Breeding biology and ecological niche of the Knysna leaf-folding frog (*Afrixalus knysnae*)

F De Lange

orcid.org 0000-0001-6744-1917

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Supervisor: Prof LH Du Preez

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PREFACE

Finally, there's one other thing that I think…
every person or frog needs to be creative: friends, for me, the best part of creativity is collaborating with friends and colleagues…

Mine happen to be bears, pigs, rats and penguins, but you go with what works for you.

Kermit the Frog *(TedX, 2014)*

A tiny frog native to Africa, and specifically, the areas in and around the picturesque town of Knysna, is the focus of this study. This being exactly what its name, *Afrixalus knysnae* means. At a diminutive size of approximately 2,5 cm and with an almost insect-like screech when calling, this small anuran is vulnerable within an environment under constant anthropogenic pressure.

Human developmental and recreational expansion claiming more and more of its habitat thereby confirming this species place on the International Union for Conservation of Nature, Red List of Species as Endangered (IUCN, 2016). The Knysna leaf-folding frog being the only such species within the Southern Cape region making this infamous list. With an Extent of Occurrence of just more than 27 km^2 and Area of Occupancy at approximately 100 km^2, it is constantly battling for its survival.

Vocalisation in the species is almost subdued amongst its sympatric relatives, while having to employ various modes of vocal signalling to attract females and ward of like-minded suiters. Selection pressure is further heightened given the small sizes of its habitat localities. Only seven such sites are known with the search for more populations ongoing but tedious and mostly fruitless.

Being able to only produce small numbers of egg clutches at a time, it has however evolved ingeniously to protect the brood and developing pollywogs by enclosing it in a protecting leaf-sheath. Maximising the offspring is paramount for sustained survival – that and some human intervention! Protecting the populations where they currently reside must be prioritized and so too possible future distributions, naturally or otherwise.

ACKNOWLEDGEMENTS

I hereby wish to express my deepest thanks to the following people:

Professor Louis du Preez, my supervisor, in being my teacher, mentor and friend through a journey that brought me to this place in my life.

My wife and two daughters, putting up with many strange outings into the field, helping catch frogs, treading through mud with flashlights in hand and waiting up for my return after nightly excursions. In the process also becoming amphibian rescuers and conservationists!

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Christa du Preez and the rest of the du Preez family for always welcoming me to their home when visits to the University was required, feeding me and always making me feel like part of the family.

ABSTRACT

Amphibians as a vertebrate class are under tremendous extinction risk. At the time of finalising this document, 41% of all amphibians worldwide face this prospect (IUCN, 2018). CHAPTER ONE discusses these threats and challenges worldwide and places them in a South African context. The realities of extinction are discussed as they relate to *Afrixalus knysnae* and its habitats within the Southern Cape region of South Africa.

CHAPTER TWO expands on the localities of *A. knysnae* as reported in literature, metadata and local information. Investigating historical sites and employing Ecological Niche Modelling (ENM) as a technique to determine other probable localities are described. Surveys using Passive Acoustic Monitoring are discussed with its applicability within the region and as tool for a sole investigator to verify ENM results. Historical sites are reviewed, recent verified sites updated to the IUCN and a new site reported.

CHAPTER THREE describes and analyses the call structure of *A. knysnae*. The two-part call structure is spectrally and temporally measured to determine finer scale attributes and then compared to other conspecific and congeneric species within relatively close geographic areas. Analysis of this aspect may assist in future taxonomic studies and also more comprehensively understand reproductive behaviour and biology.

CHAPTER FOUR is wholly dedicated to the description of the tadpole with some ancillary notes on the early larvae development and resource use by breeding adults. This description is the first known detailed description of the tadpole of *A. knysnae*, and as such was published in the peer reviewed journal, ZOOTAXA, November 2018.

CHAPTER FIVE reaches some conclusions regarding the current status of the habitats of the species, new localities and possible discovery of future sites using modern technology. The call mode and structures are compared to sympatric species with which it shares habitat while the habitat suitability for tadpoles are placed in context with the vegetation available. All of this is discussed with a view to emphasise conservation efforts to be undertaken and expanded.

Key terms: *Afrixalus knysnae*, Knysna leaf-folding frog, Habitat, Conservation, Tadpole

OPSOMMING

Amphibieërs as vertebrate-klas is onder geweldige uitwissingsdruk, en ten tye van voltooing van hierdie document is dit die lot van 41% van alle amphibieërs wêreldwyd (IUCN, 2018). HOOFSTUK EEN bespreek hierdie bedreigings en uitdagings wêreldwyd en plaas dit in konteks met Suid Afrikaanse toestande. Die realiteite van uitwissing word bespreek soos dit van toepassing mag wees op *Afrixalus knysnae* en die spesie se habitatte in die Suidkaap streek van Suid Afrika.

HOOSTUK TWEE fokus op die lokaliteite van *A. knysnae* soos dit geraporteer word in literatuur, metadata en plaaslike inligting. Ondersoeke van histories geraporteerde habitatte en die toepassing van Ekologiese Nis-Modelleering (ENM) as tegniek om ander moontlike habitatte te identifiseer, word beskryf. Passiewe Akoestiese Monitering (PAM) as wyse van data opname word bespreek asook die toepaslikheid daarvan binne die streek en as hulpmiddel vir n ondersoeker wat aleen werk om die ENM resultate te bevestig. Historiese lokaliteite word hersien, onlangse lokaliteite word opdateer by die IUCN en n nuwe lokaliteit geraporteer.

HOOFSTUK DRIE beskryf en analiseer die roep-strukture van *A. knysnae*. Die twee-komponent roep is spektraal asook temporaal gemeet om die fyner skaal eienskappe te bepaal en dan te vergelyk met naverwante generiese spesies in nabygeleë geografiese gebiede. Analises van die aard mag toekomstige taksonomiese studies behulpsaam wees en mag reproduktiewe gedrag en biologie meer volledig beskryf.

HOOFSTUK VIER is in geheel gewy aan die beskrywing van die spesie se paddavis met bygaande notas oor die vroeë stadiums van paddavisontwikkelling en hulpbronbenutting deur teel-paar volwassennes. Hierdie beskrywing is die eerste bekende gedetaileerde beskrywing van die *A. knysnae* paddavis en as sulks gepubliseer in die joernaal ZOOTAXA, November 2018.

HOOFSTUK VYF bereik n paar gevolgtrekkings ten opsigte van die huidige status van bestaande habitatte, nuwe lokaliteite en moontlike opspoor van toekomstige habitatte met die hulp van moderne tegnologie. Die roep-komponente en strukture word vergelyk met spesies wat gemeenskaplike habitatte benut terwyl hierdie habitatte se paslikheid vir voortbestaan in konteks geplaas word met beskikbare plantegroei. Ten laaste word bewaringspogings en uitbreiding daarvan bespreek.

Sleutelwoorde: *Afrixalus knysnae*, Knysna-blaarvoupadda, Habitat, Bewaring, Paddavis

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

What a queer bird, the frog are
When he sit he stand (almost)
When he hops he fly (almost)
When he talk he cry (almost)
He ain't got no sense, hardly
He ain't got no tail, neither, hardly
He sit on what he ain't got hardly   Anonymous

1.1 Amphibians in the Anthropocene

Amphibians make up in excess of 7900 species and are divided into three orders, Anura, Caudata and Gymnophiona (Frost, 2018). While only frogs (Anura) occur in South Africa, they play an ecologically significant role in the environment. Masses of frog eggs and tadpoles are important food sources while adults keep invertebrate numbers in check as major predators within ecosystems (Halliday, 2008). Invertebrates in both aquatic and terrestrial environments are mostly preyed upon by both amphibian adults and larvae alike, so prolific that the daily consumption of insects by amphibians may actually keep this class of invertebrates manageable for humans (Greenlees *et al.*, 2006). As primary consumers, amphibians are a major defence against agricultural pests, disease carrying vectors and thus a natural bio-agent assisting humans within its living environment. Larvae on the other hand often mostly consume various modes of algae (phytoplankton and periphyton) and assist to control algal blooms (Ranvestel, 2004). Larvae and adults alike furthermore function as prey for terrestrial and aquatic predators, and as such form a high protein food source in the ecosystem where other amphibians, herpetofauna, avifauna and ichthyofauna prey on them. Amphibians can in certain geographical areas form the bulk of the biomass of the terrestrial environment (Gibbons *et al.*, 2006).

The evolutionary significance of amphibians is furthermore evident from their biphasic lifestyle, this being indicative of the all-important aquatic tetrapod ancestors that first made the transition to land somewhere during the late Carboniferous era around 315 million years ago (Roelants *et al.*, 2007). This immense evolutionary step for life on earth makes it obvious why this monophyletic group commands such a vital role in the earth's history and its future (San Mauro, 2010). Although frogs make up the bulk of amphibians (7040 species), salamanders (717 species) and caecilians (212 species) add to the vast diversity of this class (Amphibiaweb.org, 2018). New species of all three orders are being described almost continuously. As many as 60% of the recognised species have been described since 1985 (Amphibiaweb, 2018).

Amphibians however, are mostly undetected during our normal daily lives and in the environments we live, work and play. More often than not, conservation focus is mainly on larger mega fauna, being easily identifiable and being seen as iconic species within landscapes. In the same way, the active lifestyles of birds flying around and rodents scurrying about in natural or man-made environments, attracts more attention than the nocturnal calls of frogs. Smaller invertebrates such as insects also grab the attention of humans more regularly due to its nuisance factor as pests and human health considerations.

Characteristics pertaining to amphibian physiology, ecology and life-histories such as the semi-permeable skin, biphasic life cycle and their low vagility makes them excellent indicators of bio-health and the state of the environment around us (Carey and Bryant, 1995; Berzins and Bundy, 2002). Ecological changes influenced by anthropogenic and natural phenomena concerning climatic conditions, toxicity in water bodies and fragmentation of habitats place tremendous pressure on the environment, both terrestrial and aquatic systems (Waddle, 2006). Both these environments are extensively utilised by amphibians thus changes affecting these systems directly influences amphibian life (Blaustein and Kiesecker, 2002). The pivotal role it plays in the ecosystem have many ancillary effects that directly impact other species of animals and plants.

1.2 Amphibian species declines

At the time of completing this dissertation, the Amphibian Species of the World database (Frost, 2018) list 7906 extant species with 88% being frogs. With 33 amphibian species already being extinct, and two extinct in the wild, a staggering 41% of all known species are listed as being threatened (Categories: Near Threatened - NT, Threatened -T, Endangered - EN and Critically Endangered - CE). Only about 30% are listed as Least Concern (IUCN, 2018). As bio-indicator species, the massive decline of amphibian populations is seen as an indication of declining ecosystem functioning (Gascon *et al.*, 2007). The result of the increased awareness about the plight of amphibians caused studies concerning amphibian declines to intensify since the late 1980's when the phenomenon became apparent (Wake, 1991; Houlahan *et al.*, 2000; Petrovan and Schmidt, 2016). Assessments of the declines gathered momentum after they were reported in 1990, the focus of these assessments being on how widespread the declines actually were. A joint initiative by the IUCN, Conservation International and Nature Conserve, culminated in the first global assessment completed in 2004 known as the Global Amphibian Assessment (GAA) (Measey, 2011).

In South Africa, the matter was also attracting the attention of scientists and in the early part of 1994, Les Minter and Phil Bishop pioneered the South African Frog Atlas Project in conjunction with the Animal Demography Unit at the University of Cape Town. This survey culminated in the Atlas and Red Data Book for frogs of South Africa, Lesotho and Swaziland (hereafter referred to as “The SA Frog Atlas”) (Minter *et al.*, 2004). This work ultimately added to the work initiated by the GAA.

Anurans are the only amphibians in South Africa and as such local assessments are therefore only done with regard to frogs. Comparisons with the work done by the GAA must also be done with this fact considered and with the understanding that approximately 88% of all amphibians globally are within the *Anura* order (Measey, 2011; Frost, 2018). Assessments carried out for The SA Frog Atlas up to 2004 were re-assessed during 2010 by the South African Frog Re-assessment Group (SA-FRoG). The status of South African frogs in 2010 indicated that approximately 18% of species are considered to be near threatened (NT), threatened (T), endangered (EN) or critically endangered (CE).

The underlying causes of the declines may be much more complicated but an understanding of the distribution and endemism may halt or at least slow down extinction rates and create practical and proper conservation plans and programmes (Stuart *et al.*, 2004; Mendelson *et al.*, 2006; Gascon *et al.*, 2007). The figures obtained from the frog assessments carried out in South Africa suggest that local species are faring a little bit better than the global trends. Cause for concern still exists however as it is often the most threatened species which has limited distributions. Globally, this limited distributions are coupled with areas of high endemicity (Brum *et al.*, 2013). This is noteworthy given that

63% of South Africa’s frog species are endemic and therefore research must focus on the distribution of our endemics. Graphically illustrated, species diversity in South Africa is highest towards the Eastern coastal areas in the Kwa-Zulu Natal Province while higher endemicity occurs to the South Western parts of the country, in the Western Cape Province (Fig.1-1). The Western Cape also contain many of the country’s more threatened category species (Turner and Baard, 2017).

