and amphibian trypanosomes are transmitted by insect or leech vectors (Acosta *et al.* 2014). According to Martin & Desser (1990), the development of *Trypanosoma fallisi* Martin & Desser, 1990 in the frog host *Anaxyrus americanus* (Holbrook, 1836) (syn. *Bufo americanus*) passes through two distinctive developmental transformations. The first alteration is the maturation of the metacyclic trypomastigotes into robust

bloodstream trypomastigotes, which are characterised by an increased surface area and undulating membrane. This occurs over a period of eight to ten days post infection. The second alteration, is the gradual replacement of the robust forms into shorter and slender trypomastigotes, often with a well-developed undulating membrane and larger kinetoplast, this takes approximately eight to 12 weeks.

Other extracellular blood parasites include the microfilariae, filarial worms or nematodes often found within the blood of amphibian, reptilian, avian and mammalian tissues. Until proven otherwise, filarial worms are assumed to have haematophagous arthropod vectors. Once transmitted through feeding on a potential host, the larval stages migrate to areas such as the subcutaneous tissues, muscle tissue, the body cavity and lymph nodes. After migration these stages develop into adult male and female nematodes which occur in the body cavity of the host. Once sexual maturity is reached, fertilized eggs develop into sheathed microfilariae within the adult female nematode. The microfilariae are then released and migrate to the lymph and blood vessels, where they remain and can survive up to two years awaiting ingestion of a vector (see Baker 2008). The life cycle is completed once the vector feeds on the infected host (Benach & Crans 1973; Van As 2012; Hamer *et al.* 2013).

4.1.3 Intraerythrocytic and extracellular blood parasites of anurans of Africa

Since haemogregarines are the most frequently recorded intraerythrocytic parasites of anurans, making up a large portion of the blood parasite fauna, many species have been reported from African frogs in the past. All of these species are of the genus *Hepatozoon* with no species of *Hemolivia* having been reported as of yet (see Smith 1996). According to Netherlands *et al.* (2014), the majority of African anuran *Hepatozoon* species have been recorded from the family Bufonidae, with only a few descriptions from the families Ptychadenidae, Pyxicephalidae and Hyperolidae (see Smith 1996; Netherlands *et al.* 2014). Of the approximately 15 *Hepatozoon* species described from African frogs, *Hepatozoon theileri* (Laveran, 1905), originally described as *Haemogregarina theileri* Laveran, 1905, remains currently the only known frog *Hepatozoon* species recorded from South Africa (Netherlands *et al.* 2014). *Hepatozoon theileri* and those infecting the frog families mentioned above will be discussed in further detail in the following chapter.

Although globally, several different frog species have been recorded to be parasitised with *Babesiosoma* species, in Africa only fish have been recorded with *Babesiosoma* species (see Barta 1991; Smit *et al.* 2003). *Dactylosoma* on the other hand has been reported from the following

anurans and areas, namely from unidentified frog species in Tunisia (Billet 1904); *Hyperolius marmoratus* Rapp, 1842 (syn. *Rappia marmorata*), *Hylarana galamensis* (Duméril & Bibron, 1841) (syn. *Rana galemensis*), *Ptychadena oxyrhynchus* (syn. *Rana oxyrhynchus*), *Ptychadena mascareniensis* (Duméril & Bibron, 1841) (syn. *R. macarensis*), and in *A. regularis* from The Gambia (Dutton *et al.* 1907); *A. regularis* (invalid species, is most likely *Amietophrynus gutturalis* [Power, 1927]) in South Africa (Fantham *et al.* 1942); *Hylarana albolabris* (Hallowell, 1856) (syn. *Rana albilabris*) somewhere in Africa (specific locality not reported, but possibly Uganda) and *Pelophylax ridibundus* (Pallas, 1771) (syn. *Rana ridibunda*) from North Africa (see Walton 1947, 1949). All these reports referred to the species as *D. ranarum* (see Barta 1991).

Other, less common African anuran blood parasites include species such as *Lankesterella minima* (Chaussat, 1850), *described from P. ridibunda* in North Africa (Walton 1947), *Lankesterella bufonis* Mansour & Mohammed, 1962, described from the toad *A. regularis* in Egypt (Mansour & Mohammed 1962), *Lankesterella ptychadeni* Paperna & Ogara, 1996 and *Lankesterella dicroglossi* Paperna & Ogara, 1996, from the frogs *Ptychadena mascareniensis* Duméril & Bibron, 1841 and *Hoplobatrachus occipitalis* (Günther, 1859) (syn. *Dicroglossus occipitalis*) respectively, in Kenya (Paperna & Ogara 1996).

Viral and viral-like infections have been recorded from a cosmopolitan distribution of amphibians (see Desser, 1987). Unfortunately, very little is known about the identity, classification and effect these organisms have on their hosts (see Desser 1987; Davies & Johnston 2000). Alves de Matos & Paperna (1993) presented the most recent study of uncertain erythrocyte virus infections from *Ptychadena anchietae* (Bocage, 1868) in South Africa. These virus-like infections were found to be similar to several different viruses of the FEV group such as *Toddia*, *Pirhemocyton* and other rickettsia-like protozoans.

In Africa, most of the recorded *Trypanosoma* species from anuran hosts were described in the early 19th century, such as *Trypanosoma bocagei* França, 1910, in *A. regularis* from Guinea-Bissau; *Trypanosoma elegans* França & Athias, 1906, in *A. regularis* from Congo; *Trypanosoma inopinatum* Sergent & Sergent, 1904, in *Pelophylax saharicus* (syn. *P. esculentus*) from Algeria; *Trypanosoma karyozeukton* Dutton & Todd, 1903, in various frog species from The Gambia; *Trypanosoma loricatum* (Mayer, 1843) in *A. regularis* from The Gambia; *Trypanosoma mega* Dutton & Todd, 1903, in various frog species from The Gambia; *Trypanosoma nelspruitense* Laveran, 1904, in *Amietia quecketti* (Boulenger, 1895) from South Africa; *Trypanosoma parroti* Brumpt, 1923, in *Discoglossus pictus* Otth, 1837, from Algeria; *Trypanosoma rotatorium* (Mayer, 1843) in *A. regularis* from Sudan; *Trypanosoma sanguinis* in (syn. *Rana trinodis*) from The Gambia; *Trypanosoma sergenti* Brumpt,

1923, in *Discoglossus pictus* Otth, 1837, from Algeria; *Trypanosoma somalense* Brumpt, 1906, in *Bufo reticulatus* (invalid species, is most likely *Amietophrynus xeros* [Tandy, Tandy, Keith, & Duff-MacKay, 1976]) from Somalia; *Trypanosoma tumida* Awerinzew, 1916, in *Amietia angolensis* (Bocage, 1866) (syn. *Rana nutti*) from Usmabara, Tanzania (see Dutton & Todd 1903; Laveran 1904; Sergent & Sergent 1904; Dutton & Todd 1907; Stevenson 1911; Bardsley & Harmsen 1973). Unfortunately in the past many reports contain inadequate taxonomic descriptions of trypanosomes, and in numerous cases they are simply referred to as a *Trypanosoma* sp. without any morphological data provided for the specific parasite (see Bardsley & Harmsen 1973; Telford 2009). Trypanosomes are also known to vary in form within the same species and are thus described as being pleomorphic. Since in the past many have been described without molecular work it is likely that numerous species have been re-described under new names and new geographical localities (Telford 2009). In order to move forward, a great deal of work is required to redescribe and molecularly characterize all the currently known *Trypanosoma* species.

On the whole taxonomic research on those microfilariae parasitising frogs is a neglected area of research, particularly with regards to those species parasitising frogs from Africa. Microfilariae species of *Foleyella* Seurat, 1917, and *Icosiella* Seurat, 1917, are closely related filarial nematodes with a cosmopolitan distribution, known to infect a number of amphibian and reptile hosts (see Barta *et al.* 1989; Schacher & Crans 1973; Barta & Desser 1984). *Icosiella neglecta* (Diesing, 1851) has been reported in a variety of frogs from Europe, Asia, and Africa (see Baker, 2008).

4.2

MATERIALS & METHODS

4.2.1 Frog collection and husbandry

Frogs were collected by hand at night from three main localities, inside the NGR, from the area bordering the NGR (outside NGR), as well as from the KNC. These localities were specifically chosen as all three are located on, and are thus supplied by a permanent water source, the Phongolo River (see Figure 2.2.1).

All three of these sites were visited during the warmer and wetter months of February and November 2012 (summer), April (winter) and November 2013 (summer) and February 2014 (summer). During collection possible invertebrate vectors, either feeding on frogs or in the vicinity of sampling sites, were searched for. Captured frogs were held in disposable plastic bags and transported back to a field laboratory either at the NGR or KNC, where they were identified to species level using du Preez & Carruthers (2009).

4.2.2 Frog blood smear preparation and screening

A drop of blood was collected from each frog via cardiac or femoral venipuncture using a sterile heparinized insulin syringe. A portion of this blood was used to prepare a thin blood smear, which once air-dried in a dust-proof container was fixed immediately using absolute methanol and stained thereafter using a modified solution of Giemsa stain (FLUKA, Sigma-Aldrich, Steinheim, Germany); the remaining blood was fixed in 70% ethanol for future molecular analysis. All frogs were processed the morning after collection and were released within 24 hr of capture. This study received the relevant ethical approval (North-West University ethics approval no.: NWU-00005-14-S3), as well as approval to do research from the appropriate conservation authorities (see appendix 3)

Smears were screened using a 100× immersion oil objective on a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, Netherlands), and images were captured with an Nikon digital camera. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear (see Cook *et al.* 2009; Netherlands *et al.* 2014). Parasitaemia for extracellular parasites were calculated as number of parasites/per-slide (ps).

4.2.3 Statistical analysis

The Monte Carlo variant of the Fisher's exact test, set to 10,000 replicates with a confidence interval of 99%, was employed to investigate significance in variation of prevalence between species, families, habitat types and sampling periods. The habitat types were established based on those described by du Preez & Carruthers (2009). Frog species were classified as terrestrial (those species thriving and breeding away from a permanent water source for most of their lives), semi-terrestrial (species thriving away from a permanent water source, but needing such a source to breed), semi-aquatic (species requiring a position near a permanent water source for most of their lives in order to survive and breed) and aquatic (species permanently living and breeding in a water source, rarely leaving that source). Since the Kruskal-Wallis test is suitable for non-parametric data and does not assume normal data distribution and equal sample size, it was used to determine significance levels ($P < 0.05$) of variation between infection intensity across species, families, habitat types, and sampling periods. It was further employed to determine significance of variation of the overall intensity of *Hepatozoon* and *Trypanosoma* across frog species, families, habitats and sampling periods. A non-parametric Levenes's test was used to verify the equality of variances in the samples (homogeneity of variance, $P > 0.05$) (Nordstokke and Zumbo, 2007; Nordstokke and Zumbo, 2010). All statistical analyses were performed using IBM SPSS Statistics ver. 22 (SPSS, 2013).

4.3

RESULTS

A total of 29 frog species were collected on several different field trips between February 2012 and February 2014, in and around NGR as well as from the KNC in KZN. The blood parasite biodiversity recorded from these frogs was high, both intracellular and extracellular parasites recorded. The following section will detail the overall diversity of blood parasites recorded infecting frogs from all of the three above localities, describing these parasites briefly, with information provided on the observed prevalence and parasitaemia. The section will then conclude with the statistical findings.

4.3.1 Overall prevalence, diversity and parasitaemia

On the whole, blood smears were collected from 11 families, six genera, and 29 species from 436 individual frogs (see Table 4.3.1 and 4.3.2). Of these 15/29 (52%) of the frog species were infected with blood parasites, making up 87/436 (20%) of the total number of frogs (see Table 4.3.1 and 4.3.2). Five groups of blood parasites were recorded including intraerythrocytic haemogregarine and haemogregarine-like species of the genus *Hepatozoon* and *Dactylosoma* respectively, and intraerythrocytic organisms of a viral or bacterial nature, the species of which could not be identified; furthermore, extracellular flagellate parasite species of the genus *Trypanosoma* and microfilarid nematode species were observed (see Table 4.3.1 and 4.3.2).

The parasites are briefly described below (intracellular followed by extracellular), providing information on the type host and other hosts, along with prevalence and parasitaemia in each host. First from inside the NGR, followed by those collected outside the reserve in the outside NGR and KNC.

4.3.2 Frog blood parasites recorded from inside the Ndumo Game Reserve

Intracellular

***Hepatozoon* sp. (A)**

This parasite is oval shaped with a well-developed cap/cavity at one pole, often staining pink. Cytoplasm stained whitish-blue to purple with an irregular, dark-purplish staining nucleus (Figure 4.3.1: A-D). A more detailed description of this species as in will be provided in the following chapter.

Type host: ***Amietophrynus garmani*** prevalence: 3/23 (12%), parasitaemia: 6% of the erythrocytes parasitised.

Other hosts:

Amietophrynus maculatus prevalence: 7/9 (78%), total mean parasitemia: 19.4% of the erythrocytes parasitised. Trophozoite, meront, merozoite, immature and mature gamont stages were observed.

***Hemisus marmoratus* (Peters, 1854)** prevalence: 2/22 (9%), mean parasitemia: 0.9%. Immature gamont stages were observed (Figure 4.3.1: C).

***Phrynobatrachus mababiensis* FitzSimons, 1932** prevalence: 1/13 (8%), parasitemia: 0.3%. Only immature gamont stages were observed (Figure 4.3.1: C).

Ptychadena mascareniensis prevalence: 1/5 (20%), mean parasitemia: 0.2%. Only immature stages were observed (see Figure 4.3.1).

***Ptychadena mossambica* (Peters, 1854)** prevalence: 4/7 (57%), mean parasitemia: 3.4%. One of the frogs was parasitised with what seemed to be possible trophozoite stages, with a parasitemia of 2%. The body has an irregular to ovoid shape, often with a vacuolated cytoplasm. The nucleus had loosely arranged chromatin, staining pink to purple. No other stages were observed in this individual (see Figure 4.3.1).

***Schismaderma carens* (Smith, 1848)** prevalence: 2/7 (29%), parasitemia: 0.8% (see Figure 4.3.1).

***Hepatozoon* sp. (B)**

This parasite is slender, and elongate, often forming a short bulged tail at the posterior pole. The cytoplasm stained pinkish-purple. The irregularly shaped nucleus stained dark pink and is situated centrally, often extending the width of the parasite. In some cases the host cell was expanded and dehaemoglobinised (Figure 4.3.1: E-H).

Type host: ***Ptychadena anchietae*** prevalence: 31/47 (66%) (out of a total of 78), mean parasitemia: 2.2%.

Other hosts: This haemogregarine was found parasitising only *P. anchietae*.

***Hepatozoon* sp. (C)**

This parasite has an oval-round shape with a well-developed cap/cavity at one pole. Cytoplasm stained light blue. The irregularly shaped nucleus stained dark blue and is situated closer to the pole opposite to the cap/cavity.

Type host: ***Hildebrandtia ornata* (Peters, 1878)** prevalence: 1/6 (17%), mean parasitemia: 0.3% of the erythrocytes parasitised.

Other hosts: This haemogregarine was found parasitising only *H. ornata* (Figure 4.3.1: I).

***Dactylosoma* sp. (A)**

Two distinct stages of merogony were present in the erythrocytes and occasionally in the thrombocytes of infected frogs. The first stage is characterised by large meronts possibly producing between four and 16 merozoites. The second stage meront was seemingly smaller, with the likelihood of producing up to six merozoites. Unfortunately no late stages of development were found in both first and second stage meronts, as in Barta *et al*. (1987) (see Figure 4.3.2: C-H).

Type host: ***Ptychadena anchietae*** prevalence: 10/47 (21%), mean parasitemia: 0.7% of the erythrocytes parasitised.

Other hosts: This haemogregarine was found parasitising only *P. anchietae*.

Unknown-rickettsiae sp. (A)

An unknown intraerythrocytic rickettsiae-like organism (possibly *Aegyptianella* or *Hemobacterium* species). The parasite is slender, and elongated with a slightly bent body, extending the entire width of the erythrocyte. The cytoplasm stained a dark purple with Giemsa stain (see Figure 4.3.2: B).

Type host: ***Ptychadena anchietae*** prevalence: 1/47 (2%), parasitemia: 0.5%.

Other hosts: This species was found parasitising only *P. anchietae*.

Frog Erythrocytic Virus (FEV) sp. (A)

These parasites appeared as circular inclusions staining purple, observable in the infected erythrocytes. These circular inclusions seem to be concurrent with a strange enveloped albuminoid vacuole within the host cell cytoplasm (see Figure 4.3.2: I-J).

Type host: ***Ptychadena anchietae*** prevalence: 3/47 (6%), mean parasitemia: 66.7%.

Other hosts: This FEV was found parasitising only *P. anchietae*.

Frog Erythrocytic Virus (FEV) sp. (B)

Small single circular inclusions, staining pink, were observed in large numbers of erythrocytes. Infected erythrocytes contained a seemingly disrupted cytoplasm with abruptly distinct light and dark areas (albuminoid vacuoles), and the host cell nucleus was displaced to the side.

Type host: ***Ptychadena mossambica* (Peters, 1854)** prevalence: 2/7 (29%), parasitemia: 99%.

Other hosts: This FEV was found parasitising only *P. mossambica*.

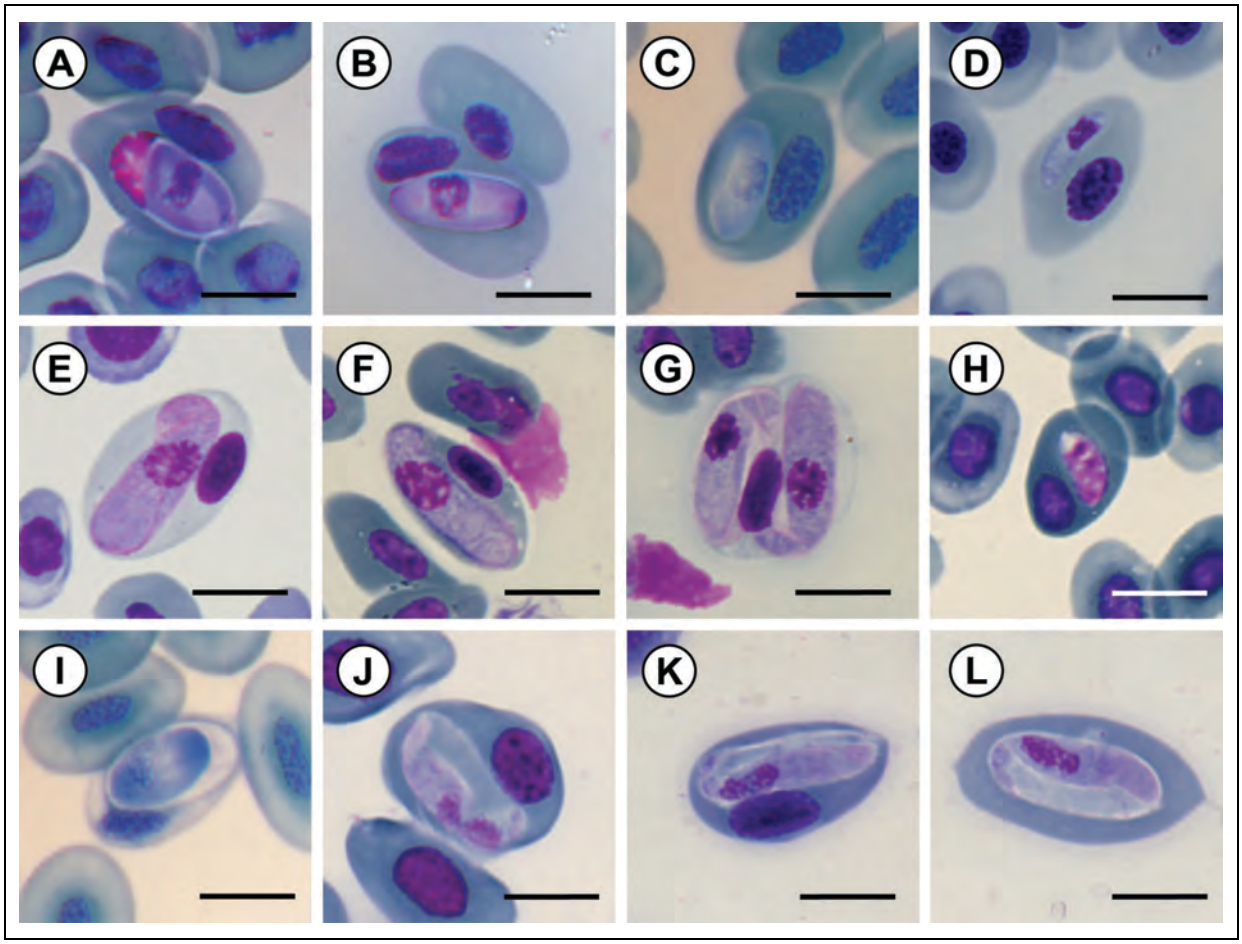


Figure 4.3.1: *Hepatozoon* species (A-L) observed in the peripheral blood of various frog species.

A-D: *Hepatozoon* sp. (A), was found in eight different frog species, and seems to be a cosmopolitan anuran parasite species. **A:** Mature gamont stages were only observed in *A. garmani*, *A. gutturalis*, and *A. maculatus.* **B-C**: Immature gamont stages of *Hepatozoon* sp. (A), were observed in *A. garmani*, *A. gutturalis*, *A. maculatus*, *H. marmoratus*, *P. mababiensis*, *P. mascareniensis*, *P. mossambica*, and *S. carens*. **D**: Possible trophozoite stages of *Hepatozoon* sp. (A). **E-H**: *Hepatozoon* sp. (B), was a commonly observed blood parasite, only found in *P. anchietae*. **H**: Possible trophozoite stages of *Hepatozoon* sp. (B). **I**: *Hepatozoon* sp. (C) seems to be a rarely found parasites and was only observed in one (16.7%) out of six *H. ornata* examined **J-K**: *Hepatozoon* sp. (D) was only observed in *H. marmoratus taeniatus* from the Kwa-Nyamazane Conservancy, KZN. Scale bars: 10µm.

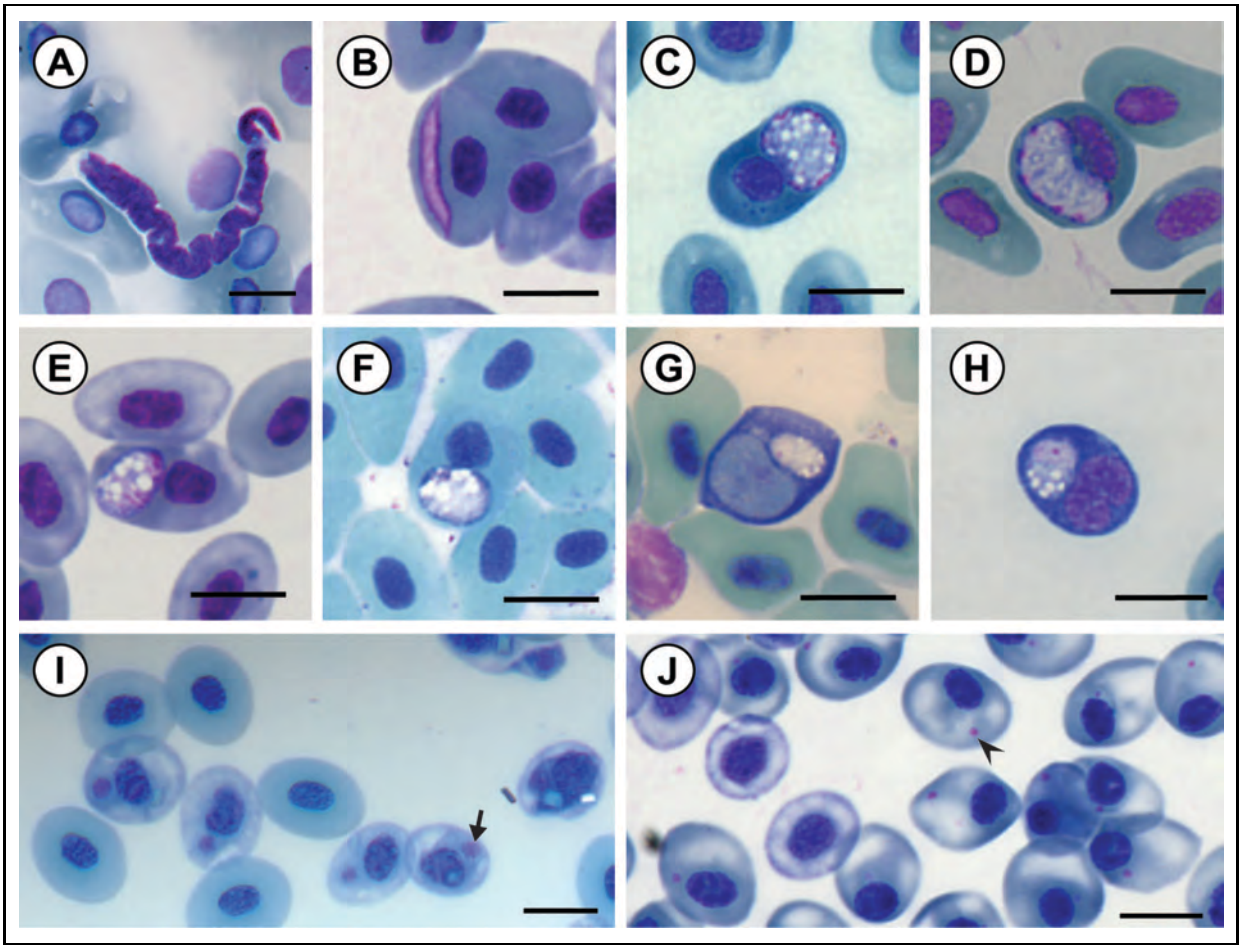


Figure 4.3.2: Various blood parasite species (A-J) observed in the peripheral blood of various frog species. A: Microfilaria sp. (A) Filarial nematode observed in the blood *S. carens*. **B**: unknown-rickettsiae sp. (A) observed in the erythrocytes of a single *P. anchietae* in the NGR. **C-H**: *Dactylosoma* sp. (A) observed in the blood of *P. anchietae* **I** FEV sp. (A) were found to infect *P. anchietae* **J**: FEV sp. (B) were found to infect *P. mossambica* Scale bars: 10µm.