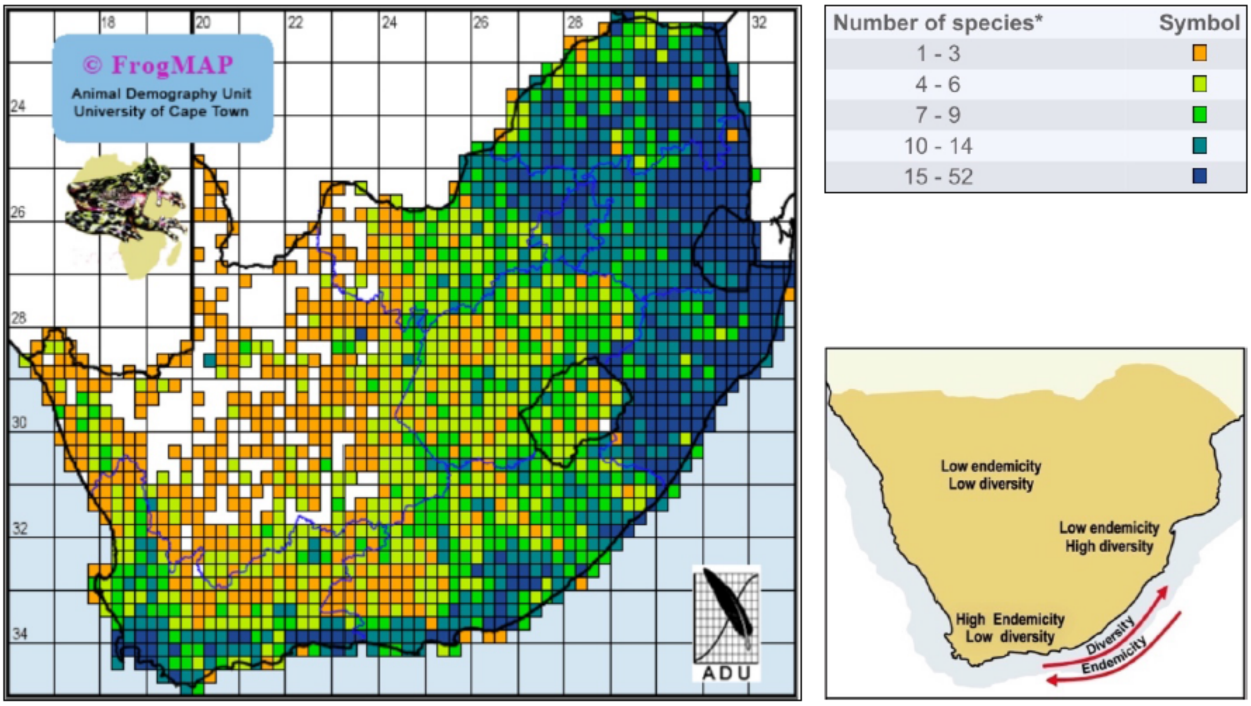


Figure 1-1: **Maps of South Africa indicating the diversity and endemicity of frogs across the country as measured by citizen scientist and other scientific observational records. Maps reproduced from Data obtained from the Animal Demography Unit online resource (Frogmap, 2018) and Du Preez and Carruthers (2009).**

1.3 Threats facing amphibians

Causes of amphibian declines are varied and multitudinous with the explanations and answers as varied as the species themselves (Crump *et al.*, 1992; Blaustein and Dobson, 2006; Lacan *et al.*, 2008). Many studies regarding causes for the decline were done in apparent undisturbed and protected areas where mass extinctions were recorded (Drost and Fellers, 1996). Occurrences of extinctions or declines within seemingly pristine environments, or protected areas and ecological healthy systems have been classed as "enigmatic declines" by Stuart *et al.* (2004) describing factors such as climate change, increased ultra violet radiation, infectious diseases and their ancillary effects (Pounds *et al.*, 2006). These causes are however difficult to explain or study and as such opens up widespread debate and requires meticulous research to verify findings and assist future conservation planning.

More easily understood causes such as habitat destruction or loss, over exploitation and impacts by alien species on endemic populations have been described and researched comprehensively. These more traditional threat interactions together with the less understood factors are increasing the complexity of the situation (Collins and Storfer, 2003). General loss of biodiversity and biomass must however be the focus when any species is in decline, as no species exists in isolation. Coherency of the natural environment makes it imperative to conserve it in totality and with it the species depending on its services (Cincotta *et al.*, 2000). Possible causes of these declines may be habitats being under threat, climate change, environmental pollution, invasive species, disease and the pet-trade, each of these being discussed herein shortly.

1.3.1 Habitats under threat

Human population on earth exceed 7.5 billion people as at June 2018 (UN, 2017), with ever increasing pressure on available resources globally (Bongaarts, 1996; Rizzo, 2017). Natural resources are under tremendous pressure due to the ever increasing need of humans for food, shelter and land. This consumption have dire consequences for other organisms also needing such resources to survive (Hero and Kriger, 2009; Cayuela *et al.*, 2015) Studies during the last decade have indicated that human-altered landscapes as a result of urbanisation and agriculture, caused changes in certain geographical areas, creating ecological traps for animal species that occupy the surrounding habitats (Rotem *et al.*, 2013; Robertson *et al.*, 2018) Metapopulations of certain amphibian species are often artificially re-ordered through boundaries such as roads, pipelines, dams and recreational sport facilities, thereby disturbing continuous and non-continuous habitat types (Downs and Horner, 2011; Puglis and Boone, 2012). Sub-populations are then cut off and these smaller population units are often subjected to decreased genetic drift, inbreeding and selection, creating increased stressors conducive to extinction.

Amphibians more often than not, also have short lives thereby increasing the selection pressures on these populations and substantially accelerating extinction threats (Pickett *et al.*, 2016).

Amphibians face deleterious consequences as a result of this habitat fragmentation and loss (Ficetola *et al.*, 2015). Dependence on fresh water for reproduction of the majority of amphibians increase extinction pressure with freshwater currently being one of the least abundant resources on earth (Olmstead, 2010). Degradation of wetlands are further among the foremost reasons for loss of breeding sites of amphibians, not only due to anthropogenic landscape alteration but also as result of industrial and agricultural chemical effluent (Hazell *et al.*, 2001; Knutson *et al.*, 2004).

1.3.2 Climate change regimes

Temperatures soared during the 20th century, making it the warmest period in the last millennium with marked peak temperatures measured during the 1980's to late 1990's (Jones *et al.*, 2001; Minter, 2011). Southern Africa is not spared from this global changes and severe climatic conditions are already having disastrous effects on our natural environments. Drier hotter summers, flooding, droughts and extreme cold winters are symptoms of these changing climate, altering the environments in all geographic regions in our country as it is globally (Davis-Reddy and Vincent, 2017).
Droughts and extreme cold weather imply less adequate aquatic systems available for amphibian breeding purposes while simultaneously placing pressure on physiological tolerances of adult frogs in terrestrial environments (Carey and Alexander, 2003). Amphibians have demonstrated the ability to adapt to change over the last 300 million years, as some of the first organisms to make the transition between the aquatic and terrestrial environments (Carroll *et al.*, 1999). Rapid changes in climatic conditions coupled with other possible causative factors (i.e. disease) occurring simultaneously in the natural environment, may actually compromise this natural adaptability of amphibians (Nyström *et al.*, 2007; Blaustein *et al.*, 2010; Wassens *et al.*, 2013).

Models are being designed and applied in an attempt to predict the outcomes of climate change regimes, using a multitude of variables affected through altered climate (Murphy and Timbal, 2008). Biotic variables such as water quality, soil conditions, plant communities within the habitat, and predator-prey interactions are taken into account and measured in time and space (Silva *et al.*, 2012; Wassens *et al.*, 2013). Impacts on physiology, life history and ultimate survival of amphibians globally may in turn have further detrimental effects on other animal and plant species relying on amphibians to perform their required ecological services (Welsh and Hodgson, 2008).

1.3.3 Environmental pollutants

While some amphibians only require a damp substrate for their reproductive activities the majority depend on water. Macrophytes and hydrophytes within water bodies assist in either mating behaviour or with egg laying and nesting strategies (Nyström *et al.*, 2007). These hydrophytes also act as deterrents or concealment of larvae against predators and as food source for newly hatched tadpoles (Axelsson *et al.*, 1997). However, studies in European water bodies with elevated nitrogenous and phosphorus compounds indicated poor water quality and low macrophyte coverage which in turn cause impaired reproductive success of resident amphibian species (Knutson *et al.*, 2004; Ortiz *et al.*, 2004). The resultant eutrophication of the aquatic habitat coupled with higher predation possibility and lower food sources leads to greater mortality and increased extinction risks (Hatch and Blaustein, 2003; Relyea, 2003; Teplitsky *et al.*, 2005).

Exposure to toxic chemicals by adult frogs and the direct link to mortality is still somewhat unknown. Tolerances to chemical substances by adult amphibians may be species-specific coupled to other environmental conditions prevalent at specific habitats. The semi-permeable skin of amphibians makes it however highly susceptible to excessive pollutants in its environment (Oldham *et al.*, 1997; Ortiz *et al.*, 2004).

1.3.4 Invasive species

Invasive species among both fauna and flora, are globally seen as a major threat to biodiversity (Measey *et al.*, 2017). The impact of both alien and endemic invasive species on amphibians has been studied extensively over the last decade and the results are far reaching. Results indicate that trophic networks become altered, predator-prey dynamics change, breeding systems are affected and overall ecosystem processes are influenced (Crossland and Shine, 2010; Both and Grant, 2012).

Widescale introduced amphibian species have thus far not impacted the South African landscape. Besides Antarctica, South Africa is the second least impacted region in the world in this regard. Invasive domestic species are however a cause for concern and the need to understand the pathways and impacts these may have on endemics and biodiversity is critical if we are to succeed in curbing invasions of any nature (Measey *et al.*, 2017). Invasive species have the effect that they may outcompete endemic species for breeding sites, food resources and even interbreeding where congeneric species are aligned in the same habitat (de Villiers *et al.*, 2016). Invasive species therefore have an even higher impact on endangered species, should they establish pathways and footholds in vulnerable habitats. *Xenopus laevis, Hyperolius marmoratus* and *Sclerophrys gutturalis* are the three major invasive domestic species impacting frog diversity and ecosystems outside of their natural distribution ranges. These species are currently found in the Western Cape well outside their normal summer rainfall ranges, most probably as result of human interference. The invasive nature of specifically *H. marmoratus* seem to indicate that they are adapting to the climatic changes with relative ease (Tolley *et al.*, 2007).

1.3.5 Disease

Disease, especially infectious pathogens, have over the centuries played a role in the regulation of population numbers for all species on the planet, including humans. The mass die-offs of amphibian species in some pristine areas around the globe can also be attributed to such disease outbreaks, but in these cases, the outbreaks seem unusual, and the same disease are affecting large numbers of different species (Carey, 2000). The main culprits in these instances are *Batrachochytrium dendrobatidis* (Bd) and ranaviruses, leading to the World Organisation for Animal Health (OIE) giving special importance to these pathogens (OIE, 2018).
Ranaviruses infects many ectothermic organisms and occur on all continents across the globe except Antarctica. These viruses have been instrumental in mass die-offs of amphibians (Gray and Chinchar, 2015). Chytridiomycosis, the disease caused by the *Batrachochytrium dendrobatidis* (*Bd*) fungus, has also been documented in Africa and Southern Africa and the spread thereof is constantly being studied with mitigation and preventative measures examined (Weldon *et al.*, 2004). The fact that the disease is difficult to identify and diagnosed in the early lifecycle of frogs makes it able to spread rapidly before an outbreak is noticed and contained (Annis *et al.*, 2004). Mass disease outbreaks, leading to mass mortalities further introduce stressors increasing extinction viability for amphibian species.

1.3.6 Commercial markets and pet-trade

The economic value of animals as part of normal commerce is probably as old as human economic systems themselves (Grier, 2006). Not only are animals consumed as a food source but also as ornaments or as pets with amphibians not being spared this fate. Over exploitation is on the rise, especially among the poorer nations of the world where food is a scarce commodity and ethnic histories dictates food sources.
The pet trade has also redesigned itself to become a sophisticated commodity trading machine where the value of pets increase along with its scarcity and therefor threatened and vulnerable wild species are becoming more and more sought after (Stuart *et al.*, 2004).

1.4 Amphibian Conservation in the Western Cape context

Poynton described the "Cape Fauna" as distinct, represented by the unique assemblages of amphibians coinciding with the Fynbos region (Poynton, 1964) measured total species richness of the Southwestern Cape frog assemblages and commented that "(*it) is conspicuously rich in endemics and range-restricted species, making this assemblage a unique biogeographic entity in the atlas region."* (Alexander *et al.*, 2004). Being mainly a winter rainfall region, summers are relatively hot and dry, while winters are mostly cold with snowfalls on higher lying mountains. This makes the region mostly arid except for parts of the Southern Cape and Cape Fold Mountains.

Despite this arid characteristic of the region and the highly seasonal rainfall regimes, the diversity of the frog assemblages in this province is high, with 60 indigenous South African species occurring within the province borders (Turner and Baard, 2017). This represents almost 46% of the total number of species (131) in South Africa (Amphibiaweb, 2018). All amphibian threat statuses in the Region, as within South Africa, have been standardised to follow the criteria employed by the IUCN (IUCN, 2018). The latest data shows that 36 of the 60 indigenous species to occur in the Western Cape Province are endemic to the province. This endemism is closely aligned with the Cape Fold Mountains where diversity is higher than in lowlands, and this again follows the coverage of the Cape Floristic region (Turner, 2012) This is graphically illustrated in Figure 1-2.

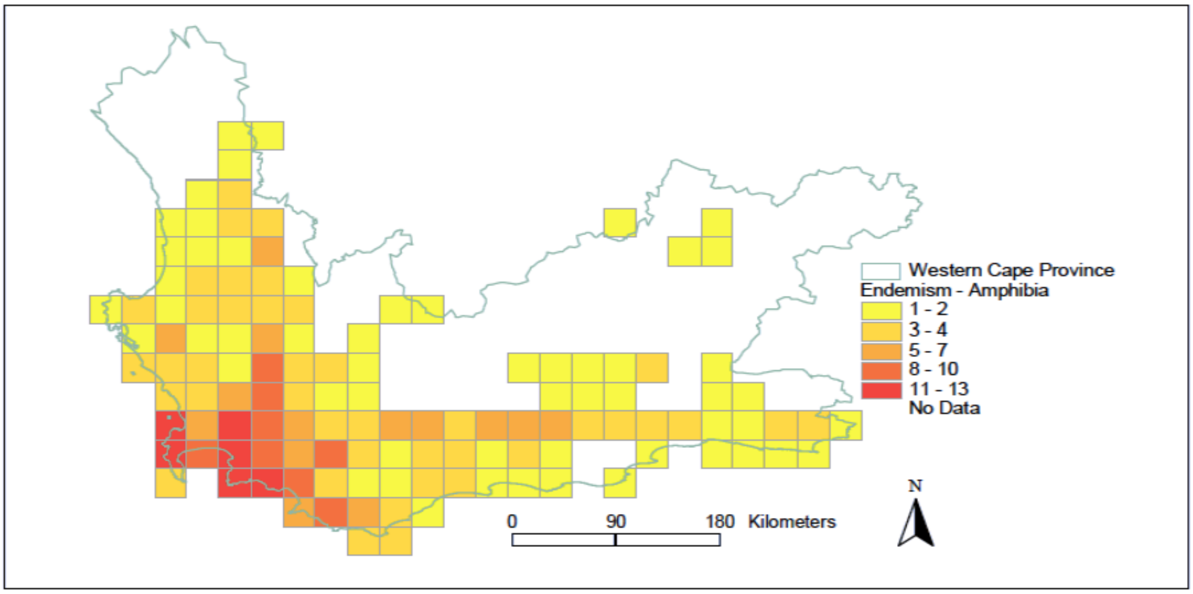


Figure 1-2: Quarter degree map of the Western Cape Province indicating the degree of endemism of frog species on a geographical scale. Map reproduced from the Western Cape Biodiversity Report (Turner, 2012)

Some of the earlier reports regarding distribution data of frogs in the Western Cape Province however lacked credibility and quality. Museum specimens were not always available, lost or incorrectly identified. This was mainly as a result of very little research being done in the area due to a lack of resources and personnel (Baard and de Villiers, 2000). However, in the last two decades reports have been more successful in establishing better distribution data, cleaning up data and updating formal assessments with the publication of The SA Frog Atlas (Minter *et al.* 2004). These more accurate reporting processes have also highlighted five species falling into the Critically Endangered (CR), four in the endangered (EN) and six in the Near Threatened (NT) categories nationally. Currently eight species need their status evaluated and are currently data deficient. This may well categorise them in one of the threatened categories (Turner and Baard, 2017).