Extracellular

***Trypanosoma* sp. (A)**

This elongated trypanosome contains a centrally placed nucleus closer to the anterior pole, and a slightly vacuolated cytoplasm. The kinetoplast is situated closer to the anterior end, with a slight undulating membrane observable extending the length of the organism. The flagellum extends slightly from the anterior pole, where the undulating membrane is slightly more defined (Figure 4.3.3: A,B).

Type host: ***Amietophrynus garmani*** prevalence: 1/23 (4%), parasitemia: 2/ps.

Other hosts:

***Kassina maculata* (Duméril, 1853)** 1/8 (13%), parasitemia: of 3/ps.

Ptychadena anchietae 4/29 (14%), mean parasitemia: 10.5/ps.

***Trypanosoma* sp. (B)**

This rather large, elongated trypanosome has a centrally placed nucleus extending the width of the parasite. The cytoplasm is coarsely granulated from the nucleus towards the posterior pole, and striated from the nucleus towards the anterior pole. The kinetoplast is situated close to the nucleus on the posterior half of the parasite. The undulating membrane is well-defined across the anterior half of the parasite (Figure 4.3.3: C).

Type host: ***Amietophrynus garmani*** prevalence: 1/23 (4%), parasitemia: 40/ps.

Other hosts: ***Ptychadena anchietae*** 3/29 (10%), mean parasitemia: 4.3/ps.

***Trypanosoma* sp. (C)**

This species is broad to narrow with a fan-like shape, and is slightly granulated, with rather thick striated bands extending into a well-defined and developed undulating membrane. The kinetoplast was situated in the centre of the cytoplasm, and in some cases the flagellum was visible (Figure 4.3.3: D-E).

Type host: ***Amietophrynus maculatus*** prevalence: 1/7 (14%)**,** parasitemia: 2/ps.

Other hosts:

***Phrynomantis bifasciatus* (Smith, 1847)** prevalence: a single individual of this species, parasitemia: 2/ps.

Ptychadena anchietae prevalence: 2/29 (7%), parasitemia: 2/ps.

***Trypanosoma* sp. (D)**

The body tapered towards the posterior pole, with a dark stained cytoplasm. A well-developed undulating membrane was clearly visible and extended from the centre of the body to the anterior

pole. Sometimes a relatively long flagellum could be observed. The kinetoplast was situated in the middle of the body, and closer to the undulating membrane (Figure 4.3.3: G-H).

Type host: ***Amietophrynus maculatus*** prevalence: 3/7 (43%)**,** mean parasitemia: 2.3/ps.

Other hosts:

***Phrynobatrachus mababiensis* FitzSimons, 1932** prevalence: 1/13 (8%), parasitemia: 8/ps.

***Phrynomantis bifasciatus* (Smith, 1847)** prevalence: a single individual of this species, parasitemia: 4/ps (see Figure 4.3.3).

Ptychadena anchietae prevalence: 19/29 (66%), mean parasitemia: 7.8/ps.

Ptychadena mascareniensis prevalence: 2/5 (40%), mean parasitemia: 8.5/ps (see Figure 4.3.3).

***Ptychadena mossambica* (Peters, 1854)** prevalence: 3/4 (75%), mean parasitemia: 2/ps.

***Trypanosoma* sp. (E)**

The body is slender and elongated, with a granulated cytoplasm, and a slightly visible undulating membrane. The kinetoplast is situated closer to the posterior end, with a centrally placed nucleus extending the width of the body. Although the flagellum was not observed, it did not exclude the possibility of there being one.

Type host: ***Chiromantis xerampelina* Peters, 1854** prevalence: 1/43 (2%), parasitemia: 26/ps.

Other hosts: This trypanosome species was only recorded in *C. xerampelina* (Figure 4.3.3: I).

***Trypanosoma* sp. (F)**

The body of this parasite is large and extended, with a coarsely granulated cytoplasm, and well-defined indents from the undulating membrane. The kinetoplast is situated closer to the posterior end, and the flagellum was not observable (Figure 4.3.3: J-K).

Type host: ***Hyperolius marmoratus* Rapp, 1842** prevalence: 1/43 (2%), parasitemia: very low with only 1/ps.

Other hosts:

Ptychadena anchietae prevalence: 3/29 (10%), parasitemia: 3.7/ps.

***Ptychadena mossambica* (Peters, 1854)** prevalence: 1/4 (25%), parasitemia: 14/ps.

***Trypanosoma* sp. (G)**

The body is fairly large, flattened, and circular in outline. The cytoplasm is both granular and striated. The nucleus is centrally positioned, along with the kinetoplast. The undulating membrane cut across the surface of the parasite without a visible flagellum (Figure 4.3.3: L-M).

Type host: ***Hyperolius tuberilinguis* Smith, 1849** prevalence: 1/12 (8%), parasitemia: 20/ps.

Other hosts:

Ptychadena anchietae prevalence: 10/29 (34%), mean parasitemia: 13/ps.

***Ptychadena mossambica* (Peters, 1854)** prevalence: 1/4 (25%), parasitemia: 4/ps (see Figure 4.3.3).

***Trypanosoma* sp. (H)**

The body is broad with a frilly outline as a result of the undulating membrane, which appeared to extend the length of the parasite. The cytoplasm is finely granulated. The kinetoplast is situated closest to what seemed to be the anterior pole of the parasite.

Type host: ***Kassina maculata* (Duméril, 1853)** prevalence: 1/8 (13%), parasitemia: 1/ps.

Other hosts: This trypanosome species was recorded in only *K. maculata* (Figure 4.3.3: N).

***Trypanosoma* sp. (I)**

The body is oddly shaped with a granulated cytoplasm, tapering towards the pole opposite the undulating membrane. The undulating membrane is located along the top pole with a sharp pointed tail-like protrusion to the side, from where the flagellum is extended. Both the nucleus and the kinetoplast were centrally positioned (Figure 4.3.3: O-P).

Type host: ***Ptychadena anchietae*** prevalence: 3/29 (10%), mean parasitemia: 14/ps.

Other hosts: This trypanosome species was recorded in only *P. anchietae.*

Microfilaria sp. (A)

These are large elongate worms, with a coarsely granulated body, which stains dark-purple (Figure 4.3.2: A).

Type host: ***Schismaderma carens* (Smith, 1848)** prevalence: 1/7 (14%), parasitaemia: 28/ps.

Other hosts: ***Ptychadena anchietae*** prevalence: 1/47 (2.1%), mean parasitemia: 1/ps.

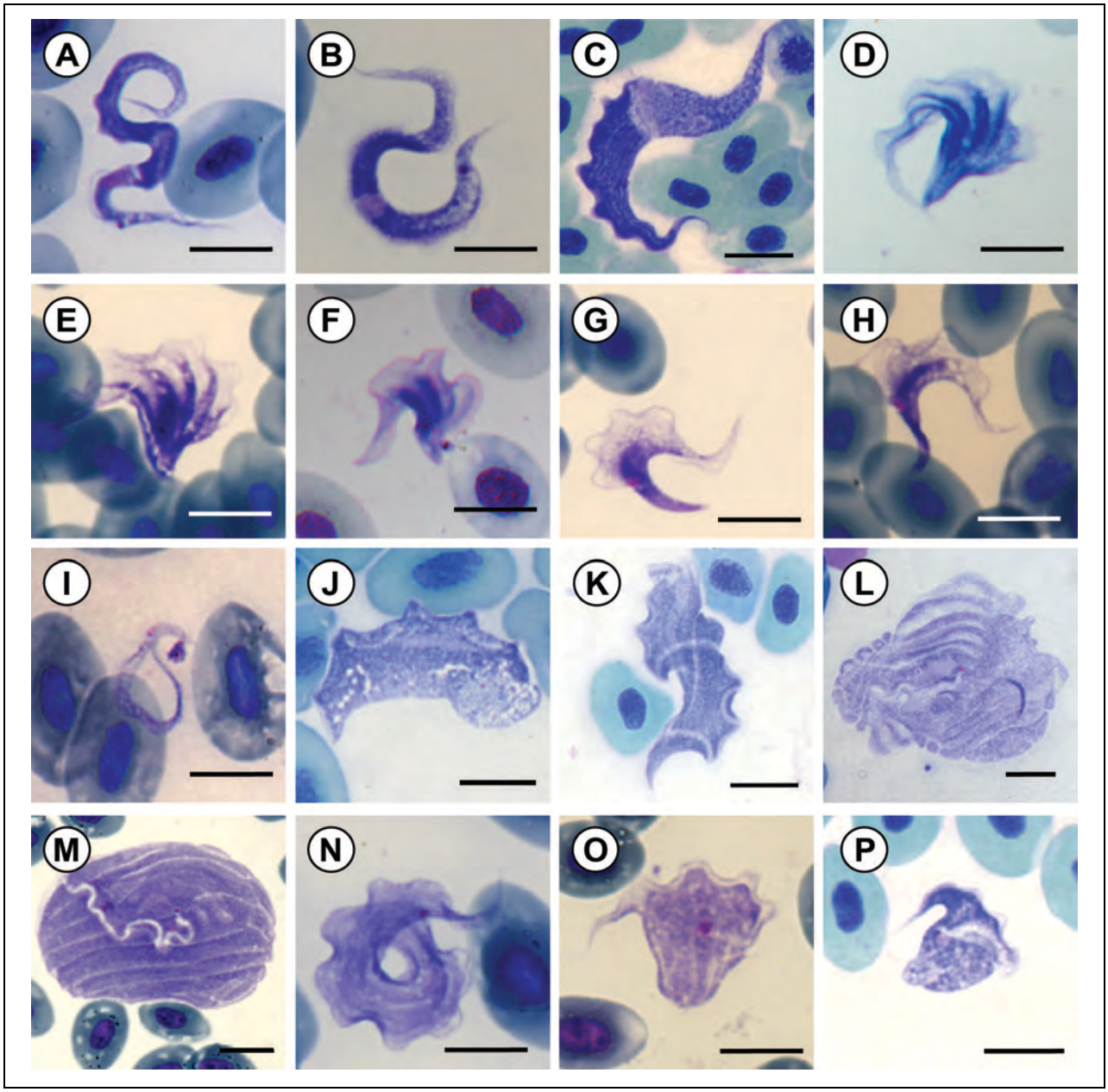


Figure 4.3.3: *Trypanosoma* species (A-P) observed in the peripheral blood of the various frog species. **A-B**: *Trypanosoma* sp. (A) was observed in *A. garmani*, *K. maculata*, and *P. anchietae*. **C**: *Trypanosoma* sp. (B) was observed in only two species, namely *A. garmani* and *P. anchietae*. **D-F:** *Trypanosoma* sp. (C) was observed in *A. maculatus*, *P. bifasciatus*, and *P. anchietae*. **G-H**: *Trypanosoma* sp. (D) was the most abundantly observed trypanosome and was found in *A. maculatus*, *P. mababiensis*, *P. bifasciatus*, *P. anchietae*, *P. mascareniensis*, and *P. mossambica*. **I**: *Trypanosoma* sp. (E) was only observed in a single specimen of *C. xerampelina* out of 43. **J-K**: *Trypanosoma* sp. (F) was found in *H. marmoratus*, *P. anchietae*, and *P. mossambica*. **L-M**: *Trypanosoma* sp. (G) was observed in three different species, namely *H. tuberilinguis*, *P. anchietae*, *P. mossambica*. **N** *Trypanosoma* sp. (H) was only observed in one out of eight individuals of *K. maculata*. **O-P** *Trypanosoma* sp. (I) this commonly encountered species was only observed in *P. anchietae*. Scale bars: 10µm.

Table 4.3.1: On the prevalence, diversity and parasitemia of blood parasites infecting frogs in the NGR.

Frog Species	Frog Prevalence	*Hepatozoon* spp.		*Trypanosoma* spp.		Microfilariae spp.		Parasites of uncertain identity		Viral or viral-like infections	
		Pre	Par (%)	Pre	Pa (p/s)	Pre	Par (p/s)	Pre	Par (%)	Pre	Par (%)
Afrixalus aureus	14										
Afrixalus delicatus	7										
Afrixalus fornasinii	2										
Amietophrynus garmani	23	1	6	2	21						
Amietophrynus gutturalis	1										
Amietophrynus maculatus	9	7	19.4	3	3						
Breviceps adspersus	4										
Chiromantis xerampelina	43			1	26						
Hemisus marmoratus	22	2	0.8								
Hildebrandtia ornata	6	1	0.3								
Hyperolius marmoratus	20			1	1						
Hyperolius pusillus	10										
Hyperolius tuberilinguis	12			1	20						
Kassina maculata	8			1	4						
Leptopelis mossambicus	2										
Phrynobatrachus mababiensis	13	1	0.0003	1	8						
Phrynomantis bifasciatus	1			1	6						
Ptychadena anchietae	77	31	2.4	29	13.3	1	1	13	1	4	66.7
Ptychadena mascareniensis	5	1	0.0002	2	8.8						
Ptychadena mossambica	19	5	3	4	6					2	99
Schismaderma carens	7	1	0.8			1	28				
Tomopterna cryptotis	6										
Tomopterna krugerensis	1										
Tomopterna natalensis	1										
Xenopus laevis laevis	1										
Xenopus muelleri	46										
Total: 14/26	**360**	**50**	**0.7**	**46**	**2.5**	**2**	**14.5**	**13**	**0.1**	**6**	**29**

Prevalence = Pre; parasitaemia = Par; per slide = p/s

4.3.3 Frog blood parasites outside the Ndumo Game Reserve

No frogs (n=54) were found to be infected in the area bordering the NGR, however those in the KNC (n=22) had the following (see Table 4.3.2):

***Hepatozoon* sp. (A)**

See above for description.

Type host: ***Amietophrynus garmani***

Hosts in this locality:

Amietophrynus garmani prevalence: 4/7 (57%), mean parasitemia: 2.4% (see Figure 4.3.1).

Amietophrynus gutturalis prevalence: 3/3 (100%), mean parasitemia: 8.3% (see Figure 4.3.1).

***Hepatozoon* sp. (D)**

This large parasite was enclosed in a parasitophorous vacuole with an ovoid shape. Excluding the parasitophorous vacuole the parasite inside often appeared to have a small protruding tail (Figure 4.3.1: K-L) although in some cases the parasite seemed to be bent at both poles (Figure 4.3.1: J). Cytoplasm stained pinkish-purple. The irregularly shaped nucleus stained dark purple and was situated closer to the allocated posterior pole, often extending the width of the parasite. The host cell nucleus was displaced to one side (Figure 4.3.1: J-L).

Type host: ***Hyperolius marmoratus*** prevalence: 3/16 (19%), mean parasitemia: 20.5%.

Other hosts: This haemogregarine was found parasitizing only *H. marmoratus taeniatus.*

Table 4.3.2: On the prevalence, diversity and parasitemia of blood parasites infecting frogs outside the NGR.

Frog Species	Frog Prevalence	*Hepatozoon* spp.		*Trypanosoma* spp.		Microfilariae spp.		Parasites of uncertain identity		Viral or viral-like infections	
		Pre	Par (%)	Pre	Par (p/s)	Pre	Par (p/s)	Pre	Par (%)	Pre	Par (%)
Amietophrynus garmani	7	4	2.4								
Amietophrynus gutturalis	3	3	8.3								
Cacosternum boettgeri	12										
Chiromantis xerampelina	1										
Hyperolius argus	24										
Hyperolius marmoratus	16	3	20.5								
Hyperolius pusillus	4										
Kassina senegalensis	3										
Ptychadena anchietae	1										
Ptychadena mascareniensis	2										
Xenopus muelleri	3										
Total: 3/11	**76**	**10**	**3.1**								

Prevalence = Pre; parasitaemia = Par; per slide = p/s

4.3.4 Remarks

Of all the frog species examined in this study *P. anchietae* harboured the highest diversity of parasite genera. This diversity included one species each of *Hepatozoon*, *Dactylosoma*, *rickettsia-like* parasite and FEV (all unique to *P. anchietae*); as well as seven *Trypanosoma* species (one of which was unique to *P. anchietae*).

Other frog species found to host unique parasites included *H. ornata*, parasitemic with a *Hepatozoon* species, *P. mossambica*, with a FEV parasite, *C. xerampelina* and *K. maculata* with a *Trypanosoma* species respectively, *S. carens* with a microfilarid species, and *H. m. taeniatus* parasitised with a *Hepatozoon* species.

Cosmopolitan parasite species (those infecting more than three host species) included one species of *Hepatozoon*, *Hepatozoon* sp. A (infecting seven of the 29 species), and five trypanosome species *Trypanosoma* A, C, D, F and G (infecting 10 of the 29 species). This diversity in trypanosome species, however, as mentioned previously, may not be an accurate interpretation, some of them possibly representing different life stages instead of different species.

4.3.5 Statistical analysis

Hepatozoon species accounted for most of the infections at 59/436 (14%), followed by *Trypanosoma* species at 46/436 (11%); viral or bacterial infections, microfilarid infections and *Dactylosoma* species, accounted for 6/436 (1%), 2/436 (0.5%) and 13/436 (3%) of the overall prevalence respectively (Table 4.3.3). As for the intensity of the groups, *Hepatozoon* showed an overall (all infected frogs pooled) intensity of 5%, the *Dactylosoma* an overall intensity of 1%, the viral or bacterial infections an overall intensity of 87%, the *Trypanosoma* an overall intensity of 11 per blood slide, and the microfilarid nematode infections an overall intensity of 15 per blood slide (Table 4.3.3). The overall prevalence of haemoparasites (all parasite groups pooled) varied significantly by frog species (χ^2 = 163.475, P < 0.01, Fisher's exact test). *Ptychadena anchietae* demonstrated the highest prevalence at 47/78 (60%) and *Chiromantis xerampelina* the lowest at 1/44 (2%) (Table 4.3.3). Upon division of the frog species into groups including aquatic, semi-aquatic, terrestrial and semi-terrestrial, it was observed that only the semi-aquatic and semi-terrestrial groups contained infected species (Table 4.3.3). These two groups varied significantly in prevalence of infection (χ^2 = 87.000, P < 0.01, Fisher's exact test), with 79% of the infected individuals from the semi-aquatic group and only 21% from the semi-terrestrial group. Of the semi-aquatic group, the genus *Ptychadena* had the highest diversity of haemoparasites, infected with all types as recorded in Table 4.3.3. Furthermore, *P. anchietae*, of all the infected frog species, revealed the highest prevalence of parasites, making up 47/87 (54%) of the total with 47/78 (60%) of the *P. anchietae* themselves found to be infected.

Hepatozoon species accounted for most of the infections followed by *Trypanosoma* species, significance of intensity calculated via the use of the Kruskal-Wallis test. *Hepatozoon* intensity across frog species (χ^2 = 17.683, P = 0.028), across families (χ^2 = 11.717, P = 0.006), and across the different habitat types (χ^2 = 7.227, P = 0.007) showed a significant difference. *Hyperolius marmoratus*, in the semi-aquatic group, and *Amietophrynus maculatus*, in the semi-terrestrial group, accounted for the highest intensities (Table 4.3.3). *Hepatozoon* intensity across the different sampling periods,

however, showed no significant variance (χ^2 = 4.177, *P* = 0.552). *Trypanosoma* intensity across frog species (χ^2 = 11.919, *P* = 0.028) showed a significant difference, however, across families (χ^2 = 3.802, *P* = 0.664), habitat types (χ^2 = 0.330, *P* = 0.585) and sampling periods (χ^2 = 6.675, *P* = 0.147), no significant difference was observed. In this case *Hyperolius tuberilinguis*, in the semi-aquatic group, and *Chiromantis xerampelina*, in the semi-terrestrial group, accounted for the highest intensities (Table 4.3.3).

Per locality, it was observed that the NGR, with 26 species examined, showed a prevalence of 77/360 (21%) as compared to outside the NGR, with 8 species examined and a prevalence of 0/54 (0%), and the KNC, with seven species examined and a prevalence of 10/22 (45%). Furthermore, the NGR had a higher diversity of haemoparasites, including 50/360 (14%) infected with *Hepatozoon* species, 11/360 (3%) with *Dactylosoma* species, 5/360 (1%) with viral or viral-like organisms, 46/360 (13%) with *Trypanosoma* species and 2/360 (0.6%) with microfilaria as compared to the KNC frogs that were only infected with *Hepatozoon*.

Table 4.3.3: All infected frog species listed alphabetically and categorized according to their habitat type. Shown are the number of frogs examined and infected, prevalence of the five haemoparasite groups (Pr) and the parasitemia of the infections (Pa or p/s).

Habitat type	Frog species	Examined	Infected	*Hep.* spp.	*Dacty.* spp.	Viral or viral-like organisms	*Tryp.* spp.	Microfi.
				Pr; Pa	**Pr; Pa**	**Pr; Pa**	**Pr; Pa**	**Pr; Pa**
Semi-terrestrial	*Amietophrynus garmani*	30	7	5/7; 8.4%			2/7; 21 p/s	
	Amietophrynus gutturalis	4	3	3/8; 2.4%				
	Amietophrynus maculatus	9	7	7/7; 19.5%			3/7; 3 p/s	
	Chiromantis xerampelina	44	1				1/1;26 p/s	
	Schismaderma carens	7	2	1/2; 0.8%				1/2; 28 p/s
Semi-aquatic	*Hemisus marmoratus*	22	2	2/2; 0.8%				
	Hildebrandtia ornata	6	1	1/1; 0.3%				
	Hyperolius marmoratus	36	4	3/4; 20.5%			1/4; 1 p/s	
	Hyperolius tuberilinguis	12	1				1/1; 20 p/s	
	Kassina maculata	8	1				1/1; 4 p/s	
	Phrynobatrachus mababiensis	13	1	1/1; 0.3%			1/1; 8 p/s	
	Phrynomantis bifasciatus	1	1				1/1; 6 p/s	
	Ptychadena anchietae	78	47	31/47; 2.4%	13/47; 1%	4/47; 75%	29/47; 13.3 p/s	1/47; 1 p/s
	Ptychadena mascareniensis	5	2	1/2; 0.2%			2/2; 8.8 p/s	
	Ptychadena mossambica	19	7	5/7; 3%		2/7; 99%	4/7; 6 p/s	
Total	**15**	**294**	**87**	**59; 5.3%**	**13; 1%**	**6; 87%**	**46; 10.6 p/s**	**2; 14.5 p/s**

Prevalence = P; parasitaemia = Pa; per slide = p/s ; *Hepatozoon* = Hep.; *Dactylosoma* = Dact.; *Trypanosoma* = Tryp.; Microfilariae = Microfi.

4.4

DISCUSSION

Among the recorded anuran blood parasite observed in and around the NGR, KZN, 20% of the frogs were infected with some harbouring representatives of all five. This was similar to previous comparable studies such as that of Readel & Goldberg (2010) in western Uganda documenting a 17% (30/180) prevalence, even though this was found to be approximately half that of other studies in Africa (see Readel & Goldberg 2010). The only blood parasites recorded outside the reserve were found in frogs collected in another protected area, the KNC, KZN, very possibly indicating the negative effect that anthropogenic influences may have on the natural ecological order at large. The apicomplexan parasites, specifically of the genera *Hepatozoon,* were the most prevalent with 60 out of the total 436 (14%) individuals examined found positive. This was equally true for the survey done in Uganda by Readel & Goldberg (2010). On the contrary, Ball (1967), during a survey completed in Tanzania and Kenya, found a considerably higher prevalence of 29%. Readel & Goldberg (2010) suggested this may be attributable to availability of insect vectors. The second most prevalent were the flagellates, specifically the trypanosomes, with 46 parasitised out of the 436 (11%) individuals examined. This was yet another comparable finding, just slightly higher than the 6% reported by Readel & Goldberg (2010). Both the present and the Readel & Goldberg (2010) studies' results are in contrast to what has been recorded in other similar studies (see Barta & Desser 1984; Barta *et al.* 1989), in which the *Trypanosoma* demonstrate a higher prevalence to that of *Hepatozoon*. Microfilarid infections from the South African frogs studied here, were also seen to occur in low numbers, similar to Readel & Goldberg (2010). Infections not reported by Readel & Goldberg (2010) and Ball (1967), but reported in this study, were a *Dactylosoma* species and viral or viral-like/rickettsial infections.

These and the rest of the observations will be discussed below with respect to the individual parasite genera, followed by a discussion on the statistical findings.

Apicomplexa

From within the Apicomplexa, only genera from the families Hepatozoidae and Dactylosomatidae were observed. The haemogregarines were the most prevalent blood parasite recorded, with four

seemingly morphologically distinct *Hepatozoon* species. Three of the four were found in the NGR, and two of the four were found in the KNC, KZN.

Of the *Hepatozoon* species found, *Hepatozoon* sp. (A) was found to be the most abundant and was the only species found inside and outside the reserve. It was found in eight different frog species, namely *Amietophrynus garmani*, *Amietophrynus gutturalis*, *Amietophrynus maculatus*, *Hemisus marmoratus*, *Phrynobatrachus mababiensis*, *Ptychadena mascareniensis*, *Ptychadena mossambica*, and *Schismaderma carens*. On the other hand *Hepatozoon* sp. (B), which was also a commonly observed blood parasite, was only found in *Ptychadena anchietae* within the NGR and seems to be host specific to this frog species. From the 78 *P. anchietae* examined, 31 (40.3%) were found to contain this parasite species. *Hepatozoon* sp. (C) seems to be a rarely found parasite and was observed in only one (16.7%) out of six *H. ornata* to be examined in the NGR. The only haemogregarine that was not found infecting any of the frog species inside the NGR was *Hepatozoon* sp. (D). This species is morphologically quite distinct in comparison to the other *Hepatozoon* species observed, and is characterised by its large transparent parasitophorous vacuole. *Hepatozoon* sp. (D) was observed in only three of the total 36 *H. marmoratus taeniatus* examined in this study. *Hepatozoon* sp. (D) was observed in only the KNC, where this parasite was discovered. Prevalence was high with 3/6 (50%) of the individuals (*H. marmoratus taeniatus*) found to be parasitised with *Hepatozoon* sp. (D). The mean parasitemia of the three infected frogs, was the highest of the entire survey totalling 20.5%. This may be due to the fact that these small frogs feed primarily on small invertebrates, such as possible mosquito vectors (du Preez & Carruthers 2009).

From the family Dactylosomatidae, a blood parasite species with similar morphological characteristics to the genera *Dactylosoma* was observed in the blood of *P. anchietae*. Due to the different life stages of these parasites in the blood, it is almost impossible to distinguish between species on a morphological level, thus all life stages observed within the peripheral blood films were preliminarily presumed those of a single species and designated *Dactylosoma* sp. (A).

Bacteria

One unknown rickettsiae-like organism (possibly *Aegyptianella* or *Hemobacterium* species) was observed in the erythrocytes of a single *P. anchietae* in the NGR. This strange bacterium was designated unknown-rickettsiae sp. (A), and further ultrastructural and molecular analyses will be needed to correctly classify this rickettsiae-like organism due to the fact that it contains very little morphological characteristics (see Figure 4.3.2 B).

Viruses

Frog Erythrocytic Virus (FEV) was only found in specimens of *P. anchietae* and *P. mossambica* in the NGR. Of the 78 examined only three individuals of *P. anchietae* were found to be infected with FEV sp. (A). Parasitemia varied between 50-99% of the erythrocytes infected. Frog Erythrocytic Virus sp. (B) was found in two of the seven *P. mossambica* individuals examined. Frog Erythrocytic Virus sp. (A) and FEV sp. (B) were morphologically very different at a superficial level. Frog Erythrocytic Virus sp. (A) seemed to develop around the host cell nucleus, compared to FEV sp. (B) which displaced the host cell nucleus to the side (see Figure 4.3.2: I-J). It is uncertain how FEV may be transmitted. Gruia-Garay *et al.* (1989) demonstrated that it is possibly transmitted to anuran hosts through interrupted feeding of amphibian-feeding Ceratopogonidae (biting midges). The classification of these iridovirus-like infections can only be completed through further ultrastructural and molecular analyses, and without these tools, categorising these organisms should be avoided (see Gruia-Gray *et al.* 1989; Telford & Jacobson 1993; Smith *et al.* 1994a).

Trypanosomes

Flagellate trypanosomes were observed in 11 (42.3%) of the 29 examined frog species. Trypanosomes were the second most prevalent blood parasite recorded, with nine morphologically distinct *Trypanosoma* species, seven of which were found within *P. anchietae*. Most of the observed trypanosome species seemed to be shared by a number of different host species, with the exception of *Trypanosoma* sp. (E), (H) and (I). This could be as a result of flying vectors, being able to more between different sites, and infecting various frog species. All of these species were found in the NGR (see Figure 4.3.3). It is possible that anthropogenic disturbances could influence the occurrence of these vectors outside the NGR

Microfilaria

Filarial nematodes were observed in the blood of two species, namely *P. anchietae* and *S. carens*. Both individuals were collected in the NGR. Unfortunately no adult nematodes were found, as these specimens were released prior to the blood smear examination. In order to correctly describe and classify these filarial nematodes, both sexes of the adult stages, as well as microfilarial stages, would have to be compared molecularly and morphologically described. These worms are assumed to be

transmitted through the feeding of a haematophagous arthropod vector on the host organism (see Baker 2008).

Statistical findings

Similar to Readel & Goldberg (2010) significant differences ($P < 0.01$) in the prevalence of parasites among frog species were recorded during the current study, with *P. anchietae* showing the highest prevalence and *C. xerampelina* the lowest. Both of these frog species prefer habitats close to water (classified in this study as being semi-terrestrial) (du Preez & Carruthers 2009). *Ptychadena anchietae* is a grass frog and is often found around the water's edge whilst *C. xerampelina* is an arboreal frog species. Since the abundance of possible vectors associated with water, such as mosquitoes and leeches, would be high in such habitats, it may explain the high prevalence of haemoparasites recorded from *P. anchietae*. The reason for such a low prevalence in *C. xerampelina*, particularly for *Hepatozoon* species, which may be mosquito transmitted in such an environment (Desser *et al.* 1995; Davies & Johnston 2000) can, as of yet not be explained. This result is particularly peculiar since both of these frog species were infected with trypanosomes (only a single individual of *C. xerampelina*), which are mosquito and leech transmitted (Barta & Desser 1984). One of the only possibilities could be, since *Hepatozoon* is transmitted via the ingestion of the infected invertebrate or vertebrate (Davies & Johnston 2000), that *C. xerampelina* prefers a diet not inclusive of mosquitoes and other frogs. Future diet studies may help to clarify this finding.

Division of the frog species into groups showed that only the semi-aquatic and semi-terrestrial groups contained infected species, these two groups varying significantly in prevalence of infection ($P < 0.01$). The semi-aquatic group had the highest prevalence, likely attributable to the *Ptychadena* species, one of them *P. anchietae*. The *Ptychadena* species also showed the highest diversity of haemoparasites, infected with all five groups. Furthermore, of all the frog species, *P. anchietae* was the only species to be parasitised with a species of *Dactylosoma*. These parasites are closely associated with water and thus are suggested to be transmitted by a leech vector (Barta 1991). Reports of *Dactylosoma* parasitising frogs in Africa are numerous, accounts of these organisms from at least five countries and approximately eight species of frog (see Barta 1991). One such report was from South Africa from the bufonid *A. regularis* (most likely *Amietophrynus gutturalis*) by Fantham *et al.* (1942). In all these reports the *Dactylosoma* species are referred to as a single species, *Dactylosoma ranarum* (see Barta 1991), however, only future molecular work will clarify if the variants here are conspecies. Furthermore, the Ptychadenidae were the only frogs found infected with viral or viral-like organisms. Viral and viral-like infections have been recorded from a

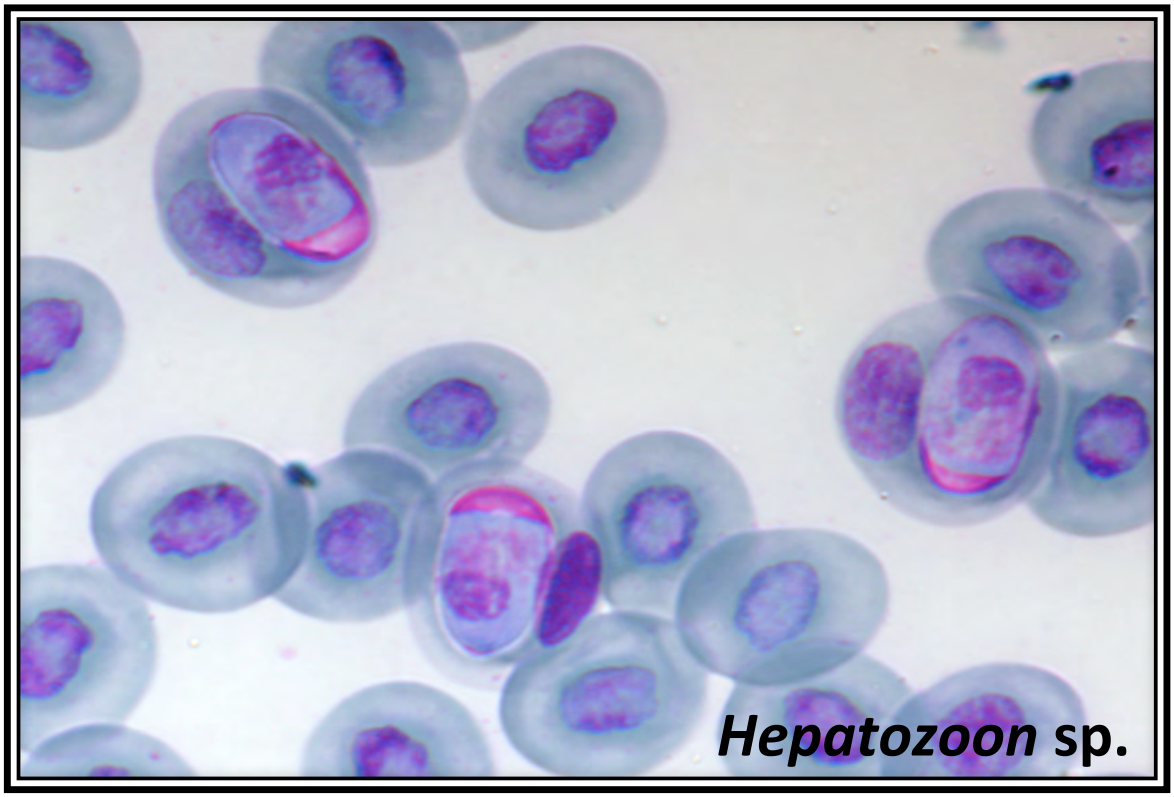
cosmopolitan distribution of amphibians (see Desser 1987). Unfortunately, very little is known about the identity, classification and effect of these organisms (see Desser 1987; Davies & Johnston 2000). Alves de Matos & Paperna (1993) presented the most recent study of uncertain erythrocyte virus infections from *P. anchietae* in South Africa. These virus-like infections were found to be similar to several different viruses of the Frog Erythrocytic Virus (FEV) group such as *Toddia*, *Pirhemocyton* and other rickettsia-like protozoans. In contrast to the above findings in both the semi-aquatic and semi-terrestrial groups, the frog species from the terrestrial as well as aquatic groups were not observably parasitemic. Since, *B. adspersus* (terrestrial) spends most of its life underground (du Preez & Carruthers 2009), contact with vectors would be rare. However, since the semi-aquatic group had the highest prevalence of parasites, most likely due to the frequent contact with vectors, it was expected that those of the aquatic group would be equally parasitised. Yet, as in Readel and Goldberg (2010), the species of *Xenopus* (aquatic) here were found to be uninfected.

Intensity of *Hepatozoon* species across frog species and families, as well as across habitat types were found to be significant ($P = 0.007$). *Hyperolius marmoratus* (Hyperolidae) of the semi-aquatic group, and *A. maculatus* (Bufonidae) from the semi-terrestrial group, had the highest intensity. *Hyperolius marmoratus* is closely associated with water, where vector abundance and contact rates are likely to be high, thus accounting for this high intensity. *Amietophrynus maculatus* appears to favour more static, shallow water bodies, which are also favoured by mosquito species, the high contact rates with possibly *Hepatozoon* infected mosquitoes would thus be high. *Hepatozoon* species have been reported and described from a few *Hyperolius* species in Africa, though the majority have been reported from bufonid species such as *A. maculatus*. *Trypanosoma* species intensities varied significantly only across species ($P = 0.028$), the highest *H. tuberilinguis* from the semi-aquatic group and the lowest *C. xerampelina* from the semi-terrestrial group. These two species are permanently associated with water, and thus always in close association with an abundance of possible vectors. A plethora of trypanosome species have been described and reported from numerous African frog species, unfortunately many reports contain inadequate taxonomic descriptions and in numerous cases they are simply referred to as a *Trypanosoma* sp. without any morphological data provided on the specific parasite (see Bardsley & Harmsen 1973; Telford 2009). Furthermore, since trypanosomes are known to be pleomorphic, the true diversity seen in this study cannot be realised, and thus future molecular work is needed to differentiate between species and life stages of these organisms. Intensity across sampling periods for both *Hepatozoon* and *Trypanosoma* species was insignificant ($P = 0.552$ and $P = 0.147$ respectively), likely due to sampling occurring only during the wet seasons.

NGR was found to harbour the highest diversity of haemoparasites as compared to the other two sites, which are impacted by rural village settlements and peripheral commercial sugar cane agriculture respectively. Anthropogenic impacts, as found in Readel & Goldberg (2010), may account for the lack of diversity in these two sites, affecting vector distributions and contact rates between frog hosts and vectors.

CHAPTER 5

HEPATOZOON: CASE STUDY



5.1

INTRODUCTION

Haemogregarines comprise a large group of apicomplexan blood parasites recorded from a wide range of tetrapod vertebrates and haematophagous invertebrates (Smith 1996; Davies & Johnston 2000). Haemogregarines are heteroxenous parasites and the group presently includes three families, namely the Haemogregarinidae Léger, 1911, Hepatozoidae Wenyon, 1926, and Karyolysidae Wenyon, 1926. Within these families there are six genera of blood parasites, differentiated on the sporogonic development in their invertebrate hosts (Telford 2009; Barta *et al.* 2012). Prior to the clarification of the haemogregarine life cycles in anuran hosts by Desser *et al.* (1995), most were placed in the genus *Haemogregarina* Danilewsky, 1885. However in 1996, with further insight into the above, Smith (1996) suggested that these haemogregarines were better suited to the genus *Hepatozoon* Miller, 1908 and thus transferred them accordingly. As a result, *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 and *Hepatozoon* are the only two haemogregarine genera with species known to parasitise anuran hosts (Davies & Johnston 2000), with the latter currently representing the most common intraerythrocytic protozoan parasites of anurans worldwide (Smith 1996).

According to Netherlands *et al.* (2014), the majority (11/15, 73%) of African anuran *Hepatozoon* species have been recorded from the family Bufonidae. Nine of the 11 (81%) species, namely *H. aegyptia* (Mohammed & Mansour, 1963), *H. assiuticus* (Abdel-Rahman, El–Naffar, Sakla & Khalifa, 1978), *H. boueti* (França, 1910), *H. faiyumensis* (Mansour and Mohammed, 1966b), *H. francai* (Abdel-Rahman, El-Naffar, Sakla & Khalifa, 1978), *H. froilanoi* (França, 1925), *H. lavieri* (Tuzet & Grjebine, 1957), *H. magni* (Hassan, 1992), and *H. pestanae* (França, 1910) Smith, 1996, were recorded from the same vertebrate host *Amietophrynus regularis* (Reuss, 1833) in Egypt, Sudan, Nigeria, Guinea-Bissau, the Congo, and from northern Angola (see França 1910; França 1925; Tuzet & Grjebine 1957; Mohammed & Mansour 1963; Mansour & Mohammed 1966a-b; Mohammed & Mansour 1966; Younis & Saoud 1969; Abdel-Rahman *et al*. 1978; Hassan 1992) (see Figure 5.1.1). The remaining two species, *H. tunisiensis* (Nicolle, 1904) described from *Amietophrynus mauritanicus* (Schlegel, 1841), and *H. moloensis* (Hoare, 1920) described from an unidentified species (likely *A. regularis*), were reported from Tunisia and Kenya respectively (Nicolle 1904; Hoare 1920)

(see Figure 5.1.1 and Table 5.3.2). These species descriptions range from 20 (1992) to more than 100 (1904) years ago and were entirely morphology-based. Furthermore, illustrations and measurements were not standardised and therefore inconsistent, and deposited voucher specimens were not mentioned in any of the descriptions and reports. With so many species described from the same host in largely the same area, the huge diversity of *Hepatozoon* species from the above two hosts may in fact be a false representation of what may truly exist.

The aim of this section of the present study is therefor to elucidate, via both traditional morphological description of peripheral blood stages and molecular techniques, the identity of the *Hepatozoon* species found to infect three *Amietophrynus* species from South Africa and to determine whether they represent a single species or three cryptic species, such as what has been recorded, solely morphologically, from other *Amietophrynus* species further north in Africa. According to the authors' knowledge the only record of an anuran haemogregarine from South Africa is *Hepatozoon theileri* (Laveran, 1905), described from the host *Amietia quecketti* (Boulenger, 1895), family Pyxicephalidae (see Netherlands *et al.* 2014). This chapter therefore presents the first morphological and molecular account of a *Hepatozoon* species parasitising members of the Bufonidae from South Africa, and establishes the phylogenetic basis for all other bufonid *Hepatozoon* species in Africa.

Furthermore, ultrastructural studies were included to attempt to explain the existence of the cap-like structure, described in detail in the following work, present in the gamont stage of many of these *Hepatozoon* species from African bufonids.

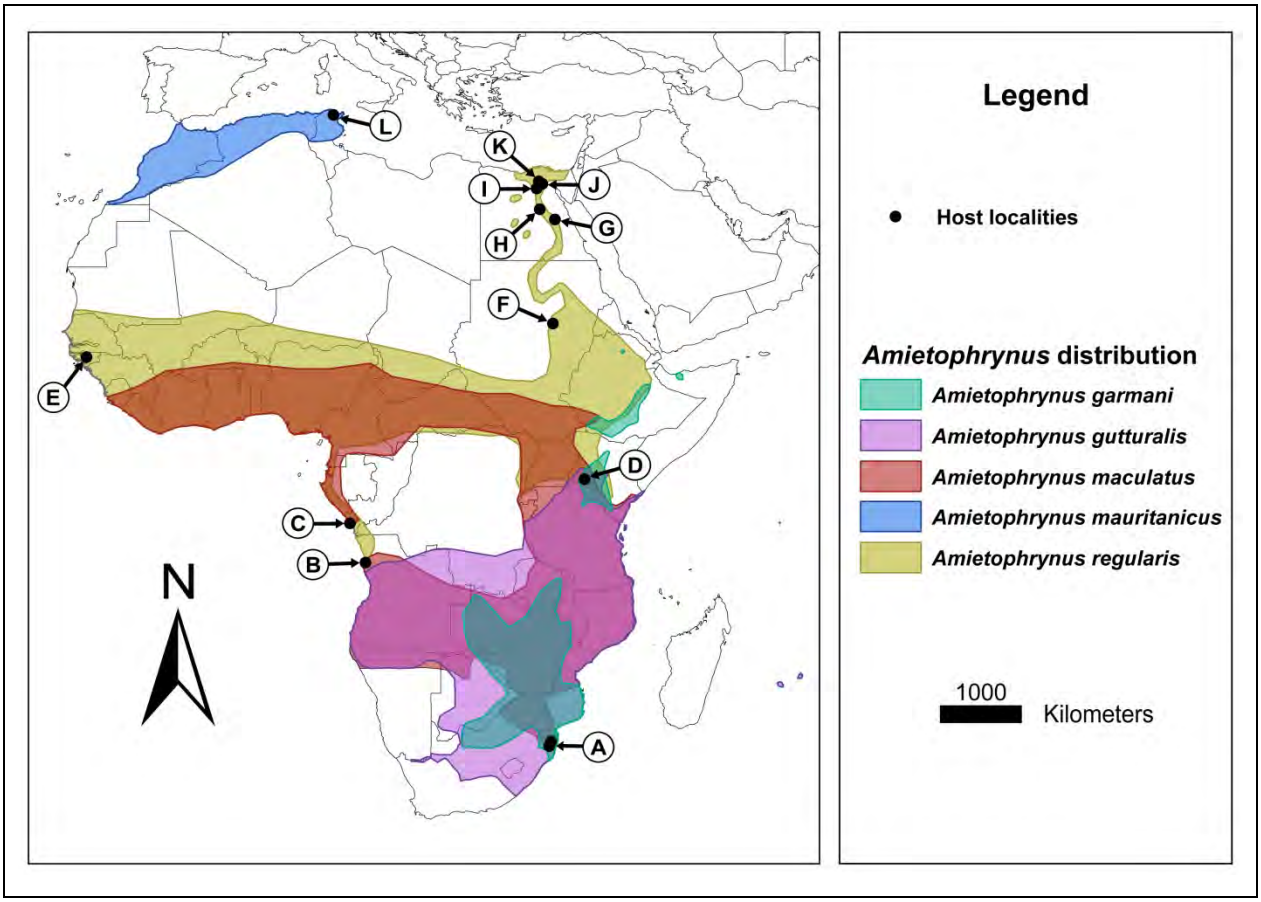


Figure 5.1.1: Map of Africa showing *Amietophrynus* species distribution pertaining to this study, with locality records of associated *Hepatozoon* species. The species *A. garmani*, *A. gutturalis*, *A. maculatus*, *A. mauritanicus* and *A. regularis* (Bufonidae) were found to be parasitised with various *Hepatozoon* species. **A:** Represents the hosts *A. garmani*, *A. gutturalis*, and *A. maculatus* infected with *Hepatozoon* sp. A. **B**: *A. regularis*, with *H. boueti* and *H. froilanoi*. **C**: *A. regularis*, with *H. lavieri*. **D**: unknown host with *H. moloensis*. **E**: *A. regularis*, with *H. boueti* and *H. pestanae*. **F**: *A. regularis*, with *H. aegyptia*. **G**: *A. regularis*, with *H. magni*. **H**: *A. regularis*, with *H. assiuticus* and *H. francai*. **I**: *A. regularis*, with *H. faiyumensis*. **J**: *A. regularis*, with *H. boueti* and *H. pestanae*. **K**: *A. regularis*, with *H. aegyptia* and *H. boueti*. **L**: *A. mauritanicus*, with *H. tunisiensis*. Map was created through ArcGIS 10.1 using spatial data downloaded from IUCN Red List of Threatened Species.

5.2

MATERIALS & METHODS

5.2.1 Frog collection and husbandry

Specimens of *Amietophrynus garmani* (Meek, 1897), *Amietophrynus gutturalis* (Power, 1927), and *Amietophrynus maculatus* (Hallowell, 1854) [21,22], were collected by hand at night in the NGR, North Eastern KZN, South Africa, from a total of eight sites. These include three temporary pans (26°51'54.5"S, 32°09'59.9"E; 26°53'51.6"S, 32°12'57.2"E; and 26°52'53.5"S, 32°15'03.4"E), one wetland (26°54'08.2"S, 32°14'15.0"E), two riverine (26°54'18.5"S, 32°19'24.7"E; and 26°52'57.8"S, 32°18'41.8"E), one lake (26°53'35.6"S, 32°17'45.2"E), and one man-made pond at the campsite (26°54'33.8"S; 32°18'50.5"E) (and therefore anthropogenically impacted); as well as from the KNC from a total of three sites, including two temporary pans (27°23'43.9"S, 32°08'33.7"E and 27°24'35.1"S, 32°08'47.8"E), and one riverine site (27°23'26.5"S, 32°08'24.2"E). Two specimens, both *A. maculatus*, collected (26°54'18.5"S, 32°19'24.7"E) in April 2013, and found to be highly parasitised with *Hepatozoon* species (3.8% and 9.2% respectively), were kept, maintained in vivarium and fed on common garden crickets (*Gryllus bimaculatus*) for over a period of a year to monitor peripheral blood parasite stages and parasitemia on a bimonthly basis.

5.2.2 Frog blood smear preparation and screening

Blood was taken from the femoral arteries or veins and thin blood smears prepared, air-dried, fixed and stained using Giemsa-stain (FLUKA, Sigma-Aldrich, Steinheim, Germany). Subsequently smears were screened at 100x, images captured and parasites measured as described previously in Netherlands *et al.* (2014). Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear, following previous methods Cook *et al.* (2009). Descriptive statistics of the length (L) and width (W) results of mature gamont stages were compared between the three frog species using one way ANOVA (IBM SPSS V22). The remaining blood, that was not used in blood smear preparation, was placed

in sterile 0.5 ml reaction tubes with an equal volume of 70% molecular grade ethanol to be processed molecularly at a future date.