Conservation efforts regarding frogs in the Western Cape Province were however lacking behind those in the Eastern Provinces of South Africa at the turn of the century. Only a small number of individuals were working in the field with both capacity and funding lacking. Priorities for future research and conservation efforts have however seen the light in recent years and public awareness and participation is on a steady increase (Measey, 2011; Turner and Baard, 2017)

1.5 Frogs of the George-Knysna area (Southern Cape of South Africa)

The towns of George and Knysna are situated within the Southern Region of the Western Cape. The area is affectionately known as the Garden Route of South Africa due to its natural beauty and diverse natural landscapes, wildlife and floral attributes. The entire area of 698 363 ha have been declared a UNESCO Biosphere wherein core areas (212 375ha), buffer zones (288 032ha) and transition areas (197 956ha) have been identified for conservation, protecting indigenous forests, wetlands, coastal areas and mountains (UNESCO, 2018) (Fig. 1-3).

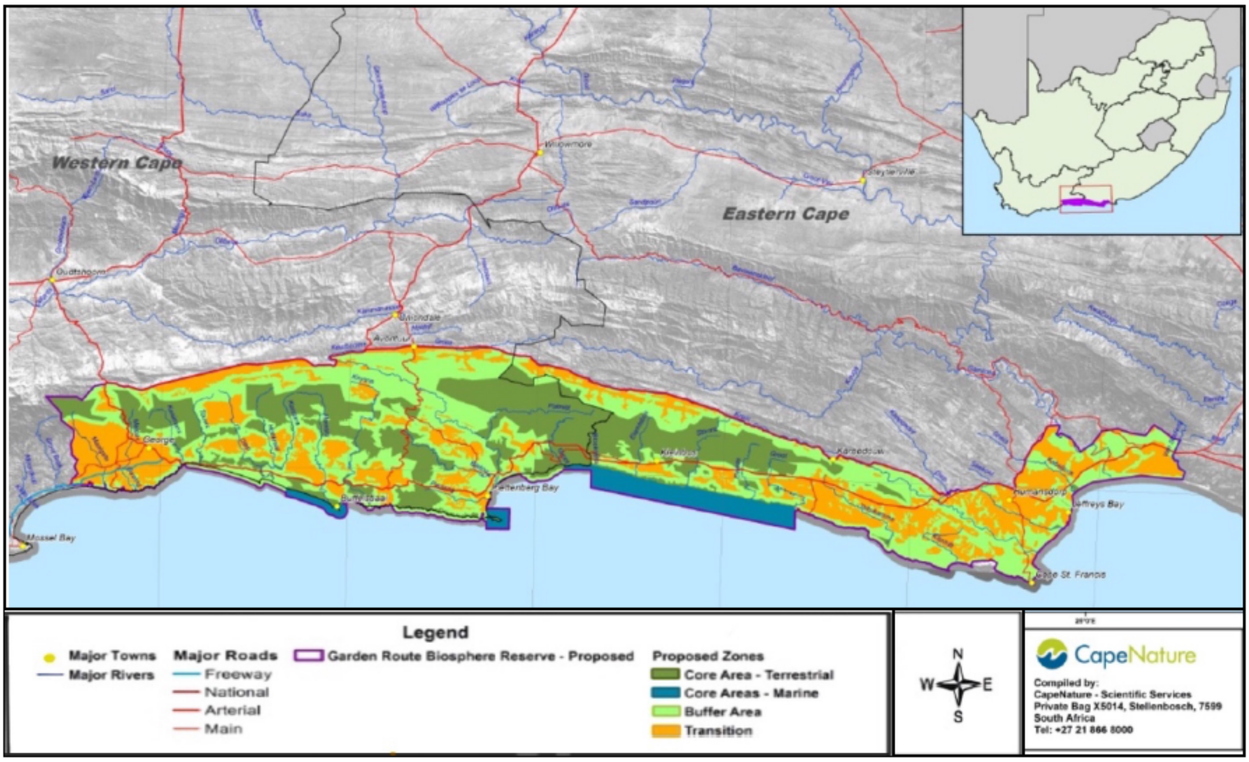


Figure 1-3: Extent of the Garden Route Biosphere reserve in the Southern Cape with descriptions of the various components of conservation areas (UNESCO, 2018).

Marine Protected Areas also forms part of these management areas. Apart from the National Parks areas, many private landowners have grouped themselves into conservatories and many other private properties are being managed by the South African National Parks Authorities and Cape Nature through stewardship programmes (Hase *et al.*, 2010)

Minter *et al* (2004) lists 19 frog species, representing six families of anurans that may be present in this Southern Cape Region. Standardized surveying methods and record keeping as well as proper scientific endeavours shaped the data contained in The SA Frog Atlas and this publication is currently the most comprehensive in mapping frog diversity and biogeography in South Africa. Du Preez and Carruthers (2009) furthermore closely followed The SA Frog Atlas in compiling their comprehensive field guide and extensive use was made of these guides during the collection of field data during the current study. The species as reported by Minter *et al.* (2004) is presented in Table1.

Although the number of species may seem relatively small, the area is within an ecotonal environment, making the species assemblages somewhat unique. This include various fossorial species, stream dwelling and wetland species, with only so-called tree dwelling species lacking in the area.

The most recent State of Knowledge report produced by Scientific Services of the Garden Route National Park, reports that 22 species occur in the park although only 19 species are listed in its Amphibian Index appendix. This information being also mainly based on the Atlas and surveys done more than three decades ago (Arendse *et al.*, 2017). The only Endangered species occurring in this region, *Afrixalus knysnae* (listed as EN by the IUCN, 2016), has a very limited or narrow range of distribution (Branch and Hanekom, 1987). This species forms the basis of this study.

Although extensive work has been done on the *Afrixalus* species of Central and Eastern Africa, very little research has been done and limited information is available on *A. knysnae,* being the only *Afrixalus* species found in the Southern Cape (Pickersgill, 1996; Channing *et al.*, 2012).

Early information on *A. knysnae* first appeared as far back as 1946 where specimens were collected at Diepwalle, Knysna, although at the time it was believed to be specimens of *Megalixalus spinifrons*, Cope (FitzSimons, 1946). Rose describes the breeding behaviour and refers to the species as *Megalixalus spinifrons* (1950). Loveridge described the species for the first time as *Hyperolius knysnae*, with the holotype collected in (1954). Poynton (1964) updated the taxonomy to *Afrixalus brachycnemis knysnae*

Table 1-1: Table depicting species recorded by Minter (2004), Du Preez & Carruthers (2009, 2017) and (Arendse et al., 2017) as being present in the Southern Cape region with current conservation status listed (EN=Endangered, LC=Least Concern)

Family	Species	Status
BREVICIPITIDAE	*Breviceps fuscus*	LC
	Breviceps rosei	LC
BUFONIDAE	*Sclerophrys capensis*	LC
	Sclerophrys pardalis	LC
	Vandijkophrynus angusticeps	LC
HELEOPHRYNIDAE	*Heleophryne regis*	LC
HYPEROLIDAE	***Afrixalus knysnae***	**EN**
	Hyperolius horstockii	LC
	Hyperolius marmoratus	LC
	Semnodactylus wealii	LC
PIPIDAE	*Xenopus laevis*	LC
PYXICEPHALIDAE	*Ametia fuscigula*	LC
	Amietia delalandii	LC
	Cacosternum nanum	LC
	Cacosternum boettgeri	LC
	Strongylopus bonaespei	LC
	Strongylopus faciatus	LC
	Strongylopus grayii	LC
	Tomopterna delalandii	LC

Figure 1-4: ***Afrixalus knysnae* – pair in amplexus (left) with male colouration much brighter than female. Young male on lilly-leaf (right) (Photographs: F. de Lange, 2016)**

Pickersgill (1996) renamed the species to *Afrixalus knysnae* and suggested that it should be grouped within the *A. spinifrons* complex as subspecies with *A. s. spinifrons*, and *A. s. intermedius*. The methods followed by Pickersgill (1996), was to use morphological and acoustic characters to distinguish between the species, with comparisons of the habitat they occupy, geographical ranges and breeding biology. Placing this species in context with each other, mention was made of *A. knysnae* and comparisons were drawn from examining museum specimens and notes from Poynton (1964), Carruthers and Robinson (1977) and collections they made of individuals along the southern and eastern Cape provinces of South Africa.

1.6 Project aims

The broad aims of this project are to investigate and document the basic breeding biology and ecological niche of the Knysna leaf-folding frog (*Afrixalus knysnae*). Data regarding its biology as well as its current true distribution and taxonomic status is in various aspects still deficient and in need of further research.

The commonly referred to "Leaf-folding Frogs", makes up the *Afrixalus* genus and contains approximately 33 taxa dispersed throughout sub-Saharan Africa (Frost, 2018). Its common name is derived from the mode they employ for oviposition, folding small leaves and depositing the eggs inside the fold (Rose 1950). *Afrixalus* are often referred to as a dwarf species, usually this name is conferred to species that seldom exceed 25 mm in length (although this is not an absolute size limitation). Currently *A. knysnae* is grouped in the *A. spinifrons* complex (Pickersgill, 1996, 2005), while the IUCN currently lists *A. knysnae* as endangered (EN) according to its criteria **B1ab(ii,iii,iv,v)**. This classification criteria indicates a species with a limited geographic range where its extent of occurrence (EOO) is less than 100 km^2, the population being severely fragmented with a continuing decline in the area of occupancy, area, extent and quality of habitat, number of locations and number of mature adults. (IUCN, 2018).

Afrixalus knysnae forms part of the Hyperolidae family of anurans and is endemic to South Africa (Pickersgill, 1996). With a body length of only approximately 25 mm and a vertical pupil it is distinguished from *Hyperolius marmoratus* and *Hyperolius horstockii*, with which it occurs sympatricaly. The latter two species are both bigger in size and have horizontal pupils. *Afrixalus knysnae* does not occur sympatricaly with any other *Afrixalus* species, with *A. spinifrons spinifrons and A. spinifrons intermedius* being its closest family members, occurring towards the Eastern Cape Province coastal area and further North into Kwa-Zulu Natal Province. *Afrixalus knysnae* inhabits mainly the Mountain Fynbos and Afromontane forests on the lower slopes of the Outeniqua mountains in the Southern Cape coastal bioregion and certain low-lying coastal areas (Branch and Hanekom, 1987).

Frog surveys in the areas of occurrence have historically focused on visual encounter surveys, sampling and monitoring. This mode of survey is very difficult for this species due to its secretive nature, small size and habitat characteristics. The occurrence of abundant numbers of sympatric species at these habitats, furthermore dominate the acoustic space making aural survey and detection of *A. knysnae* difficult.

Objectives:

1.6.1 Using passive acoustic monitoring (PAM) as method of ecological investigation:

The advancement in technology of passive sound recording instruments enables ecological research using animal vocalisations. Bioacoustic recordings were therefore used to exam and identify calls of all species at selected habitats in order to determine absence or presence of the subject species. This monitoring can therefore be done with minimal to no impact on the environment and with little bias in the habitat during sampling.

1.6.2 Detailed analysis of acoustic data:

Analysis of the data collected through sound recording equipment was to be undertaken with specialised and specific computer software in order to analyse call structure, spectral variations and comparisons with congeneric species and sympatric species.

1.6.3 Conduct Ecological Niche Modelling

Software and algorithms assist researchers to use data collected in the field over various temporal scales to create simulated models of ecological importance. In this instance, the aim is to create models to determine likely habitat areas and thus determine the Area of Occupancy and Extent of occurrence of the species. Data collected via passive acoustic methods and computer aided modelling must then be verified through actual site inspections and ground truthing exercises.

1.6.4 Describing the tadpole and basic breeding behaviour

The lack of information on the biology of this species create opportunities to investigate its breeding behaviour and tadpole morphology. This will be done through visual and photographic investigation at specific sites where populations are in abundance during the breeding season as well as collected specimens housed in collection.

CHAPTER 2 DISTRIBUTION OF *Afrixalus knysnae*

'*Their land swarmed with frogs even in the chambers of their kings.'*

Psalm 105:30 (The Bible)

2.1 Introduction

Creating suitable areas for conservation of plant and animal species in general is of paramount importance in the context of the current levels of biodiversity loss across the globe (Arntzen *et al.*, 2017) . In the light of massive declining amphibian species numbers, this is even more critical as these vertebrates inhabit all manner of habitats, which are oftentimes very niche and thus relatively small, making demarcating protected areas in this case extremely difficult (Ficetola *et al.*, 2015). Authorities need to be guided in prioritising conservation efforts in areas where vulnerable habitat may be located and in light of climate change regimes, where suitable future environmental factors will be at an optimum to ensure species survival (Guisan and Thuiller, 2005). Current geographical distributions of species must be ascertained and more often, potential geographical distributions, in order to plan future orientated conservation efforts (Araújo *et al.*, 2004). Predictive species distribution models can assist in achieving the latter objective and are being used more frequently in ecological studies, this being evident from the vast number of these applications published since 2006 (Elith *et al.*, 2011; Merow *et al.*, 2013)

Early reports on the distribution of *A. knysnae* indicated a small number of localities at only seven sites. The most eastern site being at the hamlet of Covie, near the Eastern Cape Province boundary and the most Westerly site at Groenvlei, Goukamma Nature reserve near the town of Sedgefield (Branch and Hanekom, 1987; Minter *et al.*, 2004; IUCN, 2010). A total distance between the most easterly and westerly sites being just slightly more than 70 km. *Afrixalus knysnae* has been recorded in the ecotonal areas of indigenous forests and fynbos of the Southern Cape (Du Preez and Carruthers, 2009), but the specific habitat requirements of the species are not conclusively defined. Micro habitats at the identified localities also vary and specimens have been collected in "marshy bogs, roadside pools, glades and reed-lined lakes" (FitzSimons, 1946; Minter *et al.*, 2004). This chapter intends describing these pre-existing sites as well as more recent localities where occurrences have been recorded during surveys in 2015 and 2016, and now listed in the updated IUCN assessment (IUCN, 2016). These recently recorded sites seem to be a range shift, while at the same time, recordings and observations of specimens at some of the earlier localities reported in the IUCN 2010 report could not be established.