5.2.3 DNA extraction and phylogenetic analysis

Peripheral blood, obtained from parasitised specimens of two *A. garmani*, *two A. gutturalis*, and four *A. maculatus* was transferred to sterile 0.5 ml reaction tubes. Additionally the blood of one highly parasitised *Amietia quecketti* with *Hepatozoon theileri* from a previous study (see Netherlands *et al.* 2014), was also used in order to obtain a longer sequence for comparison. DNA was extracted from the samples using the standard protocol for human or animal tissue and cultured cells as detailed in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Düren, Germany). To amplify apicomplexan parasite 18S rDNA from the total DNA extracted, polymerase chain reaction (PCR) sequence runs were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad, Hemel Hempstead, UK). Identification of *Hepatozoon* species were initially completed using the *Hepatozoon* specific SIGMA primer set HepF300: 5'-GTTTCTGACCTATCAGCTTTCGACG-3' and HepR900 5'-CAAATCTAAGAATTTCACCTCTGAC-3'. The PCR reactions were run targeting a fragment of the 18S rDNA gene (see Ujvari *et al.* 2004). Conditions for PCR are detailed according to previous methods in Netherlands *et al.* (2014). A second PCR was carried out using another apicomplexan-specific parasite SIGMA primer set 4558: 5‘-GCTAATACATGAGCAAAATCTCAA-3’ and 2733: 5‘-CGGAATTAACCAGACAAAT-3’ (Mathew *et al.* 2000), targeting a longer fragment (approximately 1,120 bp) of the 18S rDNA gene for all samples found positive with *Hepatozoon* species. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 40 cycles, entailing a 94 °C denaturation for 1 min, annealing at 55 °C for 2 min with an end extension at 72 °C for 2 min, and following the cycles a final extension of 72 °C for 10 min (Mathew *et al.* 2000). Resulting amplicons were visualised under UV on a 1% agarose gel stained with gel red using a Bio-Rad GelDoc Imaging System (Bio-Rad, Hemel Hempstead, UK). PCR products were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd. Pretoria, South Africa) for purification and sequencing in both directions. From the resulting sequences chromatogram-based contigs were generated and trimmed using Geneious Ver. 7.1 (created by Biomatters, available from http://www.geneious.com), for further analysis.

Sequences were identified as those of *Hepatozoon* using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast/), and comparative haemogregarine sequences were selected for further phylogenetic analysis. In addition to the four sequences obtained from the three *Amietophrynus* species and one *A. quecketti* in this study, 18 additional sequences were acquired from Genbank, 15 *Hepatozoon* as well as a single *Hemolivia* [GenBank: KC512766], *Haemogregarina* [GenBank: KF257925] and *Dactylosoma* [GenBank: HQ224957] (used as an outgroup) species. All phylogenetic analyses were undertaken using the Geneious (Ver. 7.1) bioinformatics software package. Sequences were aligned using the MUSCLE algorithm (Edgar 2004), for both Maximum likelihood (ML) and Bayesian inference (BI) analyses. The alignment consisted of 22 taxa and 690 bp long. ML phylogeny was completed using PhyML Ver. 2.4.4 plugin (Guindon *et al.* 2010), according to the estimated AIC criterion using jModelTest Ver. 2.1.5 (Posada 2008). The chosen parameters of the substitution model were TVM+G. Nodal support was undertaken with 1000 Bootstrap replicates. Tree topolgy search was completed using Nearest Neighbour Interchange (NNI) and Subtree Pruning and Regrafting (SPR). BI phylogeny was implemented using MrBayes Ver. 3.2.1 plugin (Huelsenbeck & Ronquist 2001), under the estimated parameters as part of the analysis. The analysis was run twice for $2x10^6$ generations; saving one tree every 1000 generations; with burn-in values of 100,000 and four chains at a temperature of 0.20. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded, no burn-in samples were retained. Remaining trees were combined in a 50% majority consensus tree, in which frequency of any particular clade represents the posterior probability (Huelsenbeck & Ronquist 2001). Strict consensus trees for both ML and BI trees were generated and visualised using Tree-view (Barta *et al.* 2012).

5.2.4 Transmission Electron Microscopy (TEM) of *Hepatozoon* sp. A., from *Amietophrynus maculatus*

Blood was collected from the femoral vein of the hind limbs using sterile 1ml insulin syringes as indicated above. Blood was immediately transferred to 1.5 ml reaction tubes and pre-fixed in Todd's fixative (see appendix 1) for 12 hours (Todd 1986).

Fixed blood cells were then transferred from the blood collection tubes and washed three times for 15 min in 0.05M Cacodilate buffer. Post fixation then took place in 1% Osmium tetroxide (in Cacodilate buffer) for one hour. Following post fixation the specimen was washed three times for 15 min in distilled water. Samples were dehydrated through a series of graded alcohols (50%, 70%, 80%, 90%, 96% and 2x 100% ethanol) for 15 min each. Samples were not exposed to air during this step. The next step was to replace the 100% ethanol with 100% LR White resin by placing the samples in a rotator for 15 min. The LR White resin was replaced with fresh 100% LR White resin two times for 45min each. Samples were again placed in a rotator for this step. Samples were subsequently left in a fridge overnight. The next step was to embed the samples in gelatine capsules with fresh 100% LR White resin, and curing in an oven at 65°C overnight.

Resin blocks were hand-trimmed using minora shaving blades and a handheld rotary tool with an abrasive cutting disc. Glass knives were prepared, with disposable plastic boats attached using hot dental wax. Semithin (0.5µm) sections were cut using glass knives on a Reichert-Jung Ultracut ultramicrotome. Emerald-tinged sections (0.5 µm) were collected from the surface of 0.2µm Millipore-filtered dH_2O using an eyelash, floated on filtered dH_2O on a clean, labelled glass slide and dried on a Fried-electric hot plate at 30 - 40°C. Sections were stained using 1% toluidine blue stain in 1% borax, subsequently rinsed using dH_2O, dried on the hot plate, rinsed in xylene and mounted with entellen and a coverslip before being viewed under a Nikon light microscope.

Ultrathin (90nm) sections of selected resin blocks were also prepared using glass knives, floated on Millipore-filtered dH_2O and collected on 75-mesh square copper grids. Ultrathin sections were stained and rinsed on droplets using syringes fitted with 0.2µm DynaGard™ nonsterile syringe filters-firstly with Reynold's lead citrate for 30 min (in close proximity to NaOH to reduce precipitation with CO_2), rinsed 6x with dH_2O, thereafter stained with 1% uranyl acetate for 1 h, rinsed 6x with dH_2O, and finally stained in a second Reynold's lead

citrate for 20 min and a final 6x rinse in dH_2O. Sections were subsequently examined with a FEI Tecnai 200kV high resolution Transmission Electron Microscope (TEM) at 120kV, and digital images captured with GATAN bottom mount digital camera with accompanying Digital micrograph software.

5.3

RESULTS

5.3.1 General observations

Three species from the family Bufonidae were sampled from the NGR and the KNC, KZN (*A. garmani* and *A. maculatus*, and *A. gutturalis* respectively). Of the 53 individuals collected 16 (30.1%) were found to be infected with haemogregarines; from the NGR, 1/23 (4.3%) from *A. garmani* and 6/9 (66.7%) from *A. maculatus*; from the KNC, 5/9 (55.6%) from *A. garmani* and 4/12 (33%) from *A. gutturalis*. These infections were recorded from frogs inhabiting mainly riverine sites with the exception of two individuals from two separate temporary pans in the KNC. It was found that frogs collected from other sites such as the wetland, lake and campsite were not infected with the parasite. Five different stages were found in the peripheral blood. Trophozoite, meront and merozoite stages were rare and were only observed within the blood smears of two individuals of *A. maculatus* collected in April 2013 from the NGR. The most frequently encountered stages were immature and mature gamont stages, the latter stage being the most abundant across all the parasitised specimens. The mature gamont was characterised by a well-developed cap, typically staining pink, situated at one pole of a delicate capsule. According to the morphological measurements (Table 5.3.1) and other morphological features (as described in the detailed description) of the gamont stages, it is suggested that the *Hepatozoon* species found parasitizing all three toads is one and the same. This was concluded in spite of the size of the mature gamonts measured from *A. garmani* being smaller, with statistical differences considered to be significant at $P < 0.05$ compared to both *A. gutturalis* and *A. maculatus* $P = 0.000$. The latter two appeared to be very similar $P = 0.628$, with no significant difference observed. Pending the molecular outcome, this was attributed to intra-species variation. Throughout the collection of the host specimens no vectors were observed feeding on the frogs.

Table 5.3.1: *Hepatozoon* sp. A., morphometrics of the *Amietophrynus* species collected in this study.

Frog hosts by species	Measurements of the different stages found in the different host species		
	Trophozoites:	Immature gamonts:	Mature gamonts:
Amietophrynus garmani (Meek, 1897)		13.2 ± 0.6 (11.6–14.0) x 5.7 ± 0.3 (5.0–6.2) µm; 5.0 ± 0.7 (3.6–6.2) x 4.1 ± 0.6 (2.6–5.1) µm (n=16)	13.8 ± 0.7 (12.2–15.5) x 7.4 ± 0.6 (6.0–8.5) µm; 3.8 ± 1.3 (2.7–5.1) x 3.5 ± 1.2 (1.4–4.7) µm (n=66)
Amietophrynus gutturalis (Power, 1927)		14.7 ± 0.5 (13.7–16.0) x 6.7 ± 0.2 (6.1–7.1) µm; 4.9 ± 0.7 (3.7–6.9) x 3.90 ± 0.9 (2.0–6.1) µm (n=26)	14.7 ± 0.9 (12.3–16.5) x 7.6 ± 0.3 (7.1–8.2) µm; 4.1 ± 1.1 (3.2–5.3) x 4.0 ± 1.1 (2.5–4.8) µm (n=20)
Amietophrynus maculatus (Hallowell, 1854)	9.5 ± 1.3(6.9–11.7) x 3.3 ± 0.5 (2.2–4.8) µm (n=23)	14.4 ± 0.4 (13.6–15.3) x 6.1 ± 0.5 (4.4–6.8) µm; 5.2 ± 0.6 (4.3–6.3) x 4.2 ± 0.5 (2.7–4.9) µm (n=26)	14.5 ± 0.5 (13.4–15.7) x 7.6 ± 0.4 (6.6–8.5) µm; 3.9 ± 0.5 (3.0–5.1) x 4.3 ± 0.5 (2.7–4.9) µm (n=46)
Total average		14.2 ± 0.7 (11.6–16.0) x 6.4 ± 0.6 (4.4–7.9) µm; 5.0 ± 1.4 (3.3–7.3) x 3.9 ± 0.8 (1.9–6.1) µm (n=102)	14.2 ± 0.77 (12.23–16.53) x 7.5 ± 0.51 (6.0–8.5) µm; 3.9 ± 1.20 (2.6–5.9) x 4.0 ± 1.05 (1.4–5.5) µm (n=132)

Measurements given in µm, mean length ± standard deviation (range) x mean width ± standard deviation (range); mean nucleus length ± standard deviation (range) x mean nucleus width ± standard deviation (range) (n=number measured).

5.3.2 Description of *Hepatozoon* sp. A.

Taxonomic summary

Phylum Apicomplexa Levine, 1970

Family Hepatozoidae Wenyon, 1926

Genus *Hepatozoon* Miller, 1908

Morphology

Trophozoite: irregular to ovoid shape, often vacuolated cytoplasm, measuring 9.5 ± 1.3 (6.9–11.7) long by 3.3 ± 0.7 µm (2.2–4.8) wide (n=23), nucleus with loose chromatin,

staining pink to purple, measuring 3.4 ± 0.8 µm (2–4.8) long by 2.2 ± 0.5 µm (1.5–43.8) wide (Figure 5.3.1: A).

Meront: globular in shape, with a foamy cytoplasm, staining white, measuring 8.2 ± 1.3 µm (7–9.5) long by 8.5 ± 2 µm (7.1–9.9) wide (n=3). The nucleus has loosely arranged chromatin, extending outwards, staining light pink, measuring 4.6 ± 1.6 µm (3.2–6.3) long by 5.1 ± 1.4 µm (3.5–5.9) wide (n=3) (Figure 5.3.1: B, C). Possible later-meront stage, with granulated cytoplasm, staining light purple, with four distinct and condensed nuclei, staining dark purple (Figure 5.3.1: C).

Merozoite: elongated in shape, tapering toward one pole (possibly anterior), rounded at the other (possibly posterior), found either free in between the erythrocytes (Figure 5.3.1: D), or possibly entering or leaving an erythrocyte, vacuolated cytoplasm, staining light blue, measuring 12.9 ± 1.9 µm (10.7–14.4) long by 3.2 ± 0.8 µm (2.5–4) wide, nucleus staining similarly light blue with a condensed appearance, measuring 4.7 ± 0.6 µm (4–5.2) long by 3.1 ± 0.8 µm (2.5–4) wide (n=3).

Immature gamonts: oval shaped without cap or cavity at truncate pole (Figure 5.3.1: F-G arrow-head), cytoplasm staining whitish-blue or purple, measuring 14.2 ± 0.7 µm (11.6–16) long by 6.4 ± 0.6 µm (4.4–7.9) wide (n=102). Irregular oval shaped nucleus, with loose chromatin, staining dark blue or purple, measuring 5 ± 1.4 µm (3.3–7.3) long by 3.9 ± 0.8 µm (1.9–6) wide. Infrequently a recurved tail was observed (Figure 5.3.1: F arrow), the nucleus lying nearer to the anterior pole (the broader pole of the parasite) (Figure 5.3.1: F).

Mature gamonts: oval shaped with a well-developed cap/cavity at the truncate pole (Figure 5.3.1: H-I arrow-head) or folded region of the parasite, often staining pink (Figure 5.3.1: H-L); seemingly encased by a thick parasitophorous vacuole or delicate capsule, gamont with cap measuring 14.2 ± 0.8 µm (12.2–16.5) long by 7.5 ± 0.5 µm (6–8.5) wide (n = 133); excluding cap measuring 11.8 ± 2.4 µm (8–14.3) long, width remaining the same. A small recurved tail seldom visible (Figure 5.3.1: I arrow). Whitish-blue to purple staining cytoplasm, with an irregular, oval-shaped, blue or dark-purple staining nucleus measuring 3.90 ± 1.20 µm (2.6–5.9) long by 4 ± 1.1 µm (1.5–5.5) wide; in some cases the gamont would cause dehaemoglobinisation of the host cell (Figure 5.3.1: J-K), as well as lysing of its nucleus (Figure 5.3.1: K).

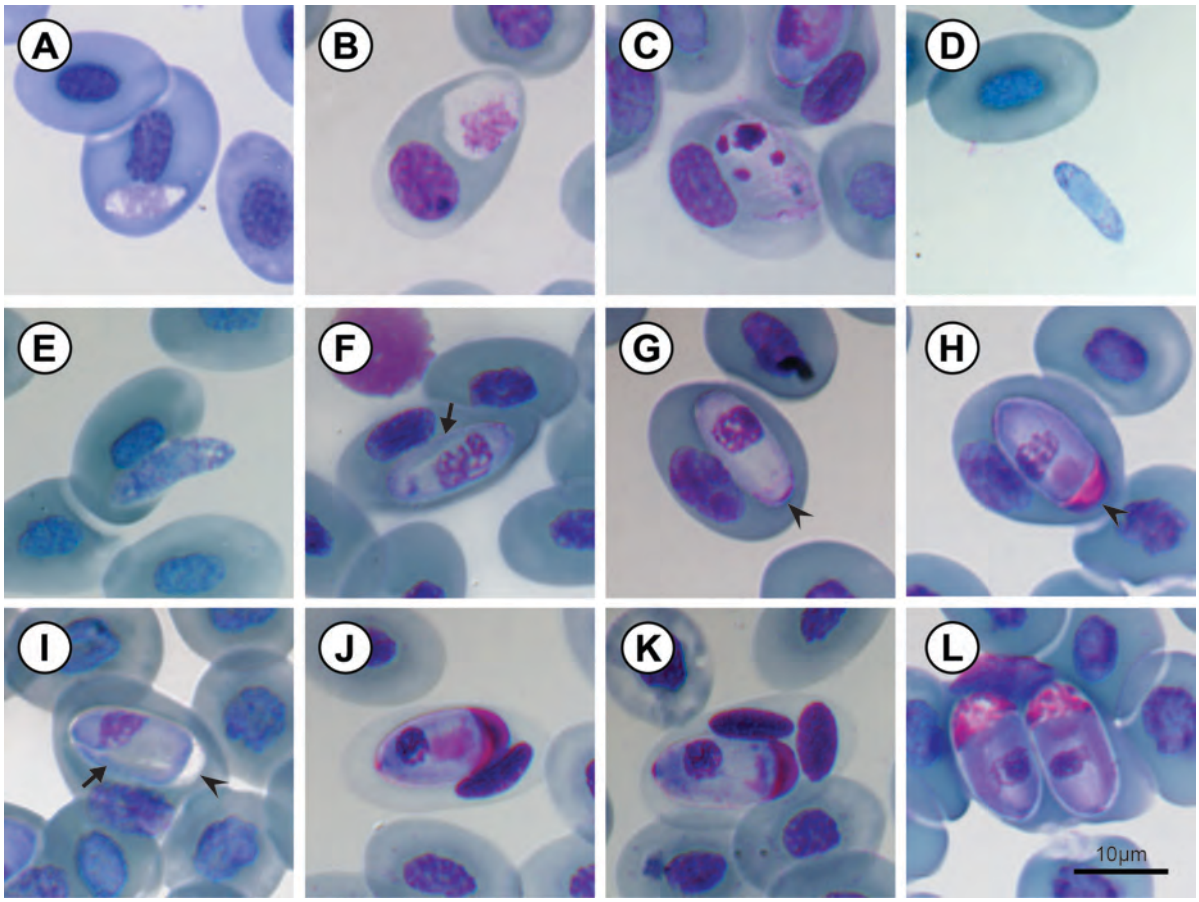


Figure 5.3.1: *Hepatozoon* sp. A. (A-L) in the peripheral blood of the frog *Amietophrynus maculatus* Hallowell, 1854 (Bufonidae). **A**: trophozoite. **B**: Possible pre-meront. **C**: Meront stage. **D**: Extracellular or free merozoite stage. **E**: Merozoite, showing likely entry into an erythrocyte. **F**: Immature gamont displaying a recurved tail (arrow) and loosely arranged nucleus. **G**: Immature gamont before development of the capsular cap at the truncate pole (arrow-head). **H-L**: Mature gamonts, note the well-developed capsular cap forming at the truncate pole (arrow-head), staining pink (**H**, and **J-L**), with a prominently visible compact nucleus. **I**: Mature gamont displaying a recurved tail (arrow), note the cap did not stain pink. **J**: Dehaemoglobinisation of host cell, often followed by, lysing of the host cell nucleus **(K)**. **L**: Double infection of a single erythrocyte. Scale bars: 10µm.

Molecular analysis

Once edited for phylogenetic analysis, four sequences of 1033 base pairs (bp), one from each of the three *Amietophrynus* species and one from an *A. quecketti* infected with *H. theileri*, were produced using the 4558 and 2733 primer sets, targeting part of the 18S rDNA gene (Mathew *et al.* 2000). The overall topology of the generated ML and BI phylogenetic

trees were identical and nodal support of each analysis is represented on the ML tree as ML/BI (Figure 5.3.2). The phylogenetic tree comprises three distinct clades. The haemogregarine isolates of all three *Amietophrynus* species, as well as that of the *A. quecketti*, were found to fall within the first clade comprising mostly anuran *Hepatozoon* species. Furthermore, that from *A. quecketti* was found to be, as expected, identical to a previously deposited isolate of *H. theileri* (Netherlands *et al.* 2014). The three isolates from the *Amietophrynus* species were all found to be identical and to form a sister taxon to *H. theileri*.

5.3.3 Bimonthly peripheral blood observations

Two specimens of highly parasitised *A. maculatus* (collected in April 2013) were kept in a vivarium and their blood screened for peripheral blood stages on a bimonthly basis, from May 2013 to and including May 2014. This was done in order to monitor their parasitaemia levels in the absence of a vector, as well as to observe any changes in the peripheral blood stages. The parasitaemia over a period of one year for the first specimen averaged 4.8% (3.6–6.6%). May 2014 had the lowest parasitemia of 3.6% and July 2013 the highest of 6.6%. The second specimen's parasitaemia averaged 12.5% (8.2–19.2%). May 2014 had the lowest parasitaemia of 8.2% and July 2013 the highest of 19.2%. In both specimens, upon first collection (April 2013), trophozoite, merozoite, immature and mature gamont stages were observed. As the observation period progressed from there on, only immature and mature gamont stages were observed, mature gamont stages being the predominant stage. Parasitaemia levels peaked within the first four months (July 2013) and then steadily decreased over the next nine months to a similar parasitaemia level as was first recorded in May 2013. No extracellular or extracapsular gamonts were ever observed during this period in the peripheral blood.

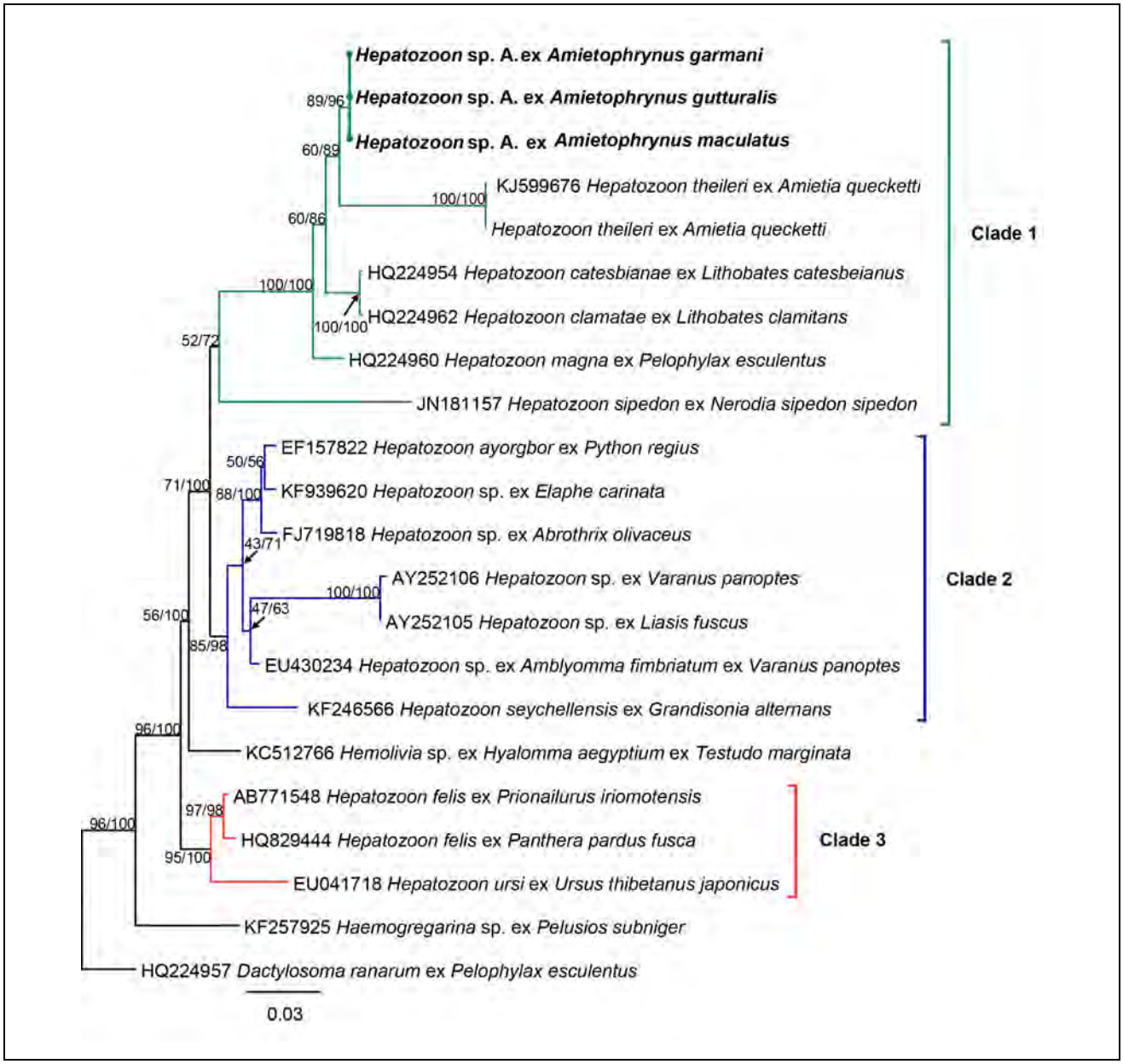


Figure 5.3.2: Phylogenetic position of *Hepatozoon* sp. A. based on 18s rDNA gene sequences.

Tree topology was identical across Maximum Likelihood (ML), and Bayesian inference (BI). Therefore the nodal support values for each analysis (ML/BI) are represented on the ML tree. ML tree was constructed using PhyML under the parameters of the substitution model being TVM+G with 1000 Bootstrap replicates, the BI tree was constructed using MrBayes under the estimated parameters as part of the analysis. New hapanotypes or parahapanotypes are represented in bold. Distinct clades are represented in different colours (green/blue/orange).

Table 5.3.2: All African *Hepatozoon* species infecting frogs from the family Bufonidae.