In order to understand any possible changes in localities or the shifts of its range, Ecological Niche Modelling (ENM) was employed to determine the optimum range of occurrence of the species. ENM is a powerful tool for modern ecologists and can greatly assist in determining Area of Occupancy (AOO) and Extent of Occurrence (EOO) (Rondinini *et al.*, 2005). This modelling tool is of specific assistance for species where information regarding its ecology and population dynamics are lacking or sometimes completely unknown (Jackson and Robertson, 2011). Results obtained through ENM are not a definitive and exact delineation of a distribution area, but give a fairly accurate indication of where a species is likely to be distributed using GIS-based software to then delineate such an area (Tarrant and Armstrong, 2013). Conservation decisions can then include these findings to plan and implement conservation policies where endangered species occur within planned conservation extensions or property acquisitions.

2.2 Methods

2.2.1 Study area

An amphibian diversity study was initially undertaken in 2014 within the confines and immediate adjacent properties of the Garden Route National Park (GRNP), situated in the Southern Cape Fynbos Biome. This is a largely fragmented conservation area of approximately 157 000 ha (Russell *et al.*, 2012) spanning a vast network of pockets of private and state-owned land with the town of George as its most western boundary and the Grootrivier mouth in the Eastern Cape Province, its most eastern boundary (Fig. 2-1). The Outeniqua mountains plateau forms its northern boundary and extends all the way southwards to the ocean. Certain areas along the coast of the Tsitsikamma Forest are also Marine Protected Areas. The nature reserve is divided into three sections, being the Wilderness Coastal Section (WCS), The Knysna Lakes Section (KLS) and the Tsitsikamma Forest and Coastal Section (TFCS). The three sections contain different micro habitat types, topography and climate and thus forms part of the entire study areaThe study area selected for the current study was designed around permanent and temporary terrestrial aquatic habitats found within this defined area.

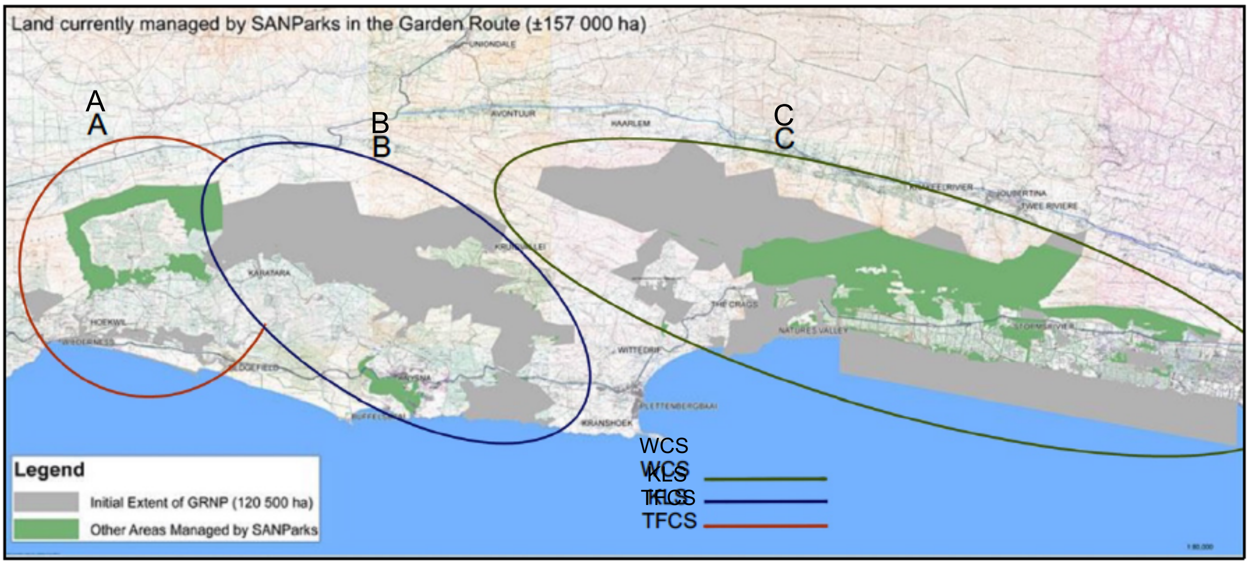


Figure 2-1: Map indicating the extent of the entire GRNP, showing its vastly fragmented nature. (A): Wilderness Coastal Section, (B): Knysna Lakes Section, (C): Tsitsikamma Forest and Coastal Section. (Arendse et al., 2017)

The GRNP incorporates the high ranking conservation status systems of the Touw River and Swartvlei. These systems has its origin in the Outeniqua Mountains, flowing and concluding into the Touw and Swartvlei estuaries, situated between Sedgefield and Knysna within the WCS (Turpie *et al.*, 2004). The Touw system is a designated RAMSAR site and specifically encompasses a vast wetland system consisting of the Serpentine River, Eilandvlei, Langvlei, Rondevlei and all the interleading channels. Together with the Swartvlei system, these lakes and wetland areas are amongst the most researched aquatic systems in South Africa. Early studies focused mainly on hydrology, chemistry, nutrient dynamics pertaining to submerged plants, ecology of estuarine fish and abundances of waterbirds. Recent studies also included dynamics of sediment in estuary mouths, fish species abundances and distribution of aquatic plants and waterbirds (Russell *et al.*, 2012).

The study area falls within a perennial rainfall zone of South Africa, with between 600–700 mm of precipitation annually while slight seasonal variations occur mainly from January to March and August to November (Robinson and De Graaff, 1994). Wind direction is mainly southwest throughout the year with some warm north-westerly winds during season changes and sometimes during the winter months. Wind speeds are normally moderate to low with 97% less than 30km/h. This further coincide with cloudy conditions being a common occurrence and relatively moderate temperatures year-round ranging between 15°C – 25°C during summer months and 7°C – 19°C in winter (Whitfield *et al.*, 1983). During June 2017 and October 2018, the average wind speeds was however dramatically breached, with speeds in excess of 90 km/h along with temperatures in excess of 38°C resulting in tremendous damaging wildfires throughout the region. The impact of these events is currently still being monitored and under observation and will most definitely have a major ecological impact in the region.

Studies into the hydrology of the area investigated rainfall run-off processes in these catchments (Russell *et al.*, 2012). Upper slopes of these river catchments are covered with fynbos vegetation while lower down, the rivers flow through forested areas. These waters are usually dark to light brown stained as result of the humic soils and matter from vegetation and is mostly acidic (Arendse *et al.*, 2017). The rivers in the systems also have perennial natural flows, although the effects of agriculture in the river catchments have altered these flows, causing zero flow conditions periodically. Natural indigenous vegetation has also been impacted substantially as result of the change in land use with many new farm dams recorded in the Touw River catchment (Filmalter and O'Keeffe, 1997).

The terrestrial vegetation in the area has been extensively described by (Moll *et al.*, 1984; Mucina *et al.*, 2014), being within the Fynbos Biome of South Africa. Indigenous forests occur intermittently within the GRNP. Restioid fynbos (or Grassy Dune Fynbos) is the major fynbos plant community represented within the GRNP boundaries and include a number of rare and endemic species (Kraaij, 2007). Many areas around the wetland and lake systems have however been invaded by alien vegetation and this is one of the major concerns threatening the ecology of the park (Jeffrey and Hilton-Taylor, 1990; Baard and Kraaij, 2014).

2.2.2 Literature study

Information regarding characteristics of typical habitat of *A. knysnae* was obtained from field guides, early records of herpetological investigation in the area and the metadata from IUCN reports (FitzSimons, 1946; Rose, 1950; Wager, 1954; Du Preez and Carruthers, 2009; IUCN, 2010; Du Preez and Carruthers, 2017). Literature on characteristics of *A. knysnae* localities are however lacking with the Atlas and Red Data book (Minter *et al*, 2004) and the field guide of Du Preez and Carruthers (2009, 2017) almost the only source of current information in this regard. Consultation with fellow scientists in the area that have a keen interest in amphibian species in the Southern Cape (pers. comm. W Matthee, NMU) and information gleaned from online resources such as iSpot, iNature, FrogMAP and IUCN Red Data List were further used to identify the habitat type and localities.

SANPARKS as custodian and managing authority of the Garden Route National Park, issues State of Knowledge reports on the park periodically. The latest of these reports was published April 2017 (Arendse *et al.*, 2017). These internal documents intend to summarise all information available to the conservation authorities within a specific conservation area pertaining to biotic and abiotic characteristics. All fauna and flora found within the area, as well as history and management aspects of the area are addressed in these reports. In previous reports produced in 2012 (Russell *et al.*, 2012) and 2015 (Baard, 2014) and the current report of 2017 (Arendse *et al*. 2017) , *A. knysnae* is only mentioned and referred to as an endangered species and a species of concern. During surveys reported in the publication leading to the 2012 report, no sightings or observations were recorded of the species and it is unclear from the 2015 report whether any observation was made prior to publication of the latter.

The 2017 report indicates that 22 species of frogs occur within the boundary of the GRNP and makes the statement that 14 species have been formally recorded. The appendix listing the amphibian species however only lists 19. The report confirms that very few publications have been made regarding amphibians in the Park and refers to the ecological knowledge contained in the Whitfield (1983) publication and work done by Carruthers and Robinson (1977) and Branch and Hanekom in (1987) and then refers any further information regarding amphibians to the work by Minter *et al.* (2004). Species that may occur around the Knysna estuary was last updated by Passmore and Carruthers (1979), when the list of Poynton (1964) was supplemented.

This study commenced in 2014 with general amphibian diversity surveys undertaken within the study area. Initial surveys regarding verification of the occurrence localities of *A. knysnae* as stipulated in the 2010 IUCN report was made during the breeding season in 2015. Occurrence records obtained from the IUCN Red Data List indicate that only seven known locations of *A. knysnae* have been recorded up to 2010. It must be noted that during this study the African Amphibian Specialist Group (AASG), updated its site locality listings of *A. knysnae* in 2016, the findings in this study therefore being reported to the AASG and the data formalised in the IUCN report (IUCN, 2016). The information collected from localities herein therefore reflects the IUCN 2010 reported data, placed in context with the new IUCN 2016 report. In this regard, the IUCN 2016 report still list seven localities, albeit different sites, confirming the updated locality information.

2.2.3 Site surveys

Using the coordinates contained within the metadata from the IUCN 2010 report together with available SANPARKS maps and fine scale GIS landcover data files, localities could be mapped and then verified in the field. Site visits were undertaken to each of these sites to visually inspect, evaluate and record the biotic habitat characteristics present. Information regarding the presence and size of the water body, depth of the water, type of vegetation present and any other factors such as proximity to indigenous or planted forests, roads, rural, agricultural or urban developments were recorded. This would also assist in confirming the suitability of sites with viable habitat for *A. knysnae*. Information in this regard would be useful for inclusion in later testing of distribution models and further surveys during ground truthing exercises. No subject species were visually or aurally encountered at these inspection times as the surveys took place during the daylight hours.

The site inspections took place over two periods of three weeks each in September and October 2015, this being an optimal time to ascertain the presence of water bodies at the sites after the first spring rains and at the height of *A. knysnae* breeding season (Du Preez and Carruthers 2009, 2017). Photographic records were made of all the IUCN sites during the visual inspections. During these surveys, new sites identified since the IUCN 2010 report were also inspected and the biotic habitat attributes for probable presence or absence of the species verified. This data was confirmed with the AASG prior to the new 2016 report being updated.

Passive Acoustic Monitoring (PAM) was the ancillary method of investigation enhancing the visual inspections at the sites. Song Meters® were used in this regard and implemented for periods of four days at a time, and every site was monitored at least twice during the study period using one Song Meter per site. The bioacoustic recorders were placed at the localities and programmed to record for the first 10 min of every hour on the hour starting at 17:00 in the evening and ending at 6:00 the following morning. While visual investigations were made of the habitat requirements during the day, nocturnal acoustic data would be collected using PAM in confirmation of species presence or absence at the sites.

2.2.4 Predictive species modelling

The most commonly used software application, MaxEnt, uses presence-only data together with environmental predictors to perform Ecological Niche Modelling (Phillips *et al.*, 2017). MaxEnt was consequently used in this study to determine possible distribution localities of *A. knysnae*. The analogue coordinates regarding the localities contained in the IUCN 2010 report and the updated locality data from the 2016 IUCN report were converted to decimal degree coordinates and entered into the MaxEnt application in order to run the model, analyse the data and determine predicted occurrence of the species by way of ENM.

Only continuous variables pertaining to environmental factors and bioclimatic variables were used in the modelling process, these pertaining to temperature, precipitation and topographic characters. Most of these environmental attributes were gleaned from international datasets, literature and personal surveys of known localities of *A. knysnae* within the study area (Minter *et al.*, 2004; Pickersgill, 2005; Du Preez and Carruthers, 2009). WorldClim was used as the source for climatic variables for purpose of the current model (www.worldclim.org, accessed on 20 October 2015). Together with an altitude variable, nineteen bioclimatic variables were used in the model pertaining to the general

South African region represented by the 30 arc-second resolution WorldClim tiles (Fig. 2-2). The bioclimatic variables initially used to fit the model are set-out in Table 2-1.