***Hepatozoon* species**	**Host species**	**Description mature gamont**	**Locality**	**References**
***Hepatozoon* sp. A.**	*Amietophrynus maculatus* (Hallowell, 1854); *Amietophrynus garmani* (Meek, 1897); *Amietophrynus gutturalis* (Power, 1927)	**Mature capped form:** 14.2 ± 0.77 (12.23–16.53) x 7.5 ± 0.51 (6.0–8.5) µm; 3.9 ± 1.20 (2.6–5.9) x 4.0 ± 1.05 (1.4–5.5) µm	NGR and KNC, South Africa	Present study
Hepatozoon aegyptia (Mohammed and Mansour, 1963) Smith, 1996	*Amietophrynus regularis* (Reuss, 1833) (syn., *Bufo regularis*)	**Mature capped form†:** 14 x 7.3 µm; 3.7 x 2.9 µm **Mature capped form*:** 13.7 (12.7–14.7) x 8.1 (6.7–8.6) µm; 3.9 (3–4.5) x 3.9 (3.6–4.2) µm	Cairo, Egypt† Khartoum, Sudan*	Mohammed & Mansour (1963)† Younis & Saoud (1969)*
Hepatozoon assiuticus (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978) Smith, 1996	*Amietophrynus regularis*	**Mature elongated form†:** 35.4 (30–38) x 4.2 (3.5–5) µm; 4.2 (3–4.7) x 4 (2.8–4.6) µm	Assuit, Egypt†	Abdel-Rahman, El-Naffar, Sakla & Khalifa (1978)†
Hepatozoon boueti (França, 1910) Smith, 1996 [syn., *Hepatozoon boneti* França, 1925 of Tuzet and Grjebine (1957)]	*Amietophrynus regularis*	**Mature elongated form†:** (16.5–18.5) x 4.5 µm; 4.5 x (3–3.5) µm **Mature elongated form*:** 22.3 (19–26) x 6 (5–6) µm; 5.2(4–6) x 4.2 (3–6) µm	Guinea-Bissau† Cairo and Giza, Egypt*	França (1910)† Mohammed & Mansour (1966)*
Hepatozoon faiyumensis (Mansour and Mohammed, 1966) Smith, 1996	*Amietophrynus regularis*	**Mature elongated form†:** 15.5 (13–17) x 4.5 (4–5) µm; 4.5 (3–5) x 3.9 (3–5) µm	Faiyum, Egypt†	Mansour & Mohammed (1966b)†

Table 5.3.2. continued

***Hepatozoon* species**	**Hosts species**	**Description mature gamont**	**Locality**	**References**
Hepatozoon francai (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978) Smith, 1996	*Amietophrynus regularis*	**Mature capped form†:** (18.5–20) x (2.7–3) µm	Assuit, Egypt†	Abdel-Rahman, El-Naffar, Sakla & Khalifa (1978)†
Hepatozoon froilanoi (França, 1925) Smith, 1996	*Amietophrynus regularis*	**Mature elongated form†:** (16.5–21) x 4.5 µm; (6–7.5) x 4.5 µm	Luanda, Angola†	França (1925)†
Hepatozoon lavieri (Tuzet and Grjebine, 1957) Smith, 1996	*Amietophrynus regularis*	**Possible gamont form†:** 20 µm in length	Pointe-Noire, Congo†	Tuzet & Grjebine (1957)†
Hepatozoon magni (Hassan, 1992) Smith, 1996	*Amietophrynus regularis*	**Capped form†:** 15.05 (14.2–16.8) x 7.7 (6.3–9.8) µm; 3.4 (2.8–4.2) x 3.4 (2.8–4.2) µm **Mature elongated form†:** 22.5 (21.2–26.6) x 8.2 (7.7–9.1) µm; 4.3 (4.1–4.6) x 4.5 (4.3–4.8) µm	Qena, Egypt†	Hassan (1992)†
Hepatozoon moloensis (Hoare, 1920) Smith, 1996	*Amietophrynus spp.*	**Mature capped form†:** 18.8 x 7.8 µm	Molo, Kenya†	Hoare (1920)†
Hepatozoon pestanae (França, 1910) Smith, 1996	*Amietophrynus regularis*	**Mature capped form†:** 12 x 4.5 µm; 3.7 µm **Mature capped form*:** 13 (12–17) x 5 (4.5–5) µm; 4.2 x 3.7 µm	Guinea-Bissau† Giza, Egypt*	França (1910)† Mohammed & Mansour (1966)*
Hepatozoon tunisiensis (Nicolle, 1904) Smith, 1996	*Amietophrynus mauritanicus* (Schlegel, 1841) [syn., Bufo *mauritanicus*]	**Mature capped form†:** (12–15) x 8 µm	Tunis, Tunisia†	Nicolle (1904)†

Measurements given where possible, mean length ± standard deviation (range) x mean width ± standard deviation (range); mean nucleus length ± standard deviation (range) x mean nucleus width ± standard deviation (range). † original species description, * redescription or other descriptions of the same species.

5.3.4 Transmission electron microscopy of *Hepatozoon* sp. A. from *Amietophrynus maculatus*

Ultrathin sections of the peripheral blood, showing a high parasitaemia of *Hepatozoon* sp. A., from *Amietophrynus maculatus* were examined by means of transmission electron microscopy (TEM) (Figure 5.4.1: A-K). Only intraerythrocytic immature and mature gamonts were observed in the blood film and ultrathin sections (Figure 5.4.1: A, B). Mature gamonts, as described by light microscopy in section 5.3.2, were characterised by the presence of a prominent dark staining cap/cavity (c/c) at the truncate pole. The substance or purpose of this feature at an ultrastructural level offers up no further information and thus still remains unknown (see Figure 5.4.1: B, C). Mature gamonts also contain a clear folding (f) region closer to the truncate pole (see Figure 5.4.1: B). This is where the recurved tail (rt) is observed to lie reflexed upon itself and in some cases the (rt) extends all the way to the anterior pole (ap) (see Figure 5.4.1: D). The gamonts seemed to be lodged within a dense capsule (c). The capsule is situated on the inside of what was identified as a parasitophorous vacuole (pv), and is closely associated with the parasite's pellicle (p) or double membrane. The pellicle of the gamont is divided by the outer plasmalemma and an inner complex of two closely disparate membranes (Figure 5.4.1: E) (see Paterson *et al.* 1988; Vargas 2010). The inner membrane complex terminates at the beginning of the conoid (co) (see Figure 5.4.1: H). In some cases a small structure, similar to that found in various *Hemolivia* species is observed and is recognised as a suture within the capsule wall (see Figure 5.4.1: C, F, G) (see Paperna & Smallridge 2001). This structure is apparent at the opposite poles of the capsule (c). The apical complex is situated at the anterior pole (ap) of the parasite within the apex (apx) of the gamont (see Figure 5.4.1: I). According to Paterson *et al.* (1988), the apical complex comprises a conoid, two pre-conoidal rings, and a polar ring complex. The conoid (co) is situated at the top of the gamont apex (see Figure 5.4.1: H, I), with the two pre-conoidal rings (pco) situated above the conoid (see Figure 5.4.1: J). The last structure to make up the apical complex is the polar ring (pr) (see Figure 5.4.1: H, I), which is positioned between the pellicle membrane and subpellicular microtubules (see Figure 5.4.1: H, I, J). The polar ring complex consists of approximately 85 subpellicular microtubules (see Figure 5.4.1: K) (see Desser *et al.* 1995).

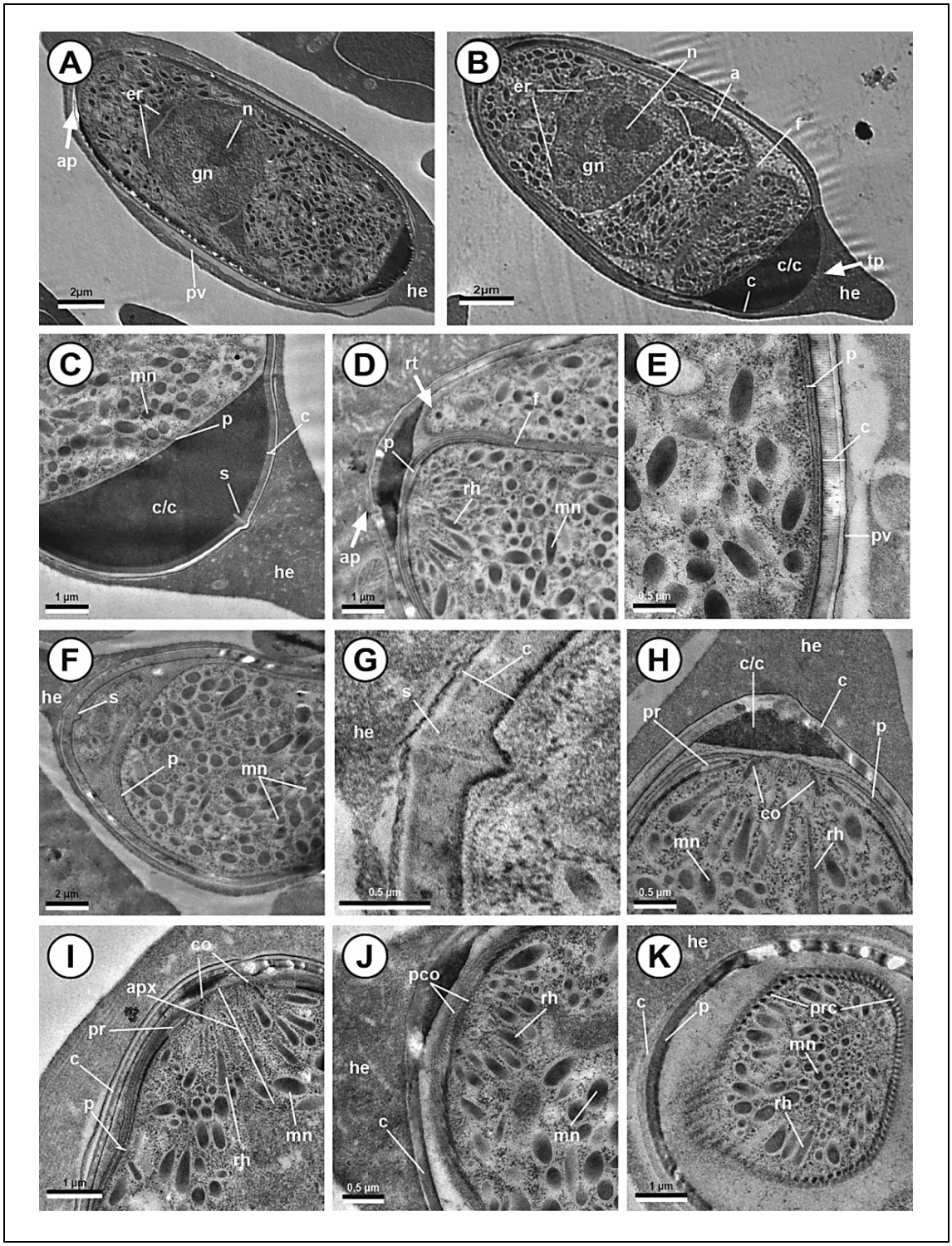


Figure 5.3.4. Transmission electron micrographs of *Hepatozoon* sp. A. (A-K) in the peripheral blood of the frog Amietophrynus maculatus Hallowell, 1854 (Bufonidae). A: immature gamont in the host erythrocyte (he) found within a slender parasitophorous vacuole (pv). With endoplasmic reticulum (er) surrounding the gamont nucleus (gn).

Figure 5.3.4. continued.

B: Mature gamont showing cap/cavity (c/c) at the truncate pole (tp), with folding (f) of the parasites tail on itself. The apicoplast (a) is situated next to the (gn) **C:** enlarged truncate pole (tp) of the mature gamont, displaying the thick capsule (c) and (c/c), along with the parasites pellicle (p) membrane, and suture (s). **D:** Shows the recurved tail (rt) extending to the anterior pole (ap) of the parasite. Along with the micronemes (mn), rhoptries (rh) and folding (f) of the (rt) across the pellicle (p) **E:** magnified cell wall, consisting of the (pv) on the outside, followed by a thick capsule (c), lined by a (p) membrane divided by the outer plasmalemma and inner complex membranes **F:** displays different positions of the (mn), (p) and (s) **G:** enlarged suture within the (c) wall **H:** Longitudinal section of the apical complex, displaying the conoid (co) and polar ring (pr) **I:** the encapsulated apex is shown in relation to the (co), (pr) (rh) and (mn) at the anterior pole of the parasite **J:** Longitudinal section of the pre-conoidal rings (pco) at the tip of the apex **K:** Transversely sectioned polar ring complex displaying approximately 85 subpellicular microtubules.

5.4

DISCUSSION

Morphological characteristics of peripheral blood parasite stages strongly suggested that all three *Amietophrynus* species were infected with the same species of haemogregarine, likely, based on the lack of peripheral division stages and the recommendations on anuran haemogregarines of Smith (1996), a species of *Hepatozoon*. This was supported molecularly, with sequences of haemogregarine isolates from all three frog species being identical and falling within a clade comprising other anuran *Hepatozoon* species.

The capped mature gamont form of the *Hepatozoon* described here was the most frequently encountered stage throughout the current study, as well as in several other studies of bufonid *Hepatozoon*, and is thus consequently considered further within the following discussion. In terms of morphometrics the capped mature gamonts of *Hepatozoon* sp. A. are most similar to the capped forms of *H. aegyptia*, *H. magni* and *H. tunisiensis* (see Table 5.3.2). Even though the present study's capped gamont form shares a number of morphological similarities to all three of the above described parasites, it does not conform entirely to any of these three. All four parasites, including the *Hepatozoon* species in this study, are characterised by being tightly enclosed in a delicate capsule that does not appear to interfere with the staining of the parasite as do those of chelonians (see Siroky et al., 2007; Cook et al., 2009, 2014), and all show some form of a cap-like structure or space at one or both poles that is filled with a dark-staining intracapsular material (Figure 5.3.1: H-L). In addition, all four species' gamonts appear to be folded-over on themselves forming two branches within the capsule, the nucleus of all four situated within the wider of the two branches. Of the four similar species , *H. tunisiensis* does not have a definite cap at the truncate end (the region of the fold) (Figure 5.3.1: G-I, arrow-head) of the parasite, the intracapsular material scattered frequently around the gamont and accumulating towards the poles of the encapsulated parasite, forming, in comparison to the other three, weak cap-like structures. Similarly, the parasite described in this study shows some scattered intracapsular material (Figure 5.3.1: H, J, K), but unlike *H. tunisiensis* it has a distinct cap similar to those of *H. aegyptia* and *H. magni*. Furthermore, Nicolle (1904) described *H. tunisiensis* to cause no hypertrophy of the host cell, this being equally true of *H. magni* described by Hassan (1992), which is in direct contrast to *H. aegyptia* and *Hepatozoon* sp. A.,

which both cause fragmentation of the host cell nucleus as well as dehaemoglobinisation of the host cell cytoplasm (see Figure 5.3.1: J-K). Even though it would appear that *Hepatozoon* sp. A. conforms closely both in size and morphological characteristics to *H. aegyptia*, during the period of a year in which peripheral blood stages were monitored bimonthly, no elongated forms, such as those of *H. aegyptia* and *H. magni*, were ever observed for *Hepatozoon* sp. A.

Geographically, the three frog species examined in this study are sympatric. The finding that the haemogregarine isolates from these three frog species represents the same *Hepatozoon* species, *Hepatozoon* sp. A., is thus not surprising. In addition, even though *A. garmani*, *A. gutturalis* and *A. maculatus* are sympatric to *A. regularis*, the closest geographical overlap to the present study's site is Kenya, which even though *A. regularis* has been examined there in the past no haemogregarine infections were ever reported (see Ball 1967). Since the above frog species are found to occur sympatrically in Kenya, it would be expected that the same is true for their parasites, *Hepatozoon* sp. A. and *H. aegyptia* respectively, as can be seen in the case of *H. catesbianae* and *H. clamatae* which along with their hosts occur sympatrically across Nova Scotia (Boulianne *et al.* 2007). It may be that the *Hepatozoon* species of African bufonids are locality specific as are some of those infecting African chelonians (see Cook et al., 2009; 2014a, 2014b), or the snakes of Florida in the U.S.A. (see Telford *et al*. 2001). As mentioned previously, *Hepatozoon* sp. A., was found parasitizing frogs inhabiting temporary pan and riverine sites as compared to no visible infections in frog hosts inhabiting wetland, lake and campsites. It may suggest that the infection is especially dependent on a vector that may be limited to a specific habitat. Hence, considering the above morphological and geographical aspects, it is strongly suggested that *Hepatozoon* sp. A. is a new species.

Molecularly, as mentioned above, *Hepatozoon* sp. A. fell within the first clade (represented in green) comprising anuran *Hepatozoon* species (Figure 5.3.2). The second clade (represented in blue) comprises mainly of reptile hosts with the exception of a *Hepatozoon* sp. [GenBank: FJ719818] from the rodent host *Abrothrix olivaceus* (Waterhouse, 1837) and surprisingly *Hepatozoon seychellensis* [GenBank: KF246566] Harris, Damas-Moreira, Maia et Perera, 2014, from the caecilian host *Grandisonia alternans* (Stejneger, 1893) (see Harris *et al.* 2013). The third clade (represented in orange) comprises *Hepatozoon* spp. from larger mammal hosts and falls outside other *Hepatozoon* spp. from amphibian and reptile hosts.

The first clade, dominated by anuran *Hepatozoon*, comprises one exception, that of *Hepatozoon sipedon* [GenBank: JN181157] Smith, Desser et Martin, 1994, which is a *Hepatozoon* species of both an anuran, *Lithobates pipiens,* as well as a snake, *Nerodia sipedon,* vertebrate host, thus forming a sister taxon on its own within the larger first clade (represented in green). Such a result is not unanticipated as it is a well-known described three-host (snake, frog and mosquito *Culex pipiens* or *Culex territans*) life cycle (see Smith *et al.* 1994b; Netherlands *et al.* 2014). Since *Hepatozoon* sp. A. falls along with *H. theileri*, it may suggest that *Hepatozoon* sp. A. follows a two-host life cycle such as that suggested for *H. theileri* in the latter's redescription by Netherlands *et al.* (2014). Furthermore, this small clade comprising *Hepatozoon* sp. A. and *H. theileri* falls within the same monophyletic sub-clade containing *H. catesbianae*, *H. clamatae* and *H. magna*. *Hepatozoon catesbianae* and *H. clamatae*, and presumably *H. magna* (Barta *et al.* 2012) follow a two-host life-cycle which does not include a cystic stage within the intermediate vertebrate host and can be experimentally transmitted to culicine mosquito definitive hosts which subsequently demonstrate sporogonic stages as described by several authors (Smith *et al*. 1994b; Desser *et al*. 1995; Kim *et al*. 1998; Barta *et al*. 2012). Once again, the positions of the above *Hepatozoon* species including that of *H. sipedon*, supports the theory of Barta et al. (2012) on the co-evolution of haemogregarines and their definitive hosts. Overall, the tree indicates with the phylogenetic placement of larger mammal hosts (clade 3), small rodent and snake hosts (clade 2) and frog, snake associations (clade 1), the potential of *Hepatozoon* host, prey and vector interactions.

Transmission electron microscopy observations on *Hepatozoon* sp. A. (Figure 5.4.1) have shown the ultrastructural morphology of this species, highlighting specific characteristics, such as this parasite's thick capsule, suture, and dark staining cap/cavity (Figure 5.4.1: A-G). The apical complex, characteristic to all apicomplexans is also illustrated in great detail (Figure 5.4.1: H-K). The Apical complex is one of the significant features of apicomplexan parasites. This complex organ consists of various conical structures at the apical end of the parasite (see Paterson *et al.* 1988). Although apicomplexan parasites are characterised by the apical complex, it exists in only part of its life cycle, forming a fundamental part in the infection or invasion of the host cell (see Okamoto & Keeling 2014). *Hepatozoon* sp. A. contains a thick capsule, which is surrounded by a slight parasitophorous vacuole (pv) (see Figure 5.4.1: A). According to Paterson *et al.* (1988), and although there are various explanations concerning the role of the (pv), the combination of the (pv) with the different

coated membranes, such as the capsule and pellicle membranes are unique to the haemogregarines. The thick capsule observed in this parasite could be due to a number of undetermined reasons. The most likely, is that the parasite has evolved its thick capsule to survive digestive acids in a host that consumes the infected intermediate host. Another reason could be to survive unfavourable and prolonged conditions, such as over hibernation within the host. The presence and purpose of the suture, a structure situated within the capsule wall on opposite poles of the parasite, is unknown (Paperna & Smallridge 2001). In the case of *Hemolivia* the suture possibly helps hold the parasite in place, but the extra thick capsule, evident too in previous ultrastructural studies of *Hemolivia,* seems to cause a problem with respect to the adequate fixing of the encapsulated parasite. The parasite within the capsule does not fix adequately and with the close association seen between the capsule wall and the parasite pellicle in both this study and previous studies, the parasite along with its capsule is seen to tear free from its pv within the host cell. However, in the case of *Hemolivia,* the capsule causes considerable shrinking of the encapsulated parasite (see Boulard *et al.* 2001; Paperna & Smallridge 2001; Široký *et al.* 2007). Further ultrastructural studies, particularly if a species of *Hemolivia* can be obtained, may be informative and should involve measuring of the *Hemolivia* species capsule thickness as compared to that of the *Hepatozoon* in the current study.

When compared on an ultrastructural scale to other amphibian *Hepatozoon* species i.e. *Hepatozoon magna*, there are many similar and comparative structures (see Paterson *et al.* 1988), and although the thick capsule and suture of *Hepatozoon* sp. A. seems to be more closely linked to that of *Hemolivia* species (see Boulard *et al.* 2001), the phylogenetic analysis (see Figure 5.3.2) confirms its placement as *Hepatozoon*. The dark cap/cavity situated at the truncate pole of the parasite (see Figure 5.4.1: B,C), stains pink when stained with Giemsa. Due to the fact that this structure or material is not engulfed by the pellicle membrane of the parasite, but only encapsulated by the thick walled capsule surrounding the parasite, it could be suggested that this is some form of waste product produced by the parasite, which is unable to escape through the thick capsule.

5.5

CONCLUSION

Future research should include the identification of possible definitive hosts or vectors such as mosquitoes as well as experimental transmission studies. Considering the close morphological resemblance of all four species of *Hepatozoon*, the present study's *Hepatozoon* sp. A. with *H. aegyptia*, *H. magni* and *H. tunisiensis*, it would be particularly beneficial to the study of *Hepatozoon* species of the Bufonidae if the latter three species could be isolated once more for molecular analysis. For instance, it can be seen that there is a small degree of intraspecies variation with regards to the size of the capped mature gamont of *Hepatozoon* sp. A. between the three host species. The size of the gamont measured from *A. garmani* is significantly smaller than the gamonts from both *A. gutturalis* and *A. maculatus*. In the past it would be suggested, from a solely morphological point, that the *Hepatozoon* from *A. garmani* may be a different species. If intraspecific variation of sporogonic stages of a single species of *Hepatozoon* can occur between different vector individuals of the same host species (see Paperna & Lainson 2003), it is possible that this could also be true of the peripheral blood stages. The findings of this study strongly advocate the molecular analysis of those *Hepatozoon* species described in the past from *A. regularis*. This, it is believed will aid in determining with more accuracy whether or not they are all of the same species or if they are in fact different species, along with providing a better indication, with the use of their phylogenetic placement, of their possible vectors and life cycle dynamics.

With regards to the ultrastructural analysis, to the author's knowledge no other ultrastructural studies on capped gamonts, such as those that have been described above, have been completed. Furthermore, the material seen contained in the cap or anything similar has not yet been observed in any other ultrastructural studies of *Hemolivia* or *Hepatozoon*. Its substance and role, however, remains unknown and possibly one of the only means to discovering its make-up and purpose may lie in biochemistry.

Footnote: The species described here as *Hepatozoon* sp. A, has subsequently been described and named by the candidate and co-authors as *Hepatozoon ixoxo* Netherlands, Cook and Smit, 2014 in a paper based on this chapter of the dissertation (see appendix 4).

CHAPTER 6

SUMMATIVE DISCUSSION

6.1

SUMMATIVE DISCUSSION

6.1.1 General discussion

In southern Africa, the anuran fauna currently comprises 13 families, 33 genera and 159 known species of frogs (see Du Preez & Carruthers 2009, Channing & Baptista 2013, Channing *et al*. 2013a; Channing *et al*. 2013b; Conradie 2014). Within this area the KZN province is known for its tropical environment and vast diversity of habitat systems, ultimately supporting the richest diversity of anurans throughout southern Africa (Alexander *et al*. 2004; Measey 2011). The only officially protected area within the Phongolo Floodplain (northern KZN) is the section of the Phongolo River and associated pans in the NGR. However, the role of this protected area as a refuge for anuran biodiversity had never before been established.

In this study, research on the biodiversity, habitat utilization and long-term monitoring of amphibians in northern KZN were undertaken. The majority of the work took place inside the NGR, with only two comparative surveys taking place outside the park. The first and second aim of the study was to record amphibian diversity and abundance, according to habitat preferences both inside and outside the NGR. This was achieved by conducting a comprehensive survey and monitoring program of amphibian species diversity and richness, using both active and passive techniques over a two year period, and comparing the results from the above three localities. In Chapter 2 the diversity of frogs recorded via the different surveying methods mentioned above were reported on, and as expected the diversity and abundance of frog species within the NGR, exceed the outer two anthropogenically impacted sites, with 32 of the 34 frog species (in the current study) occurring inside and only 11 frog species occurring outside the NGR. Habitat preferences for the expected species were divided into five habitat types, namely endorheic, lacustrine, palustrine, riverine and terrestrial. Endorheic microhabitats were found to harbour the most frog species, with 76% (26/34) of the species recorded occurring in these habitat types.

The third aim of this study was to evaluate long-term passive acoustic monitoring (PAM) of amphibians using a “song meter” recording device. This aim was achieved by recording amphibian activity at a selected locality for the duration of a year and comparing the data to the other active surveying methods applied. This data aided in the identification of species actively calling at the

particular site throughout the year, as well as their peak breeding season, preferred calling times and the intensity of their calls. This monitoring tool was able to detect 19 frog species, thus adding an additional two species to the current study's list, which had been recorded via active sampling. This was the first survey of its kind to be conducted in a protected natural environment in South Africa. The forth aim was to compare all current data collected with historical records of the same area. This was achieved by obtaining historical data, from Lambiris (1989), Ezemvelo Wildlife as well as from the amphibian atlas and red data book (Minter *et al.* 2004). The data from those records were used to create a baseline to which the current study's results could be compared. This was used to determine if the amphibians in this area are facing major declines, similar to what has been reported globally (see Stuart *et al*. 2004; Weldon & du Preez 2004; Beebee & Griffiths 2005).

The overall outcome of Chapter 2 was thus that NGR supports a high diversity of frog species that should be monitored and protected. Results from this study showed that the majority of the previously recorded species from this area are still present and abundant, with only six species not recorded in the current study. It is concluded that this is probably due to unfavourable sampling conditions or the specific niche microhabitats of these species not being surveyed during this study, rather than those species being locally extinct.

Additionally, the amphibian diversity surveys conducted during the present study highlighted the need to clarify some of the previous taxonomic confusion caused by the colour variations within the the polychromatic forms encountered between *Hyperolius argus* sub-adult male, adult male and adult female frogs. This was achieved in a case study (Chapter 3), by reporting on and subsequently testing these differences via the use of histology and call data, comparing the gonads and calls of the sub-adult males and adult males, to determine if the sub-adults are in fact mature as thought by their atypical tendency to produce mating calls. Furthermore, to determine if these sub-adult males, adult males and females were one of the same species or in deed represented a composite of species, 16S mitochondrial genetic data was used. The study supported that in certain cases this species does contain polychromatic forms and undergoes major changes in colour during its development from the sub-adult to adult. Additionally, for the first time, the unexplained phenomenon of sexually immature sub-adult males producing mating calls is reported.

Very little research has been conducted on frog blood parasites in sub-Saharan Africa, and a large degree of the true diversity of genera and species are unknown for these hosts. Thus the fifth aim of this study was to determine the amphibian blood parasite diversity and parasitaemia for the NGR and surrounds. This was achieved by means of a blood parasite survey, reporting on the diversity, prevalence and parasitaemia of any blood parasites encountered during the study. Light microscopy

and statistical analysis (on the prevalence and parasitaemia between frog species, families, habitat types and sampling periods) was completed and when possible parasite species were identified to genus level based on their basic morphology. Provided in Chapter 4 is a detailed assessment on previous work of the intraerythrocytic and extracellular blood parasites in amphibian hosts throughout Africa and South Africa, which formed the basis for comparison of the overall diversity of blood parasites recorded infecting frogs from all of the three sites both inside and outside the NGR. *Hepatozoon* and *Trypanosoma* species accounted for most of the infections, with viral or bacterial, microfilarid and *Dactylosoma* species being the least prevalent. Among the recorded frog blood parasites observed, 20% of the frogs were infected with at least one blood parasite group, and some were even infected with all five groups. Furthermore frog species were divided into four groups according to habitat preferences, showing that only the semi-aquatic and semi-terrestrial groups contained infected specimens. This study represents the first multispecies blood parasite survey done on frogs in South Africa, highlighting the fact that a lot more work needs to be done in this field of study.

Since the majority of African anuran *Hepatozoon* species have been recorded from the family Bufonidae (Netherlands *et al*. 2014), and as a case study to the above parasite diversity survey, the aim of Chapter 5 was thus to elucidate, via traditional morphological description of peripheral blood stages and molecular techniques, the identity of the *Hepatozoon* species found infecting *A. garmani, A. gutturalis* and *A. maculatus*. This was done to determine whether they represent a single species or three cryptic species, such as what has been recorded, solely morphologically, from other *Amietophrynus* species further north in Africa. Furthermore, a detailed review with morphological comparisons to each of the previously described African anuran *Hepatozoon* species was conducted, and it was concluded that the species from the current study is in fact new to science and is described as *Hepatozoon* sp. A. Such a finding makes it the first morphological and molecular account of *Hepatozoon* species within the family Bufonidae from South Africa, a study hoped to encourage the redescription and molecular analysis of those *Hepatozoon* species described in the past from *Amietophrynus* species, as well as to promote the use of both morphological and molecular characteristics in *Hepatozoon* species' descriptions. Additionally, Transmission Electron Microscopy (TEM) observations on the ultrastructure of *Hepatozoon* sp. A. have been completed, showing the unique morphology of this species, and highlighting specific characteristics, such as this parasite's thick capsule, suture, and dark staining cap/cavity.

Overall this study highlighted the importance of using various techniques in the surveying and monitoring of amphibians and their associated parasite communities. A novel approach in terms of long-term PAM was tested and compared to traditional surveying and monitoring methods, along with the recommendations for future research using this tool (Chapter 2). With regards to the *H. argus* case study (Chapter 3), although certain taxonomic questions in terms of colour dimorphism were answered, a new question was asked as to the possible advantages of advertisement calls by sub-adult frogs. The first multispecies haemoparasite survey was done on South African frogs (Chapter 4), with the full description of a *Hepatozoon* species using both morphological and molecular techniques (Chapter 5).

6.1.2 Future research

Future research should include a more comprehensive approach to the long-term PAM, looking at monitoring amphibians in various habitat types and systems. For instance, these data should be combined with environmental weather data, in order to arrive at more accurate conclusions regarding amphibian activity in different systems, throughout the year, and as a result of changing weather conditions and seasons. These data can then ultimately be used to model the breeding activity of various amphibian species in aiding in the conservation of suitable habitat types and frog communities as a whole. With regards to the *H. argus* case study, further experimental work is needed to determine or even propose a possible hypothesis on the unexplained behaviour of *H. argus* sub-adult frogs calling, answering what the possible evolutionary implications of this behaviour may be.

It is anticipated that through future work, using the skills mastered in this study for the morphological and molecular description and characterisation of *Hepatozoon* sp. A., the other reported parasites too may be described in a similar manner, subsequently elucidating the biodiversity of the northern KZN region. Furthermore, it is hoped that with this biodiversity knowledge and the identification of potential vectors, the effects human activities may have on frog blood parasite life cycles and as such their biodiversity will be clarified. With regards to the ultrastructural analysis completed on *Hepatozoon* sp. A., the material seen contained in the cap of the gamont stage has not yet been observed, nor has anything similar been reported in any other ultrastructural studies of *Hemolivia* or *Hepatozoon*. Its substance and role, however, remains unknown and possibly one of the only means to discovering its make-up and purpose may lie in biochemistry.

6.1.3 Conclusion

In the current study a number of important aspects with regards to monitoring and assessment of amphibians in their natural environment were explored, including looking at and determining diversity and prevalence of blood parasites. Furthermore, premilinary theories, based on assessing and utilising these parasite communities as part of the broader amphibian monitoring plan, have been acknowledged and will be given further consideration in future. Important data on gaining a better understanding of amphibians and their behavioural activities were also gathered, which should be able to assist in conservation actions to effectively protect South African anurans and their required habitat types.

REFERENCES

ABDEL-RAHMAN, A.M., EL-NAFFAR M.K., SAKLA A.A. & KHALIFA, R. 1978. On two species of Haemogregarine from *Bufo regularis* from Assiut Governorate, upper Egypt. *Journal of Egyptian Society of Parasitology* 8: 43–51.

ACOSTA I.C.L., COSTA A.P., NUNES P.H., GONDIM M.F.N., GATTI A., ROSSI J.L., GENNARI S.M. & MARCILI A. 2013. Morphological and molecular characterization and phylogenetic relationships of a new species of trypanosome in *Tapirus terrestris* (lowland tapir), *Trypanosoma terrestris* sp. nov., from Atlantic Rainforest of southeastern Brazil. *Parasites & Vectors* 6: 349.

ALLEN, C.E., ZWAAN, B.J. & BRAKEFIELD, P.M. 2011. Evolution of sexual dimorphism in the Lepidoptera. *Annual Review of Entomology* 56: 445–464.

ALEXANDER, G.J., HARRISON, J.A., FAIRBANKS, D.H. & NAVARRO, R.A. 2004. Biogeography of the frogs of South Africa, Lesotho and Swaziland In: Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland, (eds) L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer, SI/MAB Series #9, pp. 31–47. Smithsonian Institution, Washington, DC.

ALVES DE MATOS, A.P. & PAPERNA, I. 1993. Ultrastructure of erythrocytic virus of the South African anuran *Ptychadena anchietae. Diseases of Aquatic Organisms* 16: 105–109.

ArcGIS® software created by Esri [http://www.esri.com].

BADYAEV, A.V. & HILL, G.E. 2003. Avian sexual dichromatism in relation to phylogeny and ecology. *Annual Review of Ecology, Evolution, and Systematics* 34: 27–49.

BAKER, D. G. 2008. Flynn's parasites of laboratory animals. John Wiley & Sons, pp. 149–150.

BALL, G.H. 1967. Blood sporozoans from East African Amphibia. *Journal of Eukaryotic Microbiology* 14: 521–527.

BARDSLEY, J.E. & HARMSEN, R. 1973. The trypanosomes of Anura. *Advances in Parasitology* 11: 1–73.

BARTA, J.R. & DESSER, S.S. 1984. Blood parasites of amphibians from Algonquin park, Ontario. *Journal of Wildlife Diseases* 20: 180–189.

BARTA, J.R. & DESSER, S.S. 1986. Light and electron microscopic observations on the intraerythrocytic development of *Babesiosoma stableri* (Apicomplexa, Dactylosomatidae) in frogs from Algonquin Park, Ontario. *Journal of Protozoology* 33: 359–368.

BARTA, J.R. & DESSER, S.S. 1989. Development of *Babesiosoma stableri* (Dactylosomatidae; Adeleida; Apicomplexa) in its leech vector (*Batracobdella picta*) and the relationship of the dactylosomatids to the piroplasms of higher vertebrates. *Journal of Protozoology* 36: 241–253.

BARTA, J.R., BOULARD, Y. & DESSER, S.S. 1987. Ultrastructural observations on secondary merogony and gametogony of *Dactylosoma ranarum* Labbe, 1894 (Eucoccidiida; Apicomplexa). *Journal of Parasitology* 73: 1019-1029.

BARTA, J.R., BOULARD, Y. & DESSER, S.S. 1989. Blood parasites of *Rana esculenta* from Corsica: Comparison of its parasites with those of eastern North American ranids in the context of host phylogeny. *Transactions of the American Microscopical Society* 108: 6–20.

BARTA, J.R. 1991. The Dactylosomatidae. *Advances in Parasitology* 30: 1–37.

BARTA, J.R., OGEDENGBE, J.D., MARTIN, D.S. & SMITH, T.G. 2012. Phylogenetic position of the adeleorinid coccidia (Myzozoa, Apicomplexa, Coccidia, Eucoccidiorida, Adeleorina) inferred using 18S rDNA sequences. *Journal of Eukaryotic Microbiology* 59: 171–180.

Basic Local Alignment Search Tool [http://www.ncbi.nlm.nih.gov/Blast/].

BEEBEE, T.J.C. & GRIFFITHS, R.A. 2005. The amphibian decline crisis: A watershed for conservation biology? *Biological Conservation* 125: 271–285.

BELL, R.C. & ZAMUDIO, K.R. 2012. Sexual dichromatism in frogs: natural selection, sexual selection and unexpected diversity. *Proceedings of the Royal Society B: Biological Sciences* 279: 4687–4693.

BENACH, J.L. & CRANS, W.J. 1973. Larval Development and Transmission of *Foleyella flexicauda* Schacher and Crans, 1973 (Nematoda: Filarioidea) in *Culex territans*. *Journal of Parasitology* 59: 797–800.

BERNARD, G.W., COOPER, E.L. & MANDELL, M.L. 1969. Lamellar membrane encircled virses in the erythrocytes of *Rana pipiens*. *Journal of Ultrastructure Research* 26: 8–16.

BILLET, A. 1904. Sur le *Trypanosoma inopinatum* de la grenouille verte d'Algérie et sa relation possible avec les Drepanidium. *Comptes Rendus de la Société de Biologie* 57: 161–164.

BISHOP, P.J. 2004a. *Hyperolius acuticeps* Ahl, 1931. In: Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland, (eds) L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer, SI/MAB Series #9, pp. 136–137. Smithsonian Institution, Washington, DC.

BISHOP, P.J. 2004b. *Hyperolius argus* Peters, 1854. In: Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland, (eds) L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer, SI/MAB Series #9, pp. 137–139. Smithsonian Institution, Washington, DC.

BOULARD, Y., PAPERNA, I., PETIT, G. & LANDAU, I. 2001. Ultrastructure of developmental stages of *Hemolivia stellata* (Apicomplexa: Haemogregarinidae) in the cane toad *Bufo marinus* and in its vector tick *Amblyomma rotondatum*. *Parasitology Research* 87: 598–604.

BOULIANNE, B., RODGER, C.E. & TODD, G.S. 2007. Phylogenetic analysis of *Hepatozoon* species (Apicomplexa: Adeleorina) infecting frogs of Nova Scotia, Canada, determined by ITS-1 sequences. *Journal of Parasitology* 93: 1435–1441.

CARRUTHERS, V. & DU PREEZ, L.H. 2011. Frogs & frogging in South Africa. Struik Nature, Cape Town, South Africa.

CHANNING, A., DU PREEZ, L. & PASSMORE, N. 1994. Status, vocalization and breeding biology of two species of African bullfrogs (Ranidae: Pyxicephalus). *Journal of Zoology* 234: 141–148.

CHANNING, A. & BAPTISTA, N. 2013. *Amietia angolensis* and *A. fuscigula* (Anura: Pyxicephalidae) in southern Africa: a cold case reheated. *Zootaxa* 3640: 501–520.

CHANNING, A., HILLERS, A., LÖTTERS, S., RÖDEL, M.O., SCHICK, S., CONRADIE, W., RÖDDER, D., MERCURIO, V., WAGNER, P., DEHLING, J.M., DU PREEZ, L.H., KIELGAS, T.J. & BURGER, M. 2013a. Taxonomy of the super-cryptic *Hyperolius* nasutus group of long reed frogs of Africa (Anura: Hyperoliidae), with descriptions of six new species. *Zootaxa* 3620: 301–350.

CHANNING, A., SCHMITZ, A., BURGER, M. & KIELGAST, J. 2013b. A molecular phylogeny of African Dainty Frogs, with the description of four new species (Anura: Pyxicephalidae: Cacosternum). *Zootaxa* 3701: 518–550.

CONRADIE, W. 2014. The King of the Dwarves: a new cryptic species of Dainty Frog (Anura: Pyxicephalidae: *Cacosternum*) from the eastern Great Escarpment of South Africa. *Zootaxa* 3785: 438–452.

COOK, C.A., LAWTON, S.P., DAVIES, A.J. & SMIT, N.J. 2014. Reassignment of the land tortoise haemogregarine *Haemogregarina fitzsimonsi* Dias 1953 (Adeleorina: Haemogregarinidae) to the genus *Hepatozoon* Miller 1908 (Adeleorina: Hepatozoidae) based on parasite morphology, life cycle and phylogenetic analysis of 18S rDNA sequence fragments. *Parasitology* 141: 1611–1620.

COOK, C.A., SMIT, N.J. & DAVIES, A.J. 2009. A redescription of *Haemogregarina fitzsimonsi* Dias, 1953 and some comments on *Haemogregarina parvula* Dias, 1953 (Adeleorina: Haemogregarinidae) from southern African tortoises (Cryptodira: Testudinidae), with new host data and distribution records. *Folia Parasitologica* 56: 173–179.

COOK, C.A., SMIT, N.J. & DAVIES, A.J. 2010. Hemoproteids (Apicomplexa: Haemoproteidae) from South African tortoises (Cryptodira: Testudinidae). *Journal of Parasitology* 96: 1168–1172.

COOK, C.A., SMIT, N.J. & DAVIES, A.J. 2014. First record of an intraleucocytic haemogregarine (Adeleorina: Haemogregarinidae) from South African tortoises of the species *Stigmochelys pardalis* (Cryptodira: Testudinidae). *African Zoology* 49: 290–294.

CUNNINGHAM, M. 2004. *Bufo rangeri* Hewitt, 1935. In: Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland, (eds) L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer, SI/MAB Series #9, pp. 77–80. Smithsonian Institution, Washington, DC.

DAVIES, A.J. & JOHNSTON, M.R.L. 2000. The biology of some intraerythrocytic parasites of fishes, amphibians and reptiles. *Advances in Parasitology* 45: 1–107.

DAVIS, A.K., DEVORE, J.L., MILANOVICH, J.R., CECALA, K., MAERZ, J.C. & YABSLEY, M.J. 2009. New findings from an old pathogen: intraerythrocytic bacteria (family Anaplasmatacea) in red-backed salamanders *Plethodon cinereus*. *EcoHealth* 6: 219–228.

DESSER, S.S. 1987. *Aegyptianella ranarum* sp. n. (Rickettsiales, Anaplasmataceae): ultrastructure and prevalence in frogs from Ontario. *Journal of Wildlife Diseases* 23: 52–59.

DESSER, S.S. & BARTA, JR. 1984. An intraerythrocytic virus and rickettsia of frogs from Algonquin Park, Ontario. *Canadian Journal of Zoology* 62: 1521–1524.

DESSER, S.S. & BARTA, J.R. 1989. The morphological features of *Aegyptianella bacterifera*: an intraerythrocytic rickettsia of frogs from Corsica. *Journal of Wildlife Diseases* 25: 313–318.

DESSER, S.S., HONG, H. & MARTIN, D.S. 1995. The life history, ultrastructure, and experimental transmission of *Hepatozoon catesbianae* n. comb., an apicomplexan parasite of the bullfrog, *Rana catesbeiana* and the mosquito, *Culex territans* in Algonquin Park, Ontario. *Journal of Parasitology* 81: 212–222.

DU PREEZ, L. & CARRUTHERS, V. 2009. A Complete Guide to the Frogs of Southern Africa. Struik Nature, Cape Town.

DUTTON, J.E. & TODD, J.L. 1903. First Report of the Expedition to Senegambia. Liverpool, November.

DUTTON, J.E., TODD, J.L. & TOBEY, E.N. 1907. Concerning certain parasitic protozoa observed in Africa. *Annals of Tropical Medicine and Parasitology* 1: 285–370.

EDGAR, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and 546 high throughput. *Nucleic Acids Research* 32: 4673–4680.

ESCALANTE, A.A. & AYALA, F.J. 1995. Evolutionary origin of Plasmodium and other Apicomplexa based on rRNA genes. *Proceedings of the National Academy of Sciences of the United States of America* 92: 5793–5797.

FANTHAM, H.B., PORTER, A. & RICHARDSON, L.R. 1942. Some haematozoa observed in vertebrates in eastern Canada. *Parasitology* 34: 199–226.

FERREIRA, R.C., CAMPANER, M., VIOLA, L.B., TAKATA, C.S.A., TAKEDA, G.F. & TEIXEIRA, M.M.G. 2007. Morphological and molecular diversity and phylogenetic relationships among anuran trypanosomes from the Amazonia, Atlantic Forest and Pantanal biomes in Brazil. *Parasitology* 134: 1623–1638.

FRANÇA, C. 1910. Notes sur des hématozoaires de la Guinee Portugaise. *Archivos do Real Institute Bacteriologico Camera Pestana* 3: 229–238.

FRANÇA, C. 1925. Notes parasitologiques sur l'Angola. *Annales de Parasitologie Humaine et Comparée* 3: 255–262.

FROST, D.R., GRANT, T., FAIVOVICH, J., BAIN, R.H., HAAS, A., HADADD, C.F.B., DE SÁ, R.O., CHANNING, A., WILKINSON, M., DONNELLAN, S.C., RAXWORTHY, C.J., CAMPBELL, J.A., BLOTTO, B.L., MOLER, P.E., DREWES, R.C., NUSSBAUM, R.A., LYNCH, J.D., GREEN, D.M. & WHEELER, W.C. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History* 297: 1–370.

FROST, D.R. 2014: Amphibian Species of the World: an Online Reference. American Museum of Natural History, New York. Version 6.0, Electronic Database, [http://research.amnh.org/herpetology/amphibia/index.html].

Geneious software created by Biomatters [http://www.geneious.com].

GRUBY, M. 1843. Recherches et observations sur une nouvelle espèce d'hématozoaire, *Trypanosoma sanguinis*. *Comptes Rendus Hebdomadaire des Séances de l'Académie des Sciences* 55: 1134–1136.

GRUIA-GRAY, J., PETRIC, M. & DESSER, S.S. 1989. Ultrastructural, biochemical, and biological properties of an erythrocytic virus of frogs from Ontario, Canada. *Journal of Wildlife Diseases* 25: 497–506.

GUINDON, S., DUFAYARD, J.F., LEFORT, V., ANISIMOVA, M., HORDIJK, W. & GASCUEL, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 59: 307–321.

HADDAD, C.R. 2003. Fruit chafers (Coleoptera: Scarabaeidae: Cetoniini) of the Ndumo Game Reserve and Tembe Elephant Park, KwaZulu-Natal. *African Entomology* 11: 130–133.

HAMER, G.L., ANDERSON, T.K., BERRY, G.E., MAKOHON-MOORE, A.P., CRAFTON, J.C., BRAWN, J., DOLINSKI, A.C., KREBS, B.L., RUIZ, M.O., MUZZALL, P.M., GOLDBERG, T.L. & WALKER, E.D. 2013. Prevalence of filarioid nematodes and trypanosomes in American robins and house sparrows, Chicago USA. *International Journal for Parasitology: Parasites and Wildlife* 2: 42–49.

HAMILTON, P.B., STEVENS, J.R. & GIBSON, W.C. 2007. Patterns of co-evolution between trypanosomes and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. *Molecular Phylogenetics and Evolution* 43: 15–25.

HAN, X. & FU, J. 2013. Does life history shape sexual size dimorphism in anurans? A comparative analysis. *BMC Evolutionary Biology* 13: 27.

HARRIS, D.J., DAMAS-MOREIRA, I., MAIA, J.P.M.C. & PERERA, A. 2013. First report of *Hepatozoon* (apicomplexa: adeleorina) in caecilians, with description of a new species. *Journal of Parasitology* 100: 117–120.

HASSAN, I.M. 1992. On haemogregarine parasites of the toad *Bufo regularis* in Qena Governorate, upper Egypt with description of a new species, *Haemogregarina magni*. *Journal of Egyptian German Society of Zoology; Invertebrates and Parasitology* 9: 379–385.

HAYES, T.B. & MENENDEZ, K.P. 1999. The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic reed frog (*Hyperolius argus*: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. *General and Comparative Endocrinology* 115: 188–199.

HILL, D., FASHAM, M., TUCKER, G., SHEWRY, M. & SHAW, P. 2005. Handbook of biodiversity methods. Survey, evaluation and monitoring. Cambridge University Press, New York.

HIRAI, T. & MATSUI, M. 1999. Feeding habits of the pond frog, *rana nigromaculata*, inhabiting rice fileds in Kyoto, Japan. *Copeia* 4: 940–947.

HOARE, C.A. 1920. On some new haemogregarines from British East Africa. *Parasitology* 12: 315–327.

HUELSENBECK, J.P. & RONQUIST, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

JOHNSTON, M.R.L. 1975. Distribution of *Pirhemocyton* Chatton & Blanc and other, possibly related, infections of poikilotherms. *Journal of Protozoology* 22: 529–535.

KIM, B., SMITH, T.G. & DESSER S.S. 1998. The life history and host specificity of *Hepatozoon clamatae* (Apicomplexa: Adeleorina) and ITS1 nucleotide sequence variation of *Hepatozoon* species of frogs and mosquitoes from Ontario. *Journal of Parasitology* 84: 789–797.

KOCHER, T.D., THOMAS, W.K., MEYER, A., EDWARDS, S.V., PÄÄBO, S., VILLABLANCA, F.X. & WILSON, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA* 86: 6196–6200.

KODRIC-BROWN, A. 1998. Sexual dichromatism and temporary color changes in the reproduction of fishes. *American Zoologist* 31: 70–81.

KRUGER, D.J.D., WELDON, C. & DU PREEZ, L.H. 2011. Resolving the confusion: *Amietia vertebralis* and *A. umbraculata* tadpole morphology. *African Zoology* 46: 309–319.

LAINSON, R. 2007. *Theileria electrophori* n. sp., a parasite of the electric eel Electrophorus electricus (Osteichthyes: Cypriniformes: Gymnotidae) from Amazonian Brazil. *Memórias do Instituto Oswaldo Cruz* 102: 155–157.

LAMBIRIS, A.J.L. 1988. A review of the Amphibians of Natal. Lammergeyer Teeanem Printers, Pietermaritzburg.

LANKFORD, B., PRINGLE, C., DICKENS, C., LEWIS, F., CHHOTRAY, V., MANDER, M., GOULDEN, M., NXELE, Z. & QUAYLE, L. 2010. The impacts of ecosystem services and environmental governance on human well - being in the Pongola region, South Africa. Report to the Natural Environment Research Council. University of East Anglia and Institute of Natural Resources, London, Norwich, UK and Pietermaritzburg, South Africa.

LAVERAN, A. 1904. Sur un nouveau Trypanosome D'une grenouille. *Comptes Rendus des Séances de la Société de Biologie* 57: 158–160.

LAVERAN, A. 1905. Contribution a l'etude des grandes hemogregarines des grenouilles. *Comptes Rendus des Séances de la Société de Biologie* 59: 172–175.

LAVERAN, A. & MESNIL, F. 1907. Trypanosomes and Trypanosomiases. *W.T. Keener & CO., Chicago* pp. 1–538.