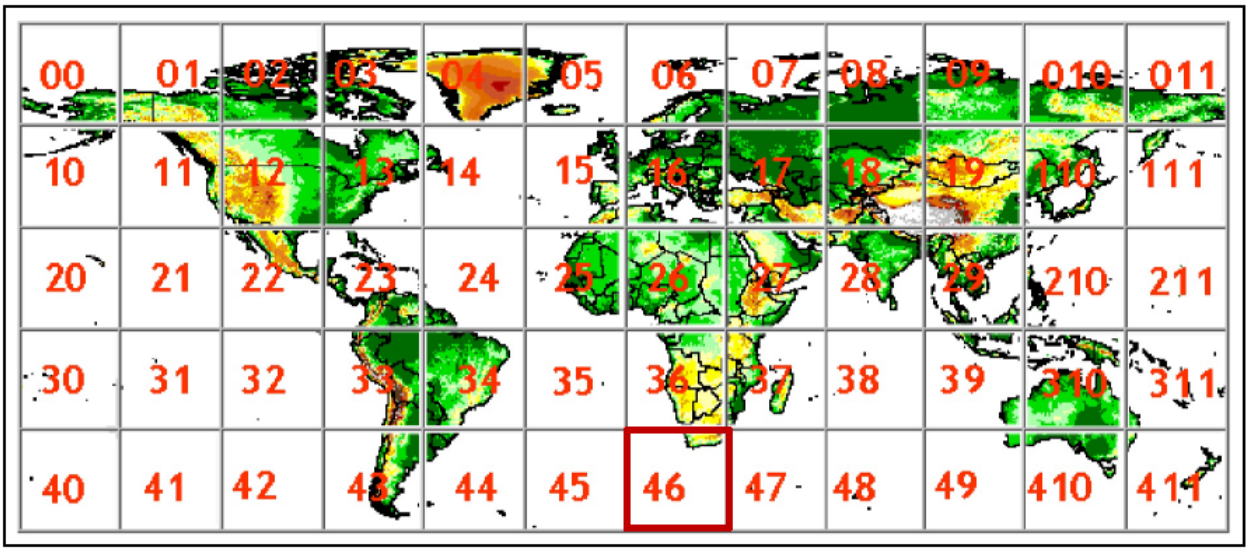


Figure 2-2: Graphical illustration of the tiles employed by WorldClim to determine variable attributes of the area within which the study area will fall. (www.worldclim.com). Tile 46 applicable to the current study

Table 2-1: The main Climatic variables associated with the BioClim tile (#46) applicable to the region within which the current study area is located.

AltSite	Altitude
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Temperature Range
BIO3	Isothermality
BIO4	Temperature Seasonality (Std dev x 10)
BIO5	Max Temp. Warmest month
BIO6	Min Temp. Coldest month
BIO7	Temperature Annual Range (BIO5 - BIO6)
BIO8	Mean Temp Wettest Quarter
BIO9	Mean Temp Driest Quarter
BIO10	Mean Temp Warmest Quarter
BIO11	Mean Temp Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation Wettest Month
BIO14	Precipitation Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation Wettest Quarter
BIO17	Precipitation Driest Quarter
BIO18	Precipitation Warmest Quarter
BIO19	Precipitation Coldest Quarter

In order to create credible modelling results using the MaxEnt algorithm, a number of replicates must be run after the first fitting of the data within the application. This first run produces a jack-knife graph indicating the bioclimatic variables relevant to the possible distribution of a species. These replicates are then run using only these most relevant bioclimatic variables. The user interface (UI) during this study was configured to allow 500 iterations in order to ensure algorithm convergence for the small number of records and limited geographical distribution of known *A. knysnae* localities records. All other settings of the application were left at default.

In order to ensure that the background samples used by MaxEnt were relevant to the region within which the species were recorded, a "mask" was created by a structured polygon within the GIS software application, QGIS® and the environmental variables clipped to this boundary. This produced a probability map indicating distribution areas of the species which could be re-imported to GIS and conventional satellite imaging. In order to best fit possible areas of occurrence, hard transformed and built environments were subtracted from the MaxEnt produced probability map.

Emphasis was then placed on the presence of wetlands or waterbodies indicative of the habitat preference by *A. knysnae* to further delimit the possible occurrence localities. Using commercially available GPS software (Garmin BaseMap®) and Google Earth Pro®, 27 possible localities where habitat may be present were identified and selected for surveys, these being in protected or natural areas and even in anthropogenic altered environments. Information regarding habitat type and vegetation occurrence at these sites together with elevational and topographic information was manually recorded each time.

All 27 sites were surveyed by way of visual surveys during the day to verify habitat viability. Where the basic criteria were present, Song Meters® were then deployed to record frog community calls. Only two Song Meters could be deployed at any given instance which resulted in a short interval setup at all the sites in order to record and collect as much possible data during the short breeding period.

2.3 Results

2.3.1 Initial localities survey and ground truthing

Information from the desk-top study regarding localities from the IUCN 2010 report indicated certain conflicts regarding metadata coordinates. Plotting the longitude and latitude references within modern day GPS software applications, placed points obtained from the metadata in locations that were clearly unable to house suitable habitats for *A. knysnae* (see Table 2-3 in this regard). Information regarding the origin of the coordinates that was obtained from the metadata could not be established with certainty. Various reasons in this regard could account for these discrepancies. Conversions from different DATUM settings between the initial GPS equipment used at time of observation and those employed for this study may be one such cause. Incorrect capturing of the data many years after original observation could also lead to errors. The dates on which the sites were first surveyed according to the IUCN (2010) are presented in Table 2-2 together with the dates on which surveys were conducted in this study. The IUCN 2010 reported localities' GPS Coordinates as well as those of the IUCN 2016 localities are listed in decimal format as required for ENM processing.

Table 2-2: Site survey information regarding the locality designation with the dates it was first surveyed and dates surveyed during this study. Coordinates are indicated in decimal degrees.

Location No	IUCN report	Lat	Long	Date: First Site Recording	Date: Surveyed for Study
Ak1	2010	-30,9333	23,1500	1940-12-02	2015-10-03
Ak2	2016	-33,9381	23,4675	2001-12-27	2015-09-26
Ak3	2010	-33,9622	23,6044	2002-09-17	2015-09-26
Ak4	2010, 2016	-33,9633	23,5964	2002-09-17	2015-09-26
Ak5	2010, 2016	-33,9614	23,6031	2001-10-04	2015-09-26
Ak6	2010	-34,0475	22,8692	2010-06-10	2015-10-03
Ak7	2010, 2016	-34,0333	22,8667	2001-11-14	2015-10-18
Ak8	2016	-33.9513	22.5262	2015-11-05	2015-11-05
Ak9	2016	-33.9445	22.5217	2015-11-05	2015-11-05
Ak10	2016	-34.0257	22.8345	2015-11-05	2015-11-05

The dates of survey of the initial records placed in context with the attributes at the sites surveyed on the dates recorded in the current study is described in Table 2-3. The account of the attributes of the environment at the sites during surveys in this study is summarised in the table. Analysing the information contained in Table 2-2 correlated with Table 2-3 indicates various anomalies and may raise questions regarding modern data capture methods and/or current state of habitat, which might be very different as a result of anthropogenic changes to the landscape since the time of the original record. These observation records are still extremely important to analyse, and even to continuously monitor to ensure that conservation efforts be implemented at the right localities in context with continued anthropogenic pressures.

Detailed analysis of the sites inspected and not containing any viable habitat are presented hereunder:

1. Observation record at the Ak1 site was obtained during 1940, according to the metadata. The identity of the observer is not known from the data but probably was FitzSimons in person during his expedition between October and December 1940 (FitzSimons, 1946) and was only uploaded by the Animal Demography Unit to their database in 2002. The correctness of the coordinates uploaded may be questionable and plotting this on the finer scale maps and satellite images available, indicated that the likelihood of suitable habitat at this site was also negligible as it was at an altitude of 640m and in a forested area with no waterbodies close by. Changes in the land use over time may account for the discrepancies too, but the topography does not lend itself as a temporary wetland habitat locality.

2. The record for Ak6 is a sighting during June, well outside the breeding season of *A. knysnae* and adults are usually not observable during this period. The desktop study further revealed that the area at Ak6 is currently a residential area and has been so since the late 1990's. This record being during 2010 would then have been within these gardens and the current survey confirmed that suitable habitat within the residential garden was not present.

3. The exact locality of Ak2 is within 30m of a natural shallow dam used only as a drinking facility for cattle being farmed within a large pasture. The water body is highly degraded, trampled and extremely churned with heavily silted water. Although this is a wetland/waterbody, no suitable habitat is available and the likelihood of the species currently occurring in the pasture very minimal.

Table 2-3: Summarised findings of surveys at the sites of reported localities from the IUCN 2010 report.

Site	Site Attributes	Verification Notes
Ak1	The coordinate is situated on top of a foothill within the Outeniqua Mountains and within an indigenous forest. However, the area is an open clearing with no water body nearby. A fast-flowing stream is the closest aquatic feature at approximately 800m from the way-point in a valley.	Dense indigenous forest with detritus leaf litter surrounds the immediate area. Steep slopes all around the area. The locality is not currently suitable habitat.
Ak2	Grass pastures with cattle and horses in fields. Some dams exist but only used by animals as drinking location.	Entire area is used as cattle farm with some dams, all trampled by cattle. *Cacosternum nanum* and *Strongylopus grayii* present at the dams.
Ak3	The exact coordinate is situated on a small rise approximately 80 m away from a hiking trail within a small fynbos reserve near the village of Covie. No water body exists at the site.	Fynbos abounds all around the site with the nearest water body from the coordinates of AK3 approximately 300m away.
Ak4	A woodcutter hut is situated at the exact coordinate. Habitat appears to be present around this area and close vicinity of the dwelling.	Area around the hut has been severely impacted by human footpaths. A small indentation exists about 80m away from the hut with a small amount of marshland. *C nanum* and *S grayii* were audible during night survey at the site.
Ak5	A water body and large enough marshland exists at the exact coordinates. The site is located within the village of Covie with maximum water depth of approximately 50mm and aquatic and semi-aquatic vegetation present.	Site is just off the road and within the boundary of a protected area of fynbos.
Ak6	The exact coordinates seem to be within the boundaries of residential properties.	The entire surrounding area have been built up and residential estates abound.
Ak7	The site could not be verified as it is within a private conservancy and access could not be gained. The surrounding area is however very close to the marine shoreline and likelihood of suitable habitat close by was not visible	Typical cape thicket and fynbos with very little marshy areas or water bodies. Close to the marine shoreline.

The survey only yielded positive correlations with suitable habitat and possible presence at the sites denoted as Ak3, Ak4 and Ak5. The entire area around the village of Covie had numerous possible marshlands and water bodies that may indeed be host to *A. knysnae*. The initial stages of the coordinate survey was to verify the exact coordinates of the IUCN 2010 data and accurately identify characteristics of suitable habitat for *A. knysnae*. These coordinates are graphically depicted in Figure 2-3. Establishing the correctness of the location data is important to understand the relevance of the IUCN records for the subject species in order to confidently use this locality information during the Ecological Niche Modelling process.

Figure 2-3: The seven site localities where observations have been recorded for the species and reported in the IUCN 2010 Metadata (represented by red/white markers) and the three sites where new populations were discovered during this study (represented by blue/white markers) (Satellite image: Google Earth®)

2.3.2 Ecological Niche Modelling: Using MaxEnt

All recorded localities as reported in the IUCN 2010 and IUCN 2016 reports have been converted to decimal coordinates for input in the MaxEnt Application enabling the algorithm to execute the Ecological Niche modelling process. These decimal coordinates of the localities as indicated in Table 2-2 and graphically depicted in figure 2-3, is situated across the study area. This area is also defined as the Landscape of Interest for purposes of ENM and masking the area demarcates it within the GIS systems used in the study, QGIS®. This furthermore delimits the background data within which the ENM model is executed.

MaxEnt uses algorithms to perform jack-knife tests on the variables entered in the application, Receiver Operating Characteristic graphs (ROC), area under the curve calculations (AUC) and bioclimatic variable plots which is analysed to evaluate the model's performance. These results then produce a graphical HTML standard probability map.

The jack-knife test performed by MaxEnt is executed to establish the most relevant variables likely to influence the probable distribution of a species. The output is in graph-form. From the altitude and 19 climatic variables from the BioClim dataset used for input in MaxEnt, the result from jack-knife testing highlighted ten variables most likely to influence the distribution probabilities of the species, this being reflected in Table 2-4 and graphically illustrated in the graph (Fig. 2-4).

These tests are done primarily by testing the variables to each locality at a time, then removing that locality data and doing the same to the next and so on. This ensures that small number of data sets are each tested for maximum probability to perform against each of the variables in the model. The variables mostly impacted on the species are then tested against all the locality data to create the outcomes.

Table 2-4: Variables relevant to the modelling of distribution of *A. knysnae* as returned by MaxEnt jack-knife testing

AltSite	Altitude
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Temperature Range
BIO4	Temperature Seasonality (Std dev x 10)
BIO6	Min Temp. Coldest month
BIO7	Temperature Annual Range (BIO5 - BIO6)
BIO9	Mean Temp Driest Quarter
BIO10	Mean Temp Warmest Quarter
BIO11	Mean Temp Coldest Quarter
BIO19	Precipitation Coldest Quarter

The jack-knife graph (Fig 2-4) indicates each variable's measure of goodness of fit during the model's training process as measured against the species presence and background data. Each variable starts at 0 and increase towards an asymptote, this indicating the probability fit of the data.

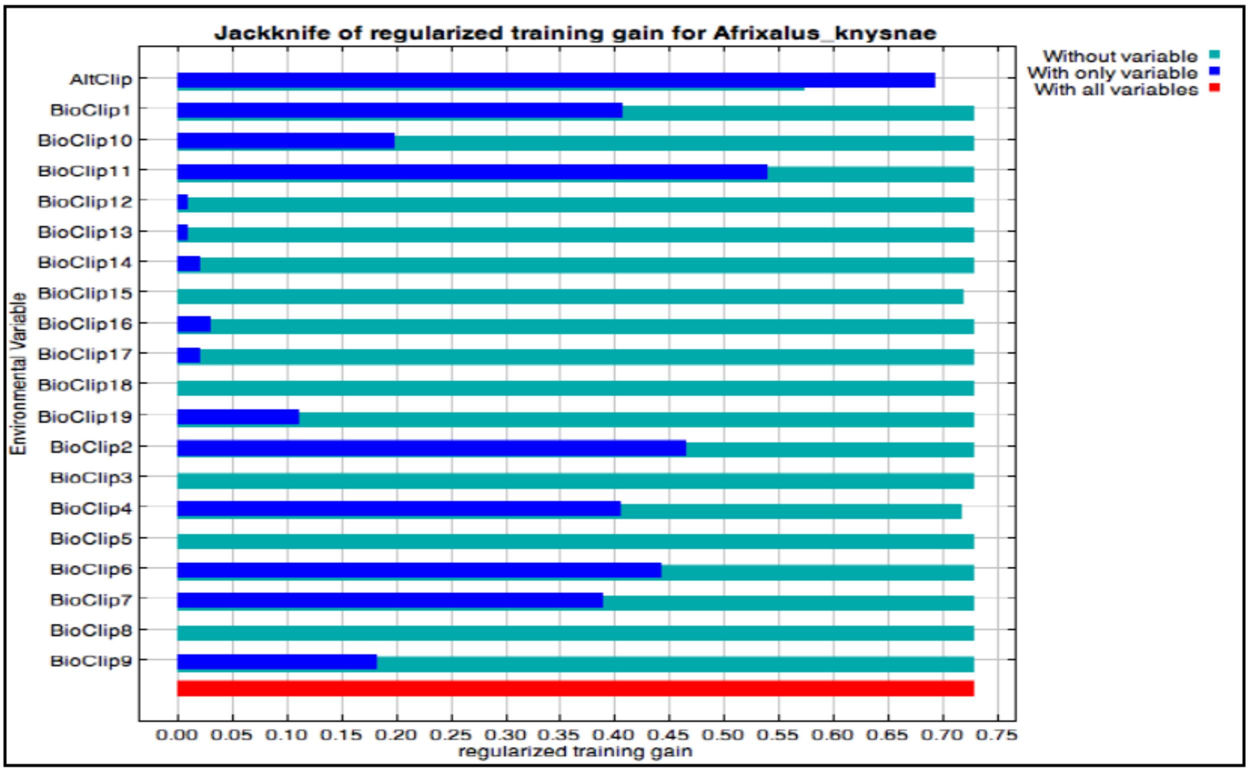


Figure 2-4: Jack-knife test graphic confirming the best-fit variables influencing the modeling process for *A. knysnae*.