LEVINE, N.D. 1985. Phylum II. Apicomplexa Levine, 1970. In: An Illustrated Guide to the Protozoa, Society of Protozoologists, (eds.) J.J. Lee, S.H. Hutner, & E.C. Bovee, pp. 322–374. Allen Press, Lawrence, Kansas.

LOVERIDGE, A. 1941. South African frogs of the genus *Hyperolius* in the Museum of Comparative Zoology, Cambridge, Massachusetts. *Annals of the Transvaal Museum* 20: 283–291.

MANSOUR, N.S. & MOHAMMED, A.H.H. 1962. *Lankesterella bufonis* sp. nov. parasitizing toads, *Bufo regularis* Reuss, in Egypt. *Journal of Protozoology* 9: 243–248.

MANSOUR, N.S. & MOHAMMED, A.H.H. 1966a. *Haemogregarina pestanae* in the toad *Bufo regularis*. *Journal of Protozoology* 13: 265–269.

MANSOUR, N.S. & MOHAMMED, A.H.H. 1966b. *Haemogregarina faiyumensis* n. sp. in the toad *Bufo regularis* in Egypt. *Journal of Protozoology* 13: 269–271.

MARTIN, D.S. & DESSER, S.S. 1991. Development of *Trypanosoma fallisi* in the leech, *Desserobdella picta*, in toads (*Bufo americanus*), and in vitro. *Parasitology Research* 77: 18–26.

MATHEW, J.S., VAN DEN BUSSCHE, R.A., EWING, S.A., MALAYER, J.R., LATHA, B.R. & PANCIERA, R.J. 2000. Phylogenetic relationships of *Hepatozoon* (Apicomplexa: Adeleorina) based on molecular, morphologic, and life-cycle characters. *Journal of Parasitology* 86: 366–372.

MEASEY, G.J. (ed.) 2011. Ensuring a future for South Africa's frogs: a strategy for conservation research. SANBI Biodiversity Series 19. South African National Biodiversity Institute, Pretoria.

MEGÍA-PALMA, R., MARTÍNEZ, J., & MERINO, S. 2013. Phylogenetic analysis based on 18S rRNA gene sequences of *Schellackia* parasites (Apicomplexa: Lankesterellidae) reveals their close relationship to the genus *Eimeria*. *Parasitology* 140: 1149–1157.

MINTER, L.R., BURGER, M., HARRISON, J.A., BRAACK, H.H., BISHOP, P.J. & KLOEPFER, D. (eds) 2004. Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

MOHAMMED, A.H.H. & MANSOUR, N.S. 1963. *Haemogregarina aegyptia* sp. nov. from African toads (*Bufo regularis*) and its relationship to *Haemogregarina tunisiensis* Nicolle, 1904. *Proceedings of the Zoological Society U.A.R*. 1: 33–46.

MOHAMMED, A.H.H. & MANSOUR, N.S. 1966. Development of *Haemogregarina boueti* in the toad *Bufo regularis*. *Journal of Protozoology* 13: 259–264.

MORRISON, D.A. 2009. Evolution of the Apicomplexa: where are we now? *Trends in Parasitology* 25: 337–382.

MOULDER, J.W. 1974. The Rickettsias. In: Bergey's Manual of Determinative Bacteriology, (eds.) R.E. Buchanan & N.E. Gibbons, 8th Ed., pp. 882–914. Williams and Wilkins, Baltimore, Maryland.

NEI, M. & KUMAR, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

NETHERLANDS, E.C., COOK, C.A., SMIT, N.J. & DU PREEZ, L.H. 2014. Redescription and molecular diagnosis of *Hepatozoon theileri* (Laveran, 1905) (Apicomplexa: Adeleorina: Hepatozoidae), infecting *Amietia quecketti* (Anura: Pyxicephalidae). *Folia Parasitologica* 61: 293–300.

NICOLLE, M.C. 1904. Sur une Hémogrégarine du Crapaud (Tunis.). *Comptes Rendus des Séances de la Société de Biologie* 56: 330–332.

NORDSTOKKE, D.W. & ZUMBO, B.D. 2007. A cautionary tale about Levene's tests for equality of variances. *Journal of Educational Research Policy Studies* 7: 1–14.

NORDSTOKKE, D.W. & ZUMBO, B.D. 2010. A new nonparametric Levene test for equal variances. *Psicologica* 31: 401–430.

NORIEGA, N.C. & HAYES, T.B. 2000. DDT congener effects on secondary sex coloration in the reed frog *Hyperolius argus*: a partial evaluation of the *Hyperolius argus* endocrine screen. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 126: 231–237.

OKAMOTO, N. & KEELING, P.J. 2014. The 3D structure of the apical complex and association with the flagellar apparatus revealed by serial TEM tomography in *Psammosa pacifica*, a distant relative of the apicomplexa. *PLoS ONE* 9: e84653.

PARKER, H.W. 1930. A collection of frogs from Portuguese East Africa. *Proceedings of the Zoological Society of London* 100: 897–905.

PASSMORE, N.I. & CARRUTHERS, V.C. 1979. South African frogs. University of the Witwatersrand, Johannesburg, South Africa.

PATERSON, W.B., DESSER, S.S. & BARTA, J.R. 1988. Ultrastructural Features of the Apical Complex, Pellicle, and Membranes Investing the Gamonts of *Haemogregarina magna* (Apicomplexa: Adeleina). *Journal of Protozoology* 35: 73–80.

PAPERNA, I. & LAINSON, R. 2003. Ultrastructural studies on the sporogony of *Hepatozoon* spp. in *Culex quinquefasciatus* Say, 1823 fed on infected *Caiman crocodilus* and *Boa constrictor* from northern Brazil. *Parasitology* 127: 147–154.

PAPERNA, I. & OGARA, W. 1996. Description and ultrastructure of *Lankesterella* species infecting frogs in Kenya. *Parasite* 4: 341–349.

PAPERNA, I. & SMALLRIDGE, C.J. 2001. Ultrastructure of developmental stages of *Hemolivia mariae* (Apicomplexa: Haemogregarinidae), natural parasite of the Australian sleepy lizard, in experimentally infected deviant hosts. *Folia Parasitologica* 48: 255–262.

POSADA, D. 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* 2: 1253–1256.

POYNTON, J.C. 1964. The amphibia of southern Africa: a faunal study. *Annals of the Natal Museum* 17: 1–334.

POYNTON, J.C. 1986. *Hyperolius argus* (Anura) in Natal: Taxonomy, biogeography and conservation. *African Zoology* 21: 149–152.

READEL, A.M. & GOLDBERG, T.L. 2010. Blood parasites of frogs from an equatorial African montane forest in western Uganda. *Journal of Parasitology* 96: 448–450.

RIKIHISA, Y., ZHANG, C. & CHRISTENSEN, B.M. 2003. Molecular characterization of *Aegyptianella pullorum* (Rickettsiales, Anaplasmataceae). *Journal of Clinical Microbiology* 41: 5294–5297.

SCHACHER J.F. & CRANS W.J. 1973. *Foleyella flexicauda* sp. n. (Nematoda: Filarioidea) from *Rana catesbeiana* in New Jersey, with a review of the genus and erection of two new subgenera. *Journal of Parasitology* 59: 685–691.

SERGENT, E. & SERGENT, E. 1904. Sur un Trypanosome nouveau, parasite de la grenouille verte. *Comptes Rendus des Séances de la Société de Biologie* 58: 123–124.

ŠIROKÝ, P., KAMLER, M., FRYE, FL., FICTUM, P. & MODRÝ, D. 2007: Endogenous development of *Hemolivia mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in the marginated tortoise *Testudo marginata* (Reptilia: Testudinidae): evidence from experimental infection. *Folia Parasitologica* 54: 13–18.

SMIT, N.J., VAN AS, J.G. & DAVIES, A.J. 2003. Observations on *Babesiosoma mariae* (Apicomplexa: Dactylosomatidae) from the Okavango Delta, Botswana. *Folia Parasitologica* 50: 85–86.

SMITH, T.G., DESSER, S.S. & HONG, H. 1994a. Morphology, ultrastructure and taxonomic status of *Toddia* sp. in northern water snakes (*Nerodia sipedon sipedon*) from Ontario, Canada. *Journal of Wildlife Diseases* 30: 169–175.

SMITH, T.G., DESSER, S.S. & MARTIN, D.S. 1994b. The development of *Hepatozoon sipedon* sp. nov. (Apicomplexa: Adeleina: Hepatozoidae) in its natural host, the northern water snake (*Nerodia sipedon sipedon*), in the culicine vectors *Culex pipiens* and *C. territans*, and in an intermediate host, the northern leopard frog (*Rana pipiens*). *Parasitology Research* 80: 559–568.

SMITH, T.G. 1996. The genus *Hepatozoon* (Apicomplexa: Adeleina). *Journal of Parasitology* 82: 565–585.

SPSS. IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

STEVENSON, A.C. 1911. Fourth report of the wellcome tropical research laboratories. *Gordon Memorial College Khartoum* A: 359–360.

STEWART, M.M. 1967. Amphibians of Malawi. Albany, NY: State University of New York Press.

STUART, S.N., CHA NSO, N.J.S., COX, N.A., YOUNG, B.E., RODRIGUES, A.S., FISCHMA, N.D.L. & WALLER R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1783–1786.

SU, Y., FENG, J., JIANG, J., GUOm Z., LIU, G. & XU, L. 2013. *Trypanosoma epinepheli* n. sp. (Kinetoplastida) from a farmed marine fish in China, the brown-marbled grouper (*Epinephelus fuscoguttatus*). *Parasitology Research* 113: 11–18.

TAMURA, K., STECHER G., PETERSON D., FILIPSKI A. & KUMAR S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725–2729.

TELFORD J.R., S.R. & JACOBSON, E.R. 1993. Lizard erythrocytic virus in east African chameleons. *Journal of Wildlife Diseases* 29: 57–63.

TELFORD, S.R., WOZNIAK, E.J. & BUTLER, J.F. 2001. Haemogregarine specificity in two communities of Florida snakes, with descriptions of six new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) and a possible species of *Haemogregarina* (Apicomplexa: Haemogregarinidae). *Journal of Parasitology* 87: 890–905.

TELFORD, S.R. 2009. Hemoparasites of the Reptilia: Color Atlas and Text. CRC Press, New York.

The IUCN Red List of Threatened Species. Conservation International & NatureServe. 2004. Version 2014.1 [http://www.iucnredlist.org].

TODD, W.J. 1986. Effects of specimen preparation on the apparent ultrastructure of microorganisms, in: Ultrastructure techniques for microorganisms. Plenum Press, New York.

TUZET, O. & GRJEBINE, A. 1957. *Haemogregarina lavieri* n. sp. Hémogrégarine parasite du *Bufo regularis* Reuss. *Society Bioligie Montpellier* 157: 966–968.

UJVARI, B., MADSEN, T. & OLSSON, M. 2004. High prevalence of *Hepatozoon* spp. (Apicomplexa: Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. *Journal of Parasitology* 90: 670–672.

VAN AS, J. 2012. Ecology, taxonomy and possible life cycles of blood protozoans infecting crag lizards (*Pseudocordylus* spp.) from the Eastern Free State highlands. P.h.D thesis, University of the Free State, Free State, South Africa.

VAN AS, J., DAVIES, A.J. & SMIT N.J. 2013. *Hepatozoon langii* n. sp. and *Hepatozoon vacuolatus* n. sp. (Apicomplexa: Adeleorina: Hepatozoidae) from the crag lizard (Sauria: Cordylidae) *Pseudocordylus langi* from the North Eastern Drakensberg escarpment, Eastern Free State, South Africa. *Zootaxa* 3608: 345–356.

VARGAS PARADA, L. 2010. The Apicoplast: An Organelle with a Green Past. *Nature Education* 3: 10.

VONESH, J.R., MITCHELL, J.C., HOWELL, K. & CRAWFORD, A.J. 2009. Rapid assessments of amphibian diversity. In: Amphibian ecology and conservation: A handbook of techniques, (ed) C.K. Dodd, pp. 263–280. Oxford University Press, New York.

WALTON, A.C. 1947. Parasites of the Ranidae (Amphibia). *Journal of Parasitology* 33: supplement, abstract no. 68.

WALTON, A.C. 1949. Parasites of the Ranidae (Amphibia). *Transactions of the American Microscopical Society* 68: 49–54.

WELDON, C. & DU PREEZ, L. 2004. Amphibian population declines and infectious disease. In: Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland, (eds) L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer, SI/MAB Series #9, pp. 11–12. Smithsonian Institution, Washington, DC.

WESOŁOWSKA, W. & HADDAD, C.R. 2009. Jumping spiders (Araneae: Salticidae) of the Ndumo Game Reserve, Maputaland, South Africa. *African Invertebrates* 50: 13–103.

YOUNIS, S.A. & SAOUD, M.F.A. 1969. *Haemogregarina aegyptia* Mohammed and Mansour 1963; a blood parasite of *Bufo regularis* in the Sudan. *Current Science* 38: 435–436

ZHANG, C. & RIKIHISA, Y. 2004. Proposal to transfer '*Aegyptianella ranarum*', an intracellular bacterium of frog red blood cells, to the family Flavobacteriaceae as '*Candidatus Hemobacterium ranarum*'comb. nov. *Environmental Microbiology* 6: 568–573.

APPENDICES

APPENDIX 1

Todd’s Fixative

Appendix 1: Todd's fixative (Todd 1986)	
Materials:	
25% Glutaraldehyde (2.5%),	100ml
Paraformaldehyde in 300 ml Na-cacodilate buffer, heated to 60°C (1.25%)	13.5g
$CaC1_2$ stock solution (0.3%)	100 ml
Picric acid stock solution (0.3g in 100 ml Na-cacodilate buffer) (0.3%)	100 ml
Fill up to the mark Na-cacodilate buffer pH 7.4 ()	1000 ml
Fix 1 – 12 hours depending on the size of the material	
Na-cacodilate buffer: (0.05M)	
Made up of 26.75g of 1NHCl made up in 2500 ml distilled water and added to 10.7g Na-cacodilate in 1000 ml distilled water till the pH is 7.4	

APPENDIX 2

Tables

Appendix 2: Showing historical data for frog species accounts, with additional data on habitat preferences.								
			Habitat preferences					
	Species	**Family**	**Micro-habitat**	**Call site**	**Oviposition**	**Tadpoles**	**Foraging**	**Retreat**
1	*Afrixalus aureus*	Hyperoliidae	Endorheic systems	Elevated on emergent vegetation	Leaf tube at water surface	Well vegetated water	Vegetation around pans	Dense bush around pans
2	*Afrixalus delicatus*	Hyperoliidae	Endorheic systems	Low sites on emergent vegetation	Leaf tube at water surface	Well vegetated water	Vegetation around pans	Vegetation around pans
3	*Afrixalus fornasinii*	Hyperoliidae	Endorheic systems	Elevated on emergent vegetation	Leaf tube at water surface	Well vegetated water	Dense vegetation around pans	Dense vegetation around pans
4	*Amietophrynus garmani*	Bufonidae	Endorheic, palustrine systems	Banks of pans /vleis	Shallow pans /vleis	Floor of shallow water	Open savanna - gardens	Under rocks or logs
5	*Amietophrynus gutturalis*	Bufonidae	Endorheic & palustrine systems	Banks of pans / vleis	Shallow pans /vleis	Floor of shallow water	Open savanna - gardens	Under rocks or logs
6	*Amietophrynus maculatus*	Bufonidae	Palustrine & riverine systems	Banks of streams, vleis	Streams / vleis	Floor of shallow water	Open savanna - gardens	Under rocks or logs
7	*Amietophrynus rangeri*	Bufonidae	Palustrine & riverine systems	Banks of streams, vleis	Streams / vleis	Floor of shallow water	Open savanna - gardens	Under rocks or logs
8	*Arthroleptis stenodactylus*	Arthroleptidae	Terrestrial systems	Concealed under leaf litter	Leaf litter on ground	Metamorphosis complete in egg	Forest floor/woodlands	Leaf litter on forest floor
9	*Breviceps adspersus*	Brevicipitidae	Terrestrial systems	Mouth of burrow	Burrows in sandy soil	Underground nest	Grassy areas	Burrow
10	*Breviceps mossambicus*	Brevicipitidae	Terrestrial systems	Mouth of burrow	Burrows in rocky soil	Underground nest	Open areas	Burrow
11	*Cacosternum boettgeri*	Pyxicephalidae	Endorheic systems	Short flooded grass	Shallow pans	Shallow water	Grassland	Cracks under logs or stones
12	*Cacosternum nanum*	Pyxicephalidae	Endorheic systems	Near shallow water	Shallow pans	Shallow water	Grassland	Cracks under logs or stones

	Species	Family	Micro-habitat	Call site	Oviposition	Tadpoles	Foraging	Retreat
13	*Chiromantis xerampelina*	Rhacophoridae	Endorheic, palustrine & riverine systems	Trees over water	Overhanging branches	Pans	Surrounding vegetation	Exposed sites
14	*Hemisus marmoratus*	Hemisotidae	Endorheic, terrestrial systems	Burrow entrance	Underground chamber	Underground chamber	Around pans	Burrows
15	*Hildebrandtia ornata*	Ptychadenidae	Endorheic systems	Concealed at water's edge	Shallow pans	Shallow water	Diverse savanna	Burrow
16	*Hyperolius argus*	Hyperoliidae	Endorheic, lacustrine & palustrine systems	Elevated sites & floating vegetation	Submerged vegetation	Temporary shallow depressions	Pans	Thick vegetation
17	*Hyperolius marmoratus*	Hyperoliidae	Endorheic, lacustrine & palustrine systems	Reeds & sedges	Submerged vegetation	Variety of water bodies	Vegetation around pans	Tall trees around pans
18	*Hyperolius poweri*	Hyperoliidae	Palustrine systems	Elevated sites on vegetation	Submerged vegetation	Vleis or in	Unknown	Unknown
19	*Hyperolius pusillus*	Hyperoliidae	Endorheic, lacustrine & palustrine systems	Emergent & floating vegetation	Between overlapping leaves	Vleis	Vegetation around pans	Vegetation around pans
20	*Hyperolius tuberilinguis*	Hyperoliidae	Endorheic, lacustrine & palustrine systems	Low on emergent vegetation	Egg mass above water level	Deep pans &pools	Vegetation around pans	Vegetation around pans
21	*Kassina maculata*	Hyperoliidae	Lacustrine, palustrine systems	Emergent vegetation in deep water	Deep water	Deep pools	Around pans	Around pans
22	*Kassina senegalensis*	Hyperoliidae	Endorheic, palustrine systems	Grass & near water	Shallow water at the edge of water	Vleis & pools	Widespread grassland	Concealed in grass tufts
23	*Leptopelis mossambicus*	Arthroleptidae	Endorheic, riverine, terrestrial systems	Low branches	Shallow burrow in mud	Shallow pool	Woodland	Burrows

	Species	Family	Micro-habitat	Call site	Oviposition	Tadpoles	Foraging	Retreat
24	*Phrynobatrachus acridoides*	Phrynobatrachidae	Endorheic systems	Concealed at water's edge	Temporary pans or pools	Shallow pool or puddle	Savanna grassland	Leaf litter
25	*Phrynobatrachus mababiensis*	Phrynobatrachidae	Endorheic systems	Base of grass tufts near water	On water surface	Shallow puddles	Grassland	Unknown
26	*Phrynobatrachus natalensis*	Phrynobatrachidae	Endorheic systems	Base of grass tufts near water	On water surface	Shallow puddles	Grassland	Unknown
27	*Poyntonophrynus fenoulheti*	Bufonidae	Endorheic systems	Base of grass tufts near water	Shallow pans	Floor of shallow water	Diverse savanna	Under rocks or logs
28	*Phrynomantis bifasciatus*	Microhylidae	Endorheic systems	Concealed at water's edge	Vegetated pools	Open water	Savanna	Under logs & stones
29	*Ptychadena anchietae*	Ptychadenidae	Endorheic & palustrine systems	Water's edge	Pans & vleis	Pans &vleis	Diverse savanna	Wide variety of habitats
30	*Ptychadena mascareniensis*	Ptychadenidae	Endorheic & palustrine systems	Water's edge	Pans & vleis	Pans &vleis	Diverse savanna	Wide variety of habitats
31	*Ptychadena mossambica*	Ptychadenidae	Endorheic & palustrine systems	Water's edge	Pans & vleis	Pans &vleis	Diverse savanna	Wide variety of habitats
32	*Ptychadena oxyrhynchus*	Ptychadenidae	Endorheic & palustrine systems	Water's edge	Pans & vleis	Pans &vleis	Diverse savanna	Wide variety of habitats
33	*Pyxicephalus edulis*	Pyxicephalidae	Endorheic & palustrine systems	Shallow water	Temporary pans & shallow vleis	Temporary shallow pans	Widely over savanna	Deep burrows
34	*Schismaderma carens*	Bufonidae	Endorheic, lacustrine & terrestrial systems	Still, shallow water	Shallow, open water	Open water bodies	Wide terrestrial ranges	Crevices in trees etc.
35	*Strongylopus fasciatus*	Pyxicephalidae	Endorheic, palustrine & riverine systems	Tall grass at pans	Shallow vleis & streams	Vleis	Rank grass	Rank grass
36	*Tomopterna cryptotis*	Pyxicephalidae	Endorheic, lacustrine & terrestrial systems	Water's edge	Diverse water bodies	Diverse water bodies	Open grassland	Burrows

	Species	Family	Micro-habitat	Call site	Oviposition	Tadpoles	Foraging	Retreat
37	*Tomopterna krugerensis*	Pyxicephalidae	Endorheic, lacustrine & terrestrial systems	Water's edge	Temporary rain pools	Temporary shallow pans	Savanna	Burrows
38	*Tomopterna natalensis*	Pyxicephalidae	Endorheic, lacustrine & terrestrial systems	Exposed mud banks	Diverse water bodies	Diverse water bodies	Open grassland	Burrows
39	*Xenopus laevis*	Pipidae	Endorheic, lacustrine, palustrine & riverine systems	Underwater	Open water	Open water	Underwater	Totally aquatic
40	*Xenopus muelleri*	Pipidae	Endorheic, lacustrine, palustrine & riverine systems	Underwater	Open water	Open water	Underwater	Totally aquatic

APPENDIX 3

Permits

ORIGINAL

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORDINARY PERMIT

Permit Fee: R 50.00
Receipt No: 447/2012

Permit No: OP 674/2012
Contact: Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 7 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 8512175087085

Mr Leon Nicolaas Meyer
North-West University
Zoology
Private Bag X6001
Potchefstroom
2530

Residential Address
3 Villa Sonica
Elm Street
Irene Park
Klerksdorp
2571

Province: KwaZulu-Natal

To Collect and Export the following species of Amphibians Invertebrates and Reptiles

CAPE TERRAPIN *(PELOMEDUSA SUBRUFA)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

RED EARED SLIDER *(TRACHEMYS SCRIPTA ELEGANS)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

YELLOW-BELLIED HINGED TERRAPI *(PELUSIOS CASTANOIDES)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 09 February 2012

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
Tel +27 33 845 1320 / 1324. Fax: +27 33 845 1747. Fax to Email: 086 529 3320
Email: permits@kznwildlife.com. Website: www.kznwildlife.com

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

MASHONA HINGED TERRAPIN *(PELUSIOS RHODESIANUS)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

SERRATED HINGED TERRAPIN *(PELUSIOS SINUATUS)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

PAN HINGED TERRAPIN *(PELUSIOS SUBNIGER)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

ALL SPECIES OF FROGS

20 (Twenty) Maximum - tissue samples per species and a maximum of 4 infected frogs showing clinical symptoms per species per locality. Collecting may take place throughout the Province including the Protected Areas BUT EXCLUDING THE COASTAL REGION. Except for Giant Bullfrog and African Bullfrog unless a Threatened or protected Species permit is obtained.

To: **ID Number: 8512175087085**

Mr L N Meyer

North-West University
Zoology
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EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

1. It is valid only:

   (i) from : 01 January 2012
   to : 31 December 2012

   (ii) in the original

   (iii) if all **5** pages are signed by the permit holder named above

   (iv) to the permit holder named above and the following Nominees :

   Dr M Badets

   Prof. L du Preez

   Mr G Everson

   Miss M Gericke

   Mr D Kruger

   Dr C Weldon

2. ALL TRAPS & BUCKETS MUST BE THOROUGHLY WASHED AND DRIED AFTER EACH SITE TO PREVENT THE TRANSMISSION OF PARASITES AND PARASITE EGGS FROM ONE SITE TO ANOTHER. The washing water must be disposed of in a manner that will not contaminate natural water bodies.

3. Permit to be returned to E KZN Wildlife, P O Box 13053, Cascades, 3202, upon expiry for renewal or cancellation.

4. Permit shall be carried by holder, or the specified nominees, at all times during use.

5. Outside of E KZN Wildlife areas, use of this permit is subject to landowner's or controlling authority's written permission.

6. Prior to collecting in areas under the control of the E KZN Wildlife the holders shall contact the Officer-in-Charge of the area at least 48 (Forty-eight) hours before commencing, and shall comply with any conditions which the Officer may impose at his discretion.

7. At least one representative specimen (preferably at least one male and one female) of each species collected from each locality must be lodged with a recognised South African museum/herbarium. Holotype specimens, and half the number of paratype specimens, of any

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ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 09 February 2012

for CHIEF EXECUTIVE

Permit Holder

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EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

new species MUST BE DEPOSITED with a recognised South African museum/herbarium, and may only leave South Africa on a loan basis. These specimens are to be deposited in the SA museums within a year of publishing the description of the new species. The holder shall provide the Chief Executive Officer, KZNNCS with the name of the museum at which the specimens have been lodged, and the accession number of each specimen. This condition relates to unavoidable by-catch of non-target organisms as well.

8. A copy or copies of any publication arising from the authority herein contained will be made available to E KZN Wildlife.

9. Should renewal of this permit be desired, a minimum of one month's notice is required.

10. (i) Reserving accommodation within E KZN Wildlife areas is entirely the responsibility of the permit holder. Booking is obtainable at the Central Booking Office, Telephone 033 8451000.(ii) Any assistance required from Board staff will be subject to other demands on the Officer's time and must be arranged in advance with him/her.

11. This permit is valid only if an export/import permit has been issued by the export/import province/country if the legislation applicable in such province/country prescribes such permit.

12. Valid for one consignment only.

13. Holders shall provide the Chief Executive, with a named list of every specimen collected (including the class, order, family, gender and age), the geographical co-ordinates (to seconds accuracy) of each collection locality and dates of collection, as laid out in the following table. A Global Positioning System with the WGS84 Datum should be used wherever possible to determine the geographical co-ordinates of the collection sites; please state the method used.

14. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE
(museum Accession) (ddmmyy) (Seconds Accuracy) (Seconds Accuracy).
Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

15. The transportation of any live specimen by air shall be done in accordance with the International Air Transport Association live animals regulations and any other Act relevant to the transport, keeping, handling, transport, care and/or welfare of the said species.

16. No collecting is permitted within the road reserve which is a strip 30 (thirty) metres either side of a public road, no matter how small or remote the road may be.

17. No collecting is permitted in the wilderness areas within the Protected Area. For confirmation

Please read the Terms and Conditions under which this Permit is issued

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EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

of boundaries of the wilderness area contact the Officer in Charge.

18. The permit holders and nominees adhere to Section 2 and Appendix 1 of the document, and other sections where applicable.

19. By signing the permit or licence the holder accepts, and agrees to comply with the conditions under which it is issued.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 09 February 2012

for CHIEF EXECUTIVE

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EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

ORDINARY PERMIT

Permit Fee: R 50.00
Receipt No: 4159/2012

Permit No: OP 5139/2012
Contact: Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 7 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 6207095025081

Prof. Louis du Preez
School of Environmental Science and Development
North West University,
Potchefstroom Campus
Private Bag X6001
Potchefstroom
2520

Residential Address
Amphibian Research Group
School of Environmental Science and Development
North West University,
Potchefstroom Campus
Potchefstroom
2522

Province: KwaZulu-Natal

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ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 18 December 2012

for CHIEF EXECUTIVE

Permit Holder

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PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
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Potchefstroom
2530

Residential Address

3 Villa Sonica
Elm Street
Irene Park
Klerksdorp
2571

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

1. It is valid only:

   (i) from : 18 December 2012
   to : 31 December 2013

   (ii) if all **4** pages are signed by the permit holder named above

   (iii) to the permit holder named above and the following Nominees :

   Mr D Kruger

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 18 December 2012

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

Mr L N Meyer

Mr E Netherlands

Dr C Weldon

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for CHIEF EXECUTIVE

Permit Holder

13. Holders shall provide the Chief Executive, with a named list of every specimen collected (including the class, order, family, gender and age), the geographical co-ordinates (to seconds accuracy) of each collection locality and dates of collection, as laid out in the following table. A Global Positioning System with the WGS84 Datum should be used wherever possible to determine the geographical co-ordinates of the collection sites; please state the method used.

14. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE
(museum Accession) (ddmmyy) (Seconds Accuracy) (Seconds Accuracy).
Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

15. The transportation of any live specimen by air shall be done in accordance with the International Air Transport Association live animals regulations and any other Act relevant to the transport, keeping, handling, transport, care and/or welfare of the said species.

16. No collecting is permitted within the road reserve which is a strip 30 (thirty) metres either side of a public road, no matter how small or remote the road may be.

17. No collecting is permitted in the wilderness areas within the Protected Area. For confirmation of boundaries of the wilderness area contact the Officer in Charge.

18. The permit holders and nominees adhere to Section 2 and Appendix 1 of the document, and other sections where applicable.

19. By signing the permit or licence the holder accepts, and agrees to comply with the conditions under which it is issued.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 18 December 2012

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE

Conservation, Partnerships & Ecotourism

ORIGINAL

ORDINARY PERMIT

Permit Fee: R 50.00
Receipt No: 422/2014

Permit No: OP 526/2014
Contact: Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 7 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 6207095025081

Prof. Louis du Preez
School of Environmental Science and Development
North West University,
Potchefstroom Campus
Private Bag X6001
Potchefstroom
2520

Residential Address
Amphibian Research Group
School of Environmental Science and Development
North West University,
Potchefstroom Campus
Potchefstroom
2522

Province: KwaZulu-Natal

To Collect and Export the following species of Amphibians Invertebrates and Reptiles

PAN HINGED TERRAPIN *(PELUSIOS SUBNIGER)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

ALL SPECIES OF FROGS

20 (Twenty) Maximum - tissue samples per species and a maximum of 4 infected frogs showing clinical symptoms per species per locality. Collecting may take place throughout the Province including the Protected Areas BUT EXCLUDING THE COASTAL REGION. Except for Giant Bullfrog and African Bullfrog unless a Threatened or protected Species permit is obtained.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 31 January 2014

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE™

Conservation, Partnerships & Ecotourism

ORIGINAL

CAPE TERRAPIN *(PELOMEDUSA SUBRUFA)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

RED EARED SLIDER *(TRACHEMYS SCRIPTA ELEGANS)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

YELLOW-BELLIED HINGED TERRAPI *(PELUSIOS CASTANOIDES)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

MASHONA HINGED TERRAPIN *(PELUSIOS RHODESIANUS)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

SERRATED HINGED TERRAPIN *(PELUSIOS SINUATUS)*

10 (Ten) adults per site Obtain blood samples, mark and release from any locality were terrepins are observed throughout KwaZulu-Natal INCLUDING KZN Wildlife protected areas

To: **ID Number: 8512175087085**

Mr L N Meyer

North-West University
Zoology
Private Bag X6001
Potchefstroom
2530

Residential Address

3 Villa Sonica
Elm Street
Irene Park
Klerksdorp
2571

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 31 January 2014

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE™

Conservation, Partnerships & Ecotourism

ORIGINAL

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

1. It is valid only:

   (i) from : 01 January 2014
   to : 31 December 2014

   (ii) if all **4** pages are signed by the permit holder named above

   (iii) to the permit holder named above and the following Nominees :

   Dr C Weldon

   Mr D Kruger

   Mr E Netherlands

2. ALL TRAPS & BUCKETS MUST BE THOROUGHLY WASHED AND DRIED AFTER EACH SITE TO PREVENT THE TRANSMISSION OF PARASITES AND PARASITE EGGS FROM ONE SITE TO ANOTHER. The washing water must be disposed of in a manner that will not contaminate natural water bodies.

3. Permit to be returned to E KZN Wildlife, P O Box 13053, Cascades, 3202, upon expiry for renewal or cancellation.

4. Permit shall be carried by holder, or the specified nominees, at all times during use.

5. Outside of E KZN Wildlife areas, use of this permit is subject to landowner's or controlling authority's written permission.

6. Prior to collecting in areas under the control of the E KZN Wildlife the holders shall contact the Officer-in-Charge of the area at least 48 (Forty-eight) hours before commencing, and shall comply with any conditions which the Officer may impose at his discretion.

7. At least one representative specimen (preferably at least one male and one female) of each species collected from each locality must be lodged with a recognised South African museum/herbarium. Holotype specimens, and half the number of paratype specimens, of any new species MUST BE DEPOSITED with a recognised South African museum/herbarium, and may only leave South Africa on a loan basis. These specimens are to be deposited in the SA museums within a year of publishing the description of the new species. The holder shall provide the Chief Executive Officer, KZNNCS with the name of the museum at which the specimens have been lodged, and the accession number of each specimen. This condition relates to unavoidable by-catch of non-target organisms as well.

8. A copy or copies of any publication arising from the authority herein contained will be made available to E KZN Wildlife.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 31 January 2014

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE™

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9. Should renewal of this permit be desired, a minimum of one month's notice is required.

10. (i) Reserving accommodation within E KZN Wildlife areas is entirely the responsibility of the permit holder. Booking is obtainable at the Central Booking Office, Telephone 033 8451000.(ii) Any assistance required from Board staff will be subject to other demands on the Officer's time and must be arranged in advance with him/her.

11. This permit is valid only if an export/import permit has been issued by the export/import province/country if the legislation applicable in such province/country prescribes such permit.

12. Valid for one consignment only.

13. Holders shall provide the Chief Executive, with a named list of every specimen collected (including the class, order, family, gender and age), the geographical co-ordinates (to seconds accuracy) of each collection locality and dates of collection, as laid out in the following table. A Global Positioning System with the WGS84 Datum should be used wherever possible to determine the geographical co-ordinates of the collection sites; please state the method used.

14. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE
(museum Accession) (ddmmyy) (Seconds Accuracy) (Seconds Accuracy).
Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

15. The transportation of any live specimen by air shall be done in accordance with the International Air Transport Association live animals regulations and any other Act relevant to the transport, keeping, handling, transport, care and/or welfare of the said species.

16. No collecting is permitted within the road reserve which is a strip 30 (thirty) metres either side of a public road, no matter how small or remote the road may be.

17. No collecting is permitted in the wilderness areas within the Protected Area. For confirmation of boundaries of the wilderness area contact the Officer in Charge.

18. The permit holders and nominees adhere to Section 2 and Appendix 1 of the document, and other sections where applicable.

19. By signing the permit or licence the holder accepts, and agrees to comply with the conditions under which it is issued.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 31 January 2014

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
Tel +27 33 845 1320 / 1324. Fax: +27 33 845 1747. Fax to Email: 086 529 3320
Email: permits@kznwildlife.com. Website: www.kznwildlife.com

APPENDIX 4

Published or submitted papers

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Redescription and molecular diagnosis of *Hepatozoon theileri* (Laveran, 1905) (Apicomplexa: Adeleorina: Hepatozoidae), infecting *Amietia quecketti* (Anura: Pyxicephalidae)

Edward C. Netherlands, **Courtney A. Cook**, **Nico J. Smit** and **Louis H. du Preez**

Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

Abstract: Blood smears prepared from the peripheral blood of 20 wild caught *Amietia quecketti* (Boulenger) from the North-West University Botanical Gardens, North West Province, South Africa, were examined for the presence of haemogregarines. A haemogregarine species comparative in morphology, host and geographical locality to that of *Haemogregarina theileri* Laveran, 1905 was detected. The original description of *H. theileri* was based solely on frog peripheral blood gamont stages. Later, further parasite stages, including trophozoites and merogonic liver stages, were recorded in a related *Amietia* sp. from equatorial Africa. This species was originally classified as a member of the genus *Haemogregarina* Danilewsky, 1885, but due to the close life cycle and morphological resemblance to those of *Hepatozoon* species, *H. theileri* was later transferred from *Haemogregarina* to *Hepatozoon* Miller, 1908. In the present study, meront and merozoite stages not described before, along with previously observed trophozoite, immature and mature gamont stages, are described from the peripheral blood of hosts. In addition, comparative phylogenetic analysis of the partial 18S rDNA sequence of *Hepatozoon theileri* to those of other haemogregarine species, including those of species of *Hepatozoon* and a *Haemogregarina*, support the taxonomic transfer of *H. theileri* to *Hepatozoon*, nesting *H. theileri* within a clade comprising species parasitising other amphibians. This is the first molecular and phylogenetic analysis of an African anuran species of *Hepatozoon*.

Keywords: Amphibia, apicomplexan, blood parasite, frog, haematozoan, haemogregarine, phylogenetic analysis, South Africa

In recent years there has been a strong focus on amphibians as the most threatened vertebrate class (Stuart et al. 2004) and as a result the number of studies and publications on amphibians increased drastically. The number of known species nearly doubled since 1992 (see Köhler et al. 2005) to the current figure of 7 198 (Frost 2014). In southern Africa, the amphibian fauna comprises currently 165 known species of frogs, with the Pyxicephalidae Bonaparte, being the most speciouse, with 48 species reported to date (du Preez and Carruthers 2009, Channing and Baptista 2013, Channing et al. 2013). Frogs are known to harbour a great variety of parasites including monogeneans, digenetic trematodes, cestodes, nematodes, acantocephalans, mites, leeches and protists (du Preez and Carruthers 2009). Despite increase of known anuran species, studies on anuran parasites did not follow the same trend and the known parasite diversity is most likely only a fraction of what exists.

Haemogregarines are among the most commonly recorded apicomplexan protozoans to parasitise frogs. Genera recorded from anurans include *Haemogregarina* Danilewsky, 1885, *Hemolivia* Petit, Landau, Baccam et Lainson, 1990, *Hepatozoon* Miller, 1908, *Lankesterella* Labbé, 1894, and *Schellackia* Reichenow, 1919 (see Davies and Johnston 2000). In the past many anuran haemogregarines were placed in *Haemogregarina* (see Smith 1996). However, Smith (1996) listed all 42 of these species as *Hepatozoon*, based on the developmental stages of these parasites being more characteristic with those of *Hepatozoon* than those of the genus *Haemogregarina*. The genus *Hepatozoon* is, in contrast to *Haemogregarina*, characterised by merogony in the vascular endothelial cells of the vertebrate host, typically without merogony in the peripheral blood erythrocytes, only intraerythrocytic or rarely intraleukocytic gamont stages being present.

Transmission of these protists occurs via the ingestion of a parasitised invertebrate host including mites, ticks, insects, and possibly, but doubtfully, leeches, in which, sporogony typically occurs in the haemocoel (Smith 1996, Davies and Johnston 2000, Van As et al. 2013). *Hepatozoon theileri* (Laveran, 1905) described from a South African frog was one such species of *Haemogregarina* transferred by Smith (1996) to *Hepatozoon*. The aim of this paper is to redescribe this haemogregarine on both morphological and molecular grounds, extending this species' distribution area along with the confirmation of its taxonomic and phylogenetic placement within the genus *Hepatozoon*.

Address for correspondence: L.H. du Preez, Unit for Environmental Sciences and Management, Potchefstroom Campus, North West University, Private Bag X6001, Potchefstroom, 2520, South Africa. Phone: 2718 299 2372; Fax: 2718 299 2370; E-mail: louis.dupreez@nwu.ac.za

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Parasites & Vectors

RESEARCH Open Access

Hepatozoon species (Adeleorina: Hepatozoidae) of African bufonids, with morphological description and molecular diagnosis of *Hepatozoon ixoxo* sp. nov. parasitising three *Amietophrynus* species (Anura: Bufonidae)

Edward C Netherlands*, Courtney A Cook and Nico J Smit

Abstract

Background: Haemogregarines comprise a large group of apicomplexan blood parasites. In 1996 all anuran haemogregarines still in the genus *Haemogregarina* Danilewsky, 1885 were reassigned to the genus *Hepatozoon* Miller, 1908. Most (11/15, 73%) African anuran *Hepatozoon* species have been recorded from the family Bufonidae, however, all these are recorded from only two host species, *Amietophrynus mauritanicus* (Schlegel, 1841) and *Amietophrynus regularis* (Reuss, 1833) from Northern and central Africa. To the authors' knowledge the only description of an anuran haemogregarine from South Africa is *Hepatozoon theileri* (Laveran, 1905), parasitising *Amietia quecketti* (Boulenger, 1895).

Methods: Thin blood smears for morphometrics and whole blood for molecular work, were collected from 32 *Amietophrynus garmani* (Meek, 1897), 12 *Amietophrynus gutturalis* (Power, 1927), and nine *Amietophrynus maculatus* (Hallowell, 1854), in Ndumo Game Reserve and Kwa Nyamazane Conservancy, KwaZulu-Natal, South Africa. Smears were Giemsa-stained, screened for haemogregarines, parasite stages measured, compared to each other and to other described African bufonid haemogregarines. Parasite 18S rDNA was amplified using two apicomplexan-specific primer sets, HepF300/HepR900 and 4558/2733. Resulting sequences of the haemogregarine isolates from the three *Amietophrynus* species were compared with each other and to comparative haemogregarine sequences selected from GenBank.

Results: Morphological characteristics of parasite stages, in particular characteristically capped mature gamont stages, and molecular findings, supported all three haemogregarine isolates from all three *Amietophrynus* species to be the same, a species of *Hepatozoon*, and furthermore different morphologically from other previously recorded bufonid *Hepatozoon* species. The haemogregarine fell within a clade comprising other anuran *Hepatozoon* species and furthermore, within a monophyletic sub-clade along with *H. theileri* and are described as *Hepatozoon ixoxo* sp. nov.

(Continued on next page)

* Correspondence: ec.netherlands@gmail.com
Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

(Continued from previous page)

Conclusions: This is the first morphological and molecular account of *Hepatozoon* species within the family Bufonidae from South Africa, a study hoped to encourage the redescription and molecular analysis of those *Hepatozoon* species described in the past from *Amietophrynus* species, as well as to promote the use of both morphological and molecular characteristics in *Hepatozoon* species descriptions. This will aid in comprehensive *Hepatozoon* descriptions, which along with the use of phylogenetic analysis will give a better indication of these parasites possible vectors and life cycle dynamics.

Keywords: Adeleorid taxonomy, Apicomplexan phylogenetics, Frog blood haematozoan, Haemogregarine, South Africa, Toad

Background

Haemogregarines comprise a large group of apicomplexan blood parasites recorded from a wide range of tetrapod vertebrates and haematophagous invertebrates [1,2]. Haemogregarines are heteroxenous parasites and the group presently includes three families, namely the Haemogregarinidae Léger, 1911, Hepatozoidae Wenyon, 1926, and Karyolysidae Wenyon, 1926. Within these families there are six genera of blood parasites, differentiated on the sporogonic development in their invertebrate hosts [3,4]. Prior to the clarification of the haemogregarine life cycles in anuran hosts by Desser *et al.* [5], most were placed in the genus *Haemogregarina* Danilewsky, 1885. However in 1996, with further insight into the above, Smith [1] suggested that these haemogregarines were better suited to the genus *Hepatozoon* Miller, 1908 and thus transferred them accordingly. As a result, *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 and *Hepatozoon* are the only two haemogregarine genera with species known to parasitise anuran hosts [2], with the latter currently representing the most common intraerythrocytic protozoan parasites of anurans worldwide [1].

According to Netherlands *et al.* [6], the majority (11/15, 73%) of African anuran *Hepatozoon* species have been recorded from the family Bufonidae. Nine of the 11 (81%) species, namely *H. aegyptia* (Mohammed and Mansour, 1963), *H. assiuticus* (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978), *H. boueti* (França, 1925), *H. faiyumensis* (Mansour and Mohammed, 1966), *H. francai* (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978), *H. froilanoi* (França, 1925), *H. lavieri* (Tuzet and Grjebine, 1957), *H. magni* (Hassan, 1992), and *H. pestanae* (França, 1910) [1], were recorded from the same vertebrate host *Amietophrynus regularis* (Reuss, 1833) in Egypt, Sudan, Nigeria, Guinea-Bissau, the Congo, and from northern Angola [7-16] (see Figure 1). The remaining two species, *H. tunisiensis* (Nicolle, 1904) described from *Amietophrynus mauritanicus* (Schlegel, 1841), and *H. moloensis* (Hoare, 1920) described from an unidentified species (likely *A. regularis*), were reported from Tunisia and Kenya respectively [17,18] (Figure 1) (Table 1). These species descriptions range from 20 to more than 100 years ago and were entirely morphology-based. Furthermore, illustrations and measurements were not standardised and therefore inconsistent, and deposited voucher specimens were not mentioned in any of the descriptions and reports. With so many species described from the same host in largely the same area, the huge diversity of *Hepatozoon* species from the above two hosts may in fact be a false representation of what may truly exist.

The aim of this study was therefor to elucidate, via traditional morphological description of peripheral blood stages and molecular techniques, the identity of the *Hepatozoon* species found to infect three *Amietophrynus* species from South Africa and to determine whether they represent a single species or three cryptic species, such as what has been recorded, solely morphologically, from other *Amietophrynus* species further north in Africa. According to the authors' knowledge the only record of an anuran haemogregarine from South Africa is *Hepatozoon theileri* (Laveran, 1905), described from the host *Amietia quecketti* (Boulenger, 1895), family Pyxicephalidae [6]. This paper therefore presents the first morphological and molecular account of a *Hepatozoon* species parasitising members of the Bufonidae from South Africa, and establishes the phylogenetic basis for all other bufonid *Hepatozoon* species in Africa.

Methods

Map (Figure 1) was created through ArcGIS 10.1 [19] using spatial data downloaded from IUCN Red List of Threatened Species [20].

Frog collection and husbandry

Specimens of *Amietophrynus garmani* (Meek, 1897), *Amietophrynus gutturalis* (Power, 1927), and *Amietophrynus maculatus* (Hallowell, 1854) [21,22], were collected by hand at night in the Ndumo Game Reserve, North Eastern KwaZulu-Natal (KZN), South Africa, from a total of eight sites. These include three temporary pans (26°51′54.5″S, 32°09′59.9″E; 26°53′51.6″S, 32°12′57.2″E; and 26°52′53.5″S, 32°15′03.4″E), one wetland (26°54′08.2″S, 32°14′15.0″E), two riverine (26°54′18.5″S, 32°19′24.7″E; and 26°52′57.8″S, 32°18′41.8″E), one lake (26°53′35.6″S, 32°17′45.2″E), and one man-made pond at

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Biodiversity of frog haemoparasites from sub-tropical northern KwaZulu-Natal, South Africa

Edward C. Netherlands *, Courtney A. Cook, Donnavan J.D. Kruger, Louis H. du Preez, Nico J. Smit

Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom, South Africa

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ABSTRACT

Since South Africa boasts a high biodiversity of frog species, a multispecies haemoparasite survey was conducted by screening the blood from 29 species and 436 individual frogs. Frogs were collected at three localities in sub-tropical KwaZulu-Natal, a hotspot for frog diversity. Twenty per cent of the frogs were infected with at least one of five groups of parasites recorded. Intraerythrocytic parasites comprising *Hepatozoon*, *Dactylosoma*, and viral or bacterial organisms, as well as extracellular parasites including trypanosomes and microfilarid nematodes were found. A significant difference ($P < 0.01$) in the prevalence of parasitaemia was found across species, those semi-aquatic species demonstrating the highest, followed by semi-terrestrial frog species. None of those species described as purely terrestrial and aquatic were infected. *Hepatozoon* and *Trypanosoma* species accounted for most of the infections, the former demonstrating significant differences in intensity of infection across species, families and habitat types ($P = 0.028$; $P = 0.006$; $P = 0.007$ respectively). Per locality, the first, the formally protected Ndumo Game Reserve, had the highest biodiversity of haemoparasite infections, with all five groups of parasites recorded. The other two sites, that is the area bordering the reserve and the Kwa Nyamazane Conservancy, had a lower diversity with no parasite infections recorded and only *Hepatozoon* species recorded respectively. Such findings could be ascribed to the anthropogenic impact on the latter two sites, the first by the rural village activities, and the second by the bordering commercial sugar cane agriculture. Future studies should include both morphological and molecular descriptions of the above parasites, as well as the identification of potential vectors, possibly clarifying the effects human activities may have on frog haemoparasite life cycles and as such their biodiversity.

1. Introduction

Amphibians are the most threatened vertebrate group, suffering large-scale declines in species diversity since at least, according to historical data, the 1970s (Stuart et al., 2004). A decade ago the IUCN's Global Amphibian Assessment indicated that a third of the estimated amphibian species had declined or become extinct (Stuart et al., 2004; Beebee and Griffiths, 2005). Such declines may be attributed to a number of factors ranging from habitat destruction, pollution and exploitation, to climate change and disease (Beebee and Griffiths, 2005). The disease known as chytridiomycosis (amphibian chytrid), caused by the fungal pathogen *Batrachochytrium dendrobatidis*, has been responsible for major global amphibian declines (Readel and Goldberg, 2010). Along with chytrid, amphibians are host to a wide variety of parasites (du Preez and Carruthers, 2009; Netherlands et al., 2014a), including intraerythrocytic and extracellular haemoparasites ranging from protozoans, comprising both intracellular apicomplexans (Davies and Johnston, 2000) and extracellular flagellates (Acosta et al., 2013), to extracellular nematode microfilariae (Baker, 2008) as well as those intracellular parasites of uncertain identity such as the viral and bacterial infections (Davies and Johnston, 2000; Davis et al., 2009). The most attention, however, has been given to those parasites of the first three groups mentioned, most likely due to the frequent findings and thus greater basis of knowledge of these organisms in anuran hosts. Furthermore, of these three groups, those of the Protozoa, particularly the apicomplexans, would appear to be the most studied of all (see Davies and Johnston, 2000; Netherlands et al., 2014a; Netherlands et al., 2014b).

However, since few parasite surveys on frogs have been carried out in sub-Saharan Africa, the degree of this haemoparasite diversity remains unknown (Readel and Goldberg, 2010; Netherlands

* Corresponding author. Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa. Tel.: +2718 299 2372; fax: +2718 299 2370.
E-mail address: ec.netherlands@gmail.com (E.C. Netherlands).

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