The application further produces an OPA graph (Omission and Predicted Area) and ROC plots. The OPA graph indicates where the model predicted unsuitable conditions for species occurrence (Fig 2-5). Possible bias can be indicated by this resultant graph while it describes the relationship between predicted occurrence probability and the proportion of actual occurrences selected. The predicted omission should therefore be 1:1 if the sample is not biased. The X-axis in this OPA graph indicates the suitability of the area to species occurrence, 0 being the whole area being unsuitable to 100 as the whole area being completely suitable. The current area has a predicted relationship of 1:1 with the entire area seemingly approximately 25% suitable for occurrence probability

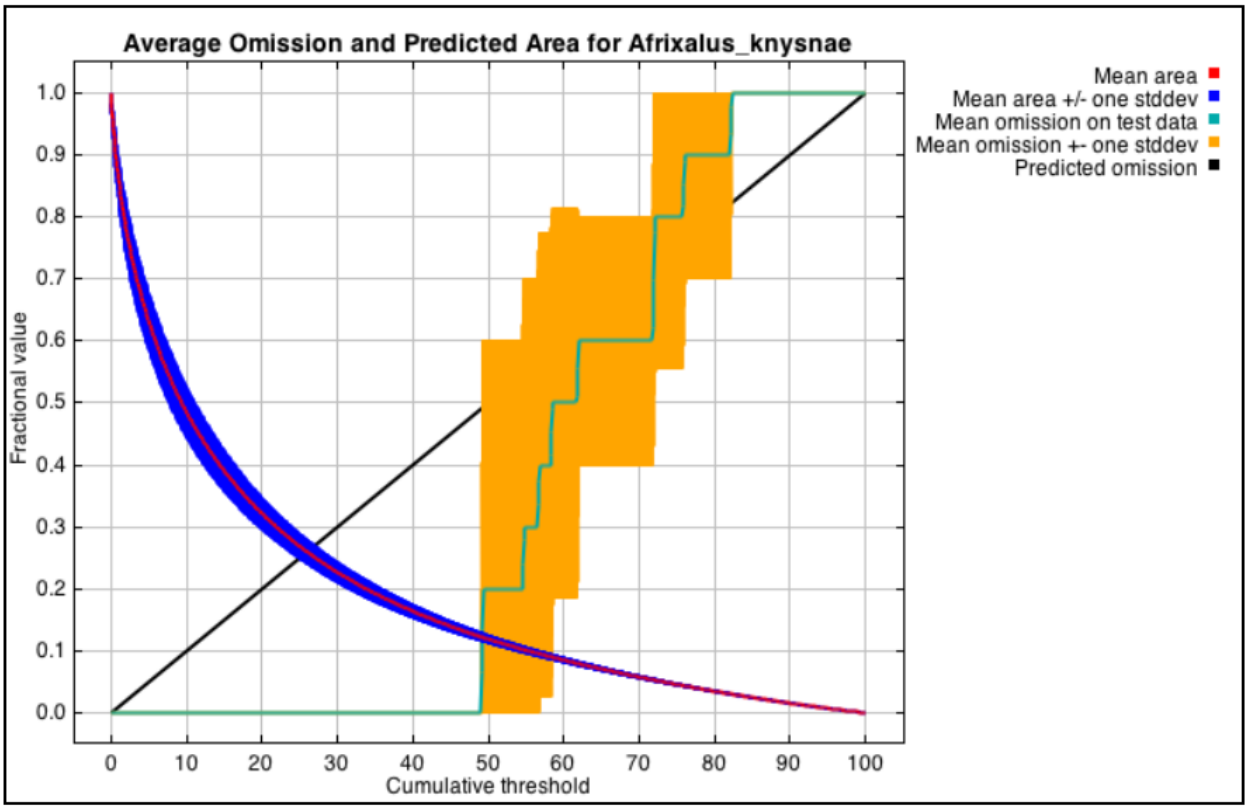


Figure 2-5: Omission and Predicted Area graph produced by MaxEnt mathematically indicating predicted area probabilities for *A. knysnae* based on known localities compared to background environmental and absence data.

The ROC plots the performance of the model (Fig 2-6). The curve created in the plot indicates the true positive rate of the occurrence against the false positives and then tested against the various threshold settings (True positives are also known as the sensitivity of the model).

The ROC also indicates the Area under the curve (AUC) which indicates the accuracy of the model to predict the probability of occurrence. A value of 1 would suggest that the entire selected area and variables are perfect matches for the distribution of the species. In the current study, the AUC returned an average value of 0,921 (±0,079 SD) indicating that the model fits well with the possible geographic distribution of the species.

MaxEnt is a powerful mathematical application with results being capable of fine statistical analysis. Ecological studies however need to be able to test results in the field and confirm that data returned by the model through ground truthing. In this instance the resultant maximum probability map of occurrences produced by MaxEnt illustrates the results from the environmental variables, OPA and ROC graphs and AUC plots, graphically.

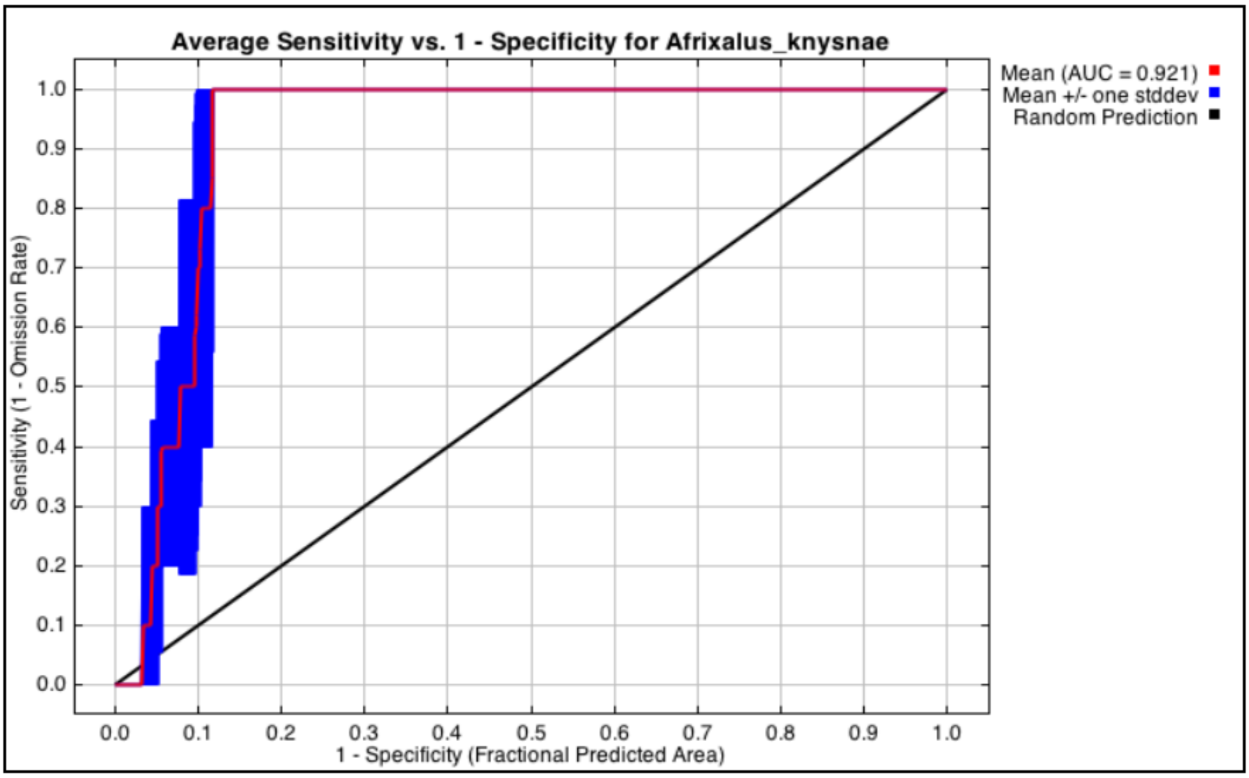


Figure 2-6: Graphical illustration of the mathematical result indicating the model's performance to predict the probability of species occurrence within the Landscape in interest (AUC = 0,921).

A map image indicating probable distribution of the subject species is produced by MaxEnt. The demarcated distribution probabilities are indicated by a colour intensity bar to assist in the the visualization of the distribution. This illustration of probable distributions and the degree of probability for *A. knysnae* is illustrated in figure 2-7. Accordingly, the predicted distribution therefore seems to indicate suitable habitats confined to the lower lying coastal areas of the Southern Cape region. The area range from the historical site at Covie in the east, hugging the coastal areas and terminating in the areas around the Town of George in the west. A high degree of probability seems more likely in the western region of its distribution.

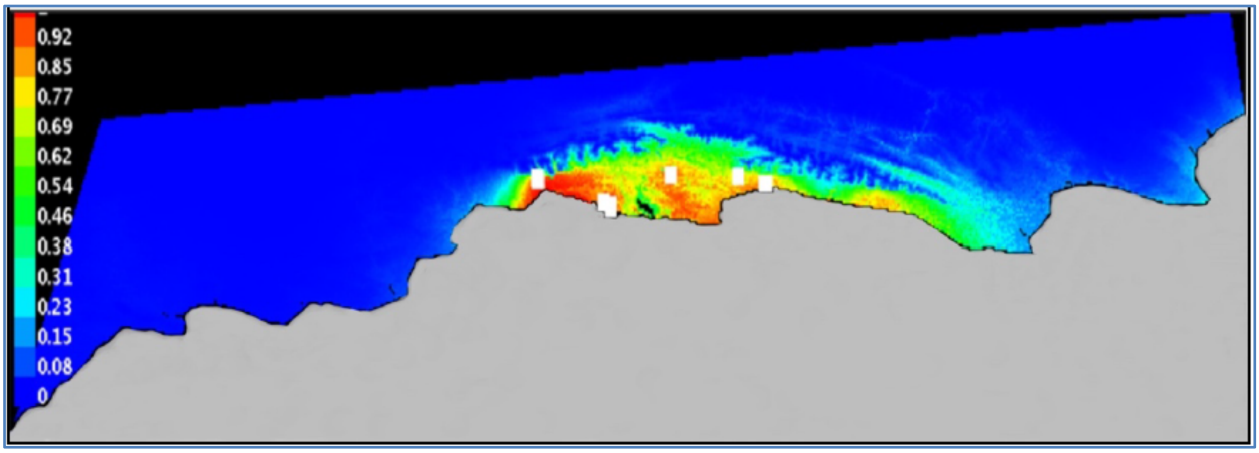


Figure 2-7: Probability map produced by MaxEnt. The colour bar (left) indicating the probability of occurrence according to colour intensity. White squares representing data of input coordinates

The application also returns variable response curves indicating the different variable's response to the input data pertaining to actual occurrence and geographic area. These plots indicate the response of input data to known locality points of *A. knysnae* and to the relevant variables. These resultant graphs then allows for finer scale analysis if required.

Analysing these plots gives a clearer grasp of the exact influence of specific variables on the distribution probabilities of the species. In this regard the different variables influencing distribution probabilities can be compared and investigated by ground truthing the recorded localities. In this regard figure 2-18 and 2-19 hereunder is an example of these graphs depicting the graphical results produced for the coldest month, coldest quarter, altitude and mean diurnal temperature. Analysis hereof depicts that temperatures around 30° C (±90 F) are conducive to species presence while localities lower than 600m are preferable for *A. knysnae.*

Graphs for all the variables influencing the probabilities are however produced by MaxEnt and can then be analysed to confirmed its effect within the landscape of interest for the subject species. Reproducing all the graphs here is however not necessary, but is a valuable tool in the field when location probabilities are examined.

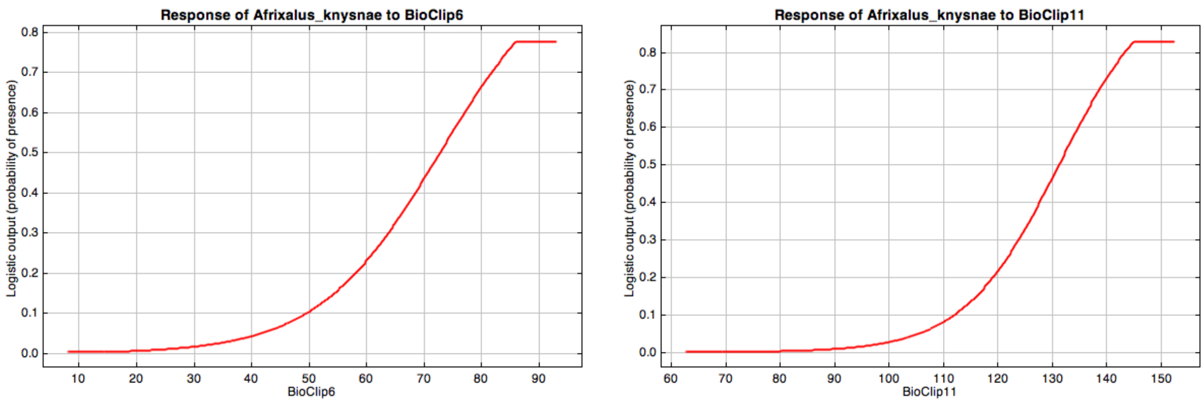


Figure 2-8: **Finer scale graphs of BioClip 6 and 11 variables, indicating influence of month with coldest temperatures and coldest quarter temperatures (respectively) on distribution probabilities.**

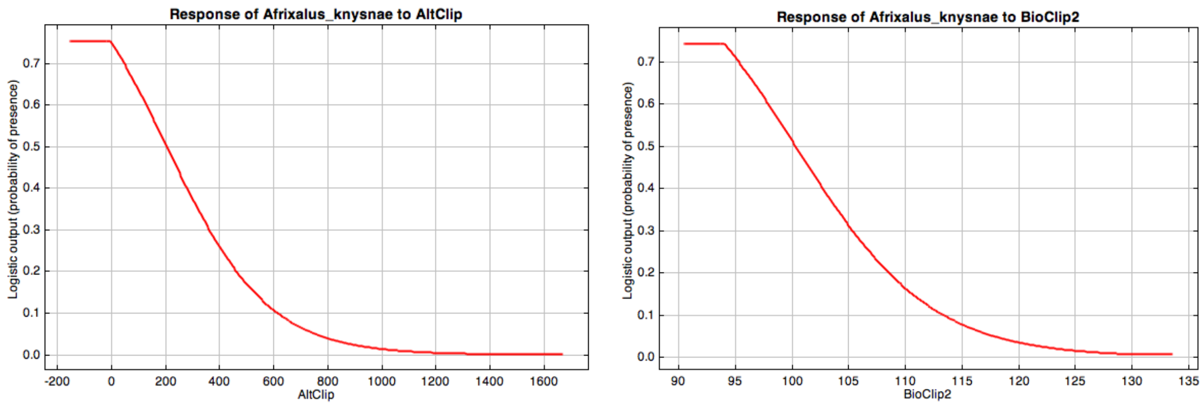


Figure 2-9: **Finer scale graphs of BioClip 2 and AltClip indicating influence of mean diurnal temperatures and Altitude (respectively) on distribution probabilities.**

2.3.3 Potential population localities

The resultant probability map was overlaid with the landcover and vegetation maps of the Western Cape within the geographic information system, QGIS®. Landcover types and vegetation units within biomes were then correlated with the current confirmed occurrence records of *A. knysnae* on the overlays. Further field surveys were undertaken based on the ENM mapping results and the GIS mask to investigate other localities identified apart from the IUCN reported sites. The 27 sites selected represented eight sites surveyed during the initial 2014/2015 frog diversity study, the ten from the IUCN reported localities and nine more sites identified after the initial model was executed. All sites where surveys were undertaken with the use of Passive Acoustic Monitoring sound recording equipment are illustrated in figure 2-10. All the localities fit well within this modelled map and confirmed the relevance of the surveys undertaken. Data from the PAM recordings of the later selected sites were analysed between November 2016 and January 2017 while the eight sites from 2014/2015 surveys had no positive recordings of *A. knysnae* at the time of its analysis.

During the current study, confirmation of presence and absence was obtained regarding the ten localities as reported in the IUCN 2016 report, updated by the African Amphibian Specialist Group. Positive recordings of calling males were recorded at all seven IUCN 2016 reported sites during the breeding period of September and October 2016, data and recordings hereof being available on digital storage.

Visual encounter surveys at the IUCN 2016 reported sites revealed rather healthy populations at these sites. Detailed individual counts and abundance calculations were not made during the study but the data from the bioacoustic recordings indicate abundant calls during the breeding seasons. Results from these recordings are reported in Chapter 3 hereof.

Figure 2-10: Location of the 27 sites selected for survey across the study area based on the ENM modeling and literature information. (Satellite image: Google Earth®

2.3.4 New locality of *A. knysnae*

Analysis of the PAM data from the further selected nine sites yielded one new site where calls were recorded. This recording was made during November 2016 and after the IUCN 2016 report was released. Recordings of only two males on only two evenings during November 2016 were captured at the site.

Figure 2-11: Map indicating location of the site discovered at Farleigh Ranger station within the GRNP, KLS where *A. knysnae* calls were recorded during the study.

This site is situated at 512m above sea level in close proximity to the Farleigh Office of SANPARKS within the GRNP. Currently this site represents the highest recorded locality for the occurrence of the species as the IUCN 2016 report indicates the upper altitude of the species being only at 300m. These calls and data are also available on digital storage. Figure 2-11 indicates the exact location of the site with GPS coordinates 33° 53' 27.20" S 22° 52'42.27" E. An image of the site where the Song Meter was installed during the recording of the calls is presented in Figure 2-12.

The site consists of a small teardrop shaped depression approximately 60 m^2 in extent, within a rocky and hard underground substrate, wholly fed by rainwater run-off. The wider part of the depression has a steep edge leading into the water body with vegetation covering the embankment. A small tree is also situated against this embankment providing a relatively large shaded area over the waterbody for most part of the day. The narrow end of the water body gradually tapers out into a flat area containing a silted bed.

The water is very clear with large-leaf lilies and other hydrophytes growing from the deeper end edge into the waterbody.

The deepest section of the depression is at approximately 60 cm while it tapers gradually towards the shallow end. The water body retracts during the drier months to only about 20 m^2 and fluctuates according to the volume of precipitation it receives. The main access road towards the SANPARKS Farleigh Ranger Station is situated within 10 m from the site with a low wooden fence running against the edge of the road, separating it from the water body.

Investigations at the site during December 2016 and again in September 2017 could however as yet not establish visual confirmation of a viable population of *A knysnae*. Since the recording of these calls, wildfires have ravaged the area and surveys in the area have been difficult. The latest fires in the area being in October 2018 during the main breeding season of the species. Fires in this area impacted not only animal life but impacted on the lives of many people living and working at this ranger station and forestry village. Impacts of fire in this area will be researched in follow-up visits with the aim of understanding this ecological impact on such a threatened species.

Figure 2-12: Image of depression at the Farleigh Ranger Station location (Photo: F.de Lange).

2.4 Discussion

2.4.1 Current condition of historical locations

Inspection at sites where the actual coordinates could be located and verified revealed large landscape modifications. Alteration in the form of forestry activities and human settlements have occurred at an increased rate over the last three decades throughout the entire Southern Cape region. Habitat degradation is seen as a main cause for declines in amphibian communities (Hocking and Semlitsch, 2007).

Localities of *A. knysnae* reported by the IUCN in its 2010 report have been found to be largely destroyed or no longer viable as habitat. Farmland, pastures and housing developments are some of the reasons for the localities no longer containing *A. knysnae* populations. The area is seen as a major tourist attraction with many recreational facilities, holiday resorts and ancillary industries being developed since the late 1990's. An increased influx of people has coincided with these developments in the area, thus placing large scale pressure on the natural environment and resources within the region. Photographs of examples of these developed locations are included in the graphics hereunder (Fig 2-13 and 2-14).

As with many other frog species within the Western Cape Province, data deficiency seems to have serious implications for the continued monitoring and protection of threatened taxa. Data anomalies and possible metadata errors regarding localities as reported in the IUCN 2010 report is further indicative of the lack of adequate scientific information regarding this endangered species. Reports from conservation authorities also lack substantive information regarding the distribution status of the species and as a result historical locality will have become under increased pressure from anthropogenic activities over the last decade leading to the ultimate loss of these habitats.

Lack of capacity and resources coupled with difficult terrain within the area further hampers adequate investigation of amphibian species in the Afro-montane and Fynbos regions of the Southern Cape.

Figure 2-13: Locality - Ak2 - Location of the cattle farming area with the heavily trampled ground dam used by cattle as drinking resource. Vegetation in and around the water body is also not typical of breeding habitat required by *A. knysnae*. *Cacosternum nanum* and *Strongylopus grayii* present at the dams. The satellite image (right) indicates the extensive agricultural development of the entire area around the historical site. (Photo F.de Lange, Satellite image: Google Earth®)

Figure 2-14: Locality Ak 6 - Although vegetation abounds within the suburbs of Knysna, many of the species are alien and very little to no water bodies exist within the boundaries of the dwellings. This area has been developed since the 1970's and is densely populated. Extensive residential development of Knysna is clear from the satellite image (right) making habitat suitability improbable. (Photo: F.de Lange, Satellite image: Google Earth®)

2.4.2 Predictive modelling incorporating climatic variables

Species occurrence records are often the only information regarding a species' actual distribution, although the species may likely occur in areas not yet known about. The seven historical localities obtained from the IUCN records together with the three update records during the 2016 surveys were available to be used as actual occurrence records during this modelling process. Potential geographical distribution of the species needed to be determined by identifying environmental conditions which may be suitable for the occurrence of *A. knysnae* and then determine where these typical environments may be located or distributed spatially (Pearson *et al.*, 2006). Potential distributions often do not contain a subject species, either as result of exclusion due to biotic interactions (e.g. competitor presence or food or other resource unavailability) The species may also not as yet have dispersed into the area due to a geographic barrier or as result of human landscape modification or the species may even have been extirpated from an area (Oswald *et al.*, 2016; Panzacchi *et al.*, 2016).

The use of an Ecological niche model or habitat suitability model in this instance was typically to correlate the presence of *A. knysnae* with relevant environmental covariates at multiple locations and then estimate habitat preferences to predict possible distributions. Ecological Niche Models (ENM's) have wide application from information regarding ecological and biogeographical theory to informing conservation planning and management for threatened environments and species. The variables used in the ENM for *A. knysnae* mainly describe physical or abiotic environmental factors (temperature, precipitation, aquatic types etc.). Actual distribution locations in environmental space could then be used to identify geographical space that may be occupied by the *A. knysnae*. This geographical space is defined as the occupied niche. Such a plotted occupied niche then indicated areas of similar environmental conditions but whereas yet, no records of *A. knysnae* currently exist. Focusing particularly on abiotic factors, produces models that more closely resemble the fundamental niche (potential distribution). Including biotic factors will result in models of occupied niches (actual distribution). In this study, biotic factors could not be considered as the study of micro-environment was not determined in detail and actual distribution was therefore determined through ground truthing within potential distribution areas.

Physical data repositories such as museums and herbaria normally house species locality data. Systematic survey data is however rarely collected and recorded, and this is the case with *A. knysnae*, rendering the species data deficient as well (Elith *et al.*, 2011). In an era of advanced technology, “Virtual museums” and on-line or “Cloud based” data servers are being used more often in storing species and environmental data (Frogmap, 2018). Such electronic format data is often more compatible to use with ENM applications and these presence only data relating to *A. knysnae* was used with regard to the species’ occurrence modelling. The maximum entropy method of generating these models is one of the current accepted approaches employed to indicate possible species distribution areas or niches, and in the current study MaxEnt was used for this purpose (Pearson *et al.*, 2006).

Predictive modelling also uses environmental data and various vegetation and land cover map overlays within GIS Software applications to predict possible distribution of species (Guisan and Thuiller, 2005; Pearson *et al.*, 2006). During the current study GIS mapping was performed by using the freely available GIS software application, QGIS®. Data layers regarding macro environmental variables and species occurrences are readily obtained from a large number of sources. These include the Global Biodiversity Information Facility (GBIF) for species distributions; WorldClim, or the Intergovernmental Panel on Climate Change (IPCC) for environmental characters; SANBI (BGIS Portal) or the Global Landcover facility for topography and the United Nations Environment Programme (UNEP) for soils and aquatic information. The data required by the QGis software to achieve the modelling results consisted of two major input matrices - firstly, the 10 known occurrence localities of *A. knysnae* and secondly, a vast array of environmental variables, known as continuous variables. These layers were obtained from BioClim® and converted to vector format files capable of being synthesised by QGIS® over the geographic space selected.

Species occur within certain geographic boundaries. Elith *et al.*, (2011) describes these geographic areas as the landscape of interest (L), with it also being the geographic area suggested by the problem and defined by the ecologist. *Afrixalus knysnae* in this instance, occurs within a sub-region of the Western Cape Province as described herein and known as the Garden Route region. Georeferencing of occurrence records was however verified before running the model. This was done by viewing the locality points in the QGIS®

software application and on Google Earth, and thereafter doing physical field inspections at the sites of occurrence records. Field investigations of recorded localities of *A*. *knysnae* revealed various important attributes regarding the habitat characteristics. This included altitude where sites were located, the size of the wetland areas, temperature during the breeding season, vegetation types at the sites and water levels of the water body during breeding seasons. Field investigations mostly ensured that the model is applied in a logistic manner. The size of the landscape of interest is important as it has implications for the model (Van Der Wal *et al.*, 2009). The distribution of *A. knysnae* in the Southern Cape as established from known data, made the landscape of interest rather limited and manageable, further ensuring that the background data for the ENM stay relevant. Surveys regarding possible sites were done within the boundaries of the GRNP and the adjacent region falling within the Garden Route biosphere reserve.

MaxEnt then uses an algorithm to predict the species occurrence results by simulating localities where *A. knysnae* is likely to respond to multiple environmental factors tested against environmental variables present at the known localities. (Pearson *et al*., 2006). Regularisation methods within the MaxEnt application enables ecologically relevant continuous variables to be correlated with each other and with the background data during the modelling process. MaxEnt has been shown to be robust when dealing with species where very little occurrence records are available, small sample sizes and little information on habitat preferences (Elith *et al.*, 2006). Modelling for probability occurrences of *A. knysnae* therefore falls neatly within this application with its small number of known localities and limited literature on habitat characteristics.

MaxEnt randomly samples background locations from the study area, also known as pseudo-absences to provide a comparative dataset of conditions under which *A. knysnae* will be absent. A landscape of interest should however not be too large relative to the spread of known occurrence data points. During this study, choosing the landscape of interest as the entire Western Cape Province would then have made the comparative dataset to be tested by MaxEnt too large. As a result, the relevant landscape of interest was defined by the known occurrence records of *A. knysnae* and limited to the most eastern and western boundaries of the GRNP. A polygon was demarcated within the QGis software application to limit the modelling process to these clipped boundaries. In order to design any future protected area network for *A. knysnae*, it will be important to understand where species actually occur. When determining sites where an endangered species such as *A. knysnae* may be located, it is more important to model areas where the species could potentially occur rather than only where it currently occurs (Jackson and Robertson, 2011). The process MaxEnt follows in executing the algorithm to predict potential localities, is simplistically depicted in figure 2-15.

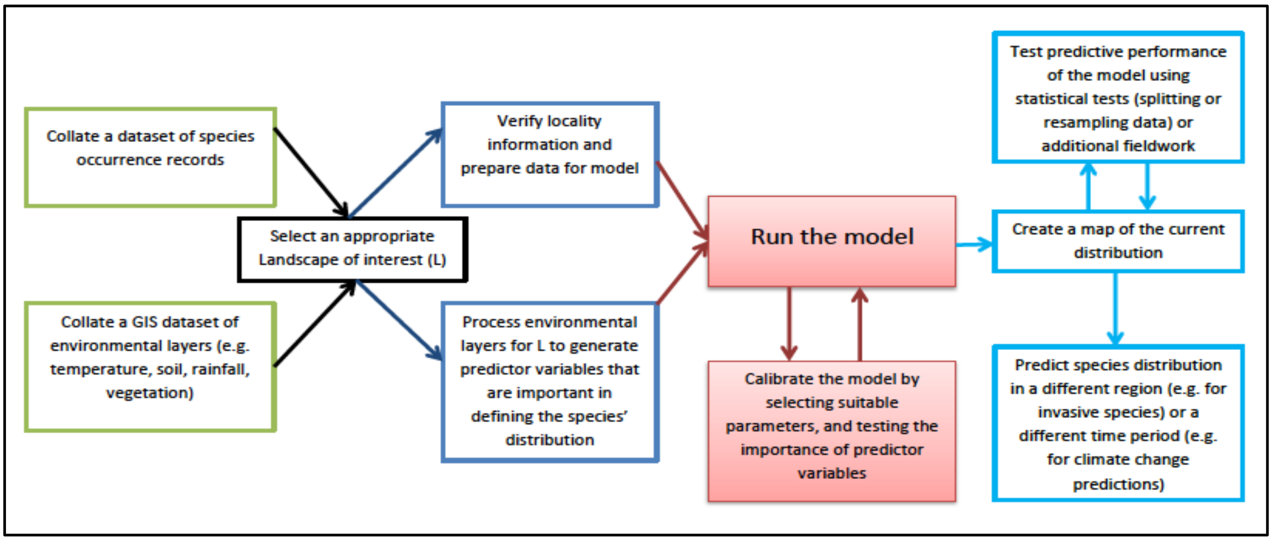
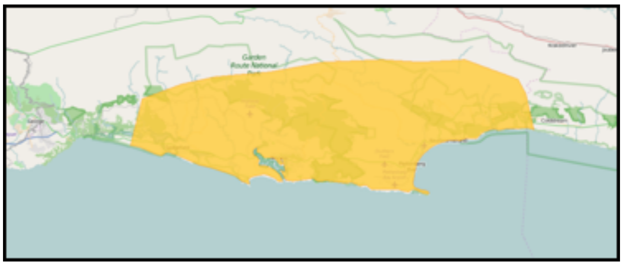


Figure 2-15: Simplistic graphical representation of the process flow when Ecological Niche Modeling is executed with the MaxEnt Application. (Reproduced from Roxburgh and Page (2015) as described in(Pearson *et al.*, 2006).

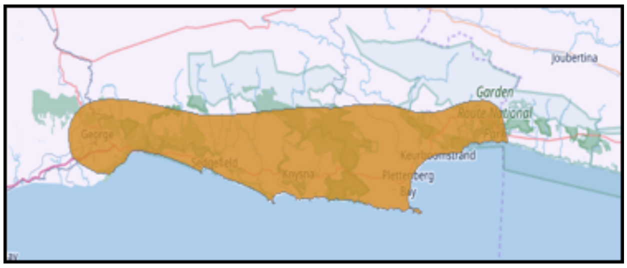
After executing the model, MaxEnt produces a visual distribution map drawn of occurrence probability of the subject species. This map identified broad areas in a landscape that have similar environments to the localities where *A. knysnae* has been actually recorded (see Fig 2-7 again in this regard). The model however does not indicate exact distribution localities of *A. knysnae*, but rather suitable environments where the they are likely to occur (Pearson, *et al*., 2006).

2.4.3 Current Area of Occupancy and Extent of Occurrence

At the commencement phase of this study in 2014, mapping the presence of species within the GRNP, the status of *A. knysnae* was Endangered (EN) (IUCN 2010). Extent of Occurrence (EOO) of the species was reported as 1756km^2 and its Area of occupancy (AOO) not having been formally calculated but reported as declining. An area of distribution was indicated graphically on a distribution map as represented by the shaded area in Figure 2-16 (A).

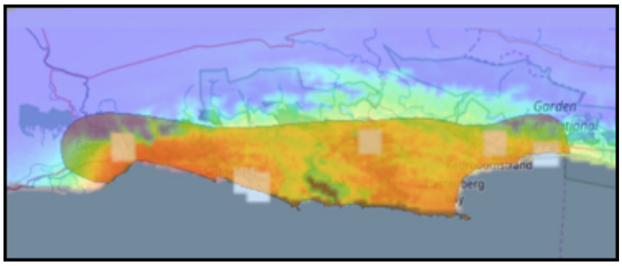


A: Area of distribution of *A. knysnae* according to IUCN 2010



B: Area of distribution (Interpreted distribution) of *A. knysnae* according to IUCN 2016

C: Graphical depiction of IUCN 2010 and 2016 EOO transposed over each other



D: ENM map of probability overlaid onto the IUCN coverage map

Figure 2-16: Distribution maps describing EOO from IUCN 2010 and 2016 asessments, relation to each other and the current study ENM map.

During the following three years of surveys and investigations into the possible habitat, fringe areas and previously unreported sightings, the information was updated by the IUCN Amphibian Specialist group in 2016. The current IUCN report indicates the EOO as 816km^2 and AOO formally calculated as 27km^2. This is now depicted by the shaded area indicated in Figure 2-16 (B). Combining these two assessment maps graphically illustrates the change in distribution (Fig 2-16 (C)).

The shift in the population records towards the west is most pronounced and initially seems an increase in distribution. Overlaying the ENM mapping output over the latest IUCN distribution imagery however clearly indicates the “hotspots” of possible localities (Fig 2-16(D)). Modelling results for *A. knysnae* clearly indicate an amendment of the fundamental niche and this again more closely resembled the occupied niche as confirmed by visual encounter surveys and acoustic monitoring. These results are clearly visible when distribution mapping is created and compared to its background data. These small pockets of possible favourable habitat illustrates the realistic occurrence probabilities and highlights the AOO in much more detail. Most concerning is the shrinking extent of occurrence by more than 50% and the accompanying minimal total area of occupancy.

2.4.4 Conservation concern in areas containing populations

Although *A. knysnae* is again listed in this latest State of Knowledge Report (2017), it is still worrying that it cannot be ascertained on what basis the listing is made and what recent scientific endeavour has caused this reporting. In this regard, the 2017 report deals with "Species of special concern" and again in this instance, no frogs are listed as fauna, either terrestrial or aquatic. In light of the fact that amphibians are the most threatened vertebrate species on the planet, that at least two alien species are invading the area and that an IUCN Red Listed Endangered species is found within the Park boundaries, increases the concern that the conservation efforts within the Park may be lacking regarding this species.

Reports regarding this species as well as the plight of frogs in general within the park was delivered to the authorities by the author and presentation was made to managers and staff at a meeting held during March 2017. Furthermore, in terms of the Research Agreement with SANPARKS, this entire document will be delivered to the Ecology Department at SANPARKS, Rondevlei office in order to act as advisory document for any future engagement and metadata analysis. Engagement with SANPARKS in this regard will be through invite to present my findings, should they wish to engage in this manner, and then make specific management recommendations at their behest. This document will however act as preliminary encouragement to assist managers in making decisions with regard to conservation efforts pertaining to the entire frog diversity in the GRNP going forward.

CHAPTER 3 *Afrixalus knysnae:* A BIOACOUSTICS ANALYSIS

"But if the heart, the stronghold of his life, is not frozen, he wakes up some warm spring day when the ferns are unrolling, and the cold is gone, and scarcely knows that he has slept more than a day. What a change in him! The long sleep, the warm, moist air, all the instincts of his being, tend to fill him with physical joy. It is such a pleasure to eat; it is so delightful to move; it is such a satisfaction to soak in the water of the spring rains. He is converted into a social creature and finds himself going with many other toads to the pond where he spent his own early days. And now at the pond and in the water, he can contain himself no longer, but bursts into that spring song, beautiful to himself and to his companions."

Mary Emily Dickerson, 1906 (The Frog Book).

3.1 Introduction

Since the observations of Mary Dickerson, science has however provided more insight and understanding into the reasons, mode and purpose of frog calls. Male frogs mainly produce calls to attract mates for reproductive purposes and at the same time to ward off other competitive suitors for the same females (Wells and Schwartz, 2007). These signals also delimit the male’s territory, acts as warning signals for approaching predators and many other possible motivations (Blackwell, 1988; Köhler *et al.*, 2017).

Recordings of animal sound, referred to as bioacoustic monitoring, are some of the most important tools used by scientists worldwide to study animal behaviour, biology, and ecology in the natural world today (Bedoya *et al.*, 2014). Ecoacoustics are furthermore becoming a major study discipline in ecology using sound as the vector to understand ecological processes and interactions within the natural world (Sueur, 2018). Machine recording of frog calls is as old as the advent of portable recording equipment although attempts to describe the calls made by frogs were attempted long before these inventions. Descriptions such as "br-wrum", "more-rum" and "jug-o-rum" were used for instance to describe characterisations of bull-frog calls in America (Wright, 1914). Pioneers in the field of frog call recordings were Arthur A. Allen and Peter Paul Kellogg, whom in 1948 produced a gramophone album of frog calls (Wells, 2007). Recordings of animal and all manner of environmental sounds are possible today through a large number of electronic devices, besides specialised recording equipment this includes cellular phones, GPS equipment, hand held tablets and laptop computers. Specialised recording equipment has also become more accessible while constantly getting smaller with improved recording abilities (Vielliard *et al.*, 2004).

Frogs in particular, make very interesting subjects where acoustics are concerned. Bogert (1960) classified frog calls into six categories: (1) mating calls, (2) territorial calls, (3) male release calls, (4) female release calls, (5) distress calls and (6) warning calls. These categories have since been refined by Littlejohn (1977) and Wells (1988) as being (1) advertisement calls, (2) male courtship calls, (3) female courtship calls, (4) aggression calls, (5) release calls and (6) distress or alarm or defensive calls. Toledo *et al.* (2015) propose an even broader classification into only three major categories being reproductive, aggressive and defensive calls. Reproductive calls are however the most frequently observed and described mode of signalling in frogs and Köhler *et al.* (2017) focuses on these in relation to taxonomical studies.

The method employed by frogs to make these varied calls are somewhat complicated, and many studies have been undertaken to examine the physiological instruments used by anurans to produce their unique sound (Gans *et al.*, 1969; Duellman and Trueb, 1986) In general, the sound is produced by air being pumped into the lungs by muscles in the buccal cavity and contracting of muscles lining the inflated vocal sac. This takes place during respiration. Air is then forced from the lungs back to the vocal sac and in the process pass over the vocal cords in the larynx where the sound is produced. Vibrations of the vocal cords are then transmitted to the inflated vocal sac that acts as a kind of amplifier. Variations in calls and the exact manner of producing the sounds are all somewhat different, but anurans use the trunk, larynx and buccal cavity and vocal sac in one way or another to produce their species-specific calls (Wells, 2007).

Acoustic behaviour and signalling are however only important if it is able to be processed by a receiver of the signal. Frogs process acoustic information firstly by detecting the signal, secondly by recognising the signals and distinguishing one from another within a specific environment and thirdly localising the signaller (Chuang *et al.*, 2017) Anurans have been shown to exhibit these three traits without exception and frogs are very adept in discriminating on a fine scale between signals during processing thereof (Leary, 2014). The mechanisms employed for these signals to be processed involves mainly the tympanum and extra-tympanic pathways such as the ear and lungs and even bones and tissue within the head itself. (Wells, 2007). Calling behaviour plays a paramount role in the evolution and survival of anuran species across the globe (Wilkins *et al.*, 2013). Pressure from increased noise pollution from anthropogenic noise sources, invasive anurans and other animal species may drive the evolutionary processes and survival gradient of frogs (Bee and Swanson, 2007). Bioacoustic surveys and the recognition of various anuran-communication is therefore of utmost importance when conservation efforts are designed to protect frogs in their natural and even anthropogenic modified habitats (Blumstein *et al.*, 2011; Both and Grant, 2012).

3.2 Objectives of recording calls from *Afrixalus knysnae.*

In the current study, calls were recorded from three more abundant populations of *A. knysnae* as surveyed during the ground truthing process discussed in the previous chapter. Data from the recordings were analysed to understand how this behavioural trait relates to the ecological significance of *A. knysnae* and to establish the extent to which it relates to environmental variables. Temporal and spectral variability can also be compared to sympatric frog populations and some basic comparison can be analysed with congeneric species. In order to completely comprehend the biology and breeding activity of *A. knysnae* within its preferred habitat, analysis of call recordings should provide valuable data in this regard. (Potamitis, 2014; Sueur, 2018).
Different recording strategies were employed to achieve optimal data collection for analysis at different localities and habitat, this being Passive Acoustic Monitoring (PAM) and Directional Microphone Recording (DMR). Data collected also contributed to confirm presence or absence of *A. knysnae* at seemingly favourable habitats. The structure of calls was further analysed to determine its finer scale attributes and differentiating characteristics and resultant inter- and intra-specific correlations. Analysis herein may lead to clearer taxonomical descriptors and groupings of closely related species populations in future.

Different call analysis software was evaluated during this process to test their effectiveness and application in this study. Various commercially available software currently exists in this regard and are user-friendly, without the investigator needing extensive experience or any coding skills. In this regard, applications such as Audacity®, Song Scope®, Kaleidoscope® and Raven Pro® were tested for this purpose. Abilities of these applications to analyse anuran calls seems to differ depending on the objective of analysis. These variations may be mainly as a result of the initial development thereof focusing predominantly on bat and bird calls (Gasc *et al.*, 2013). Developers of the various applications are however constantly improving its functionality, and this can be driven by input from scientists in the bioacoustics and eco-acoustics scientific fields. Studies in anuran acoustics have also seen a tremendous growth over the last decade assisting in understanding amphibian extinction threats, declining habitat and biological requirements (Acevedo *et al.*, 2009; Waddle *et al.*, 2009; Llusia *et al.*, 2011).

3.3 Methods and materials

3.3.1 Recording localities

Three sites were selected from the sampling sites based on chorus strength. These selected localities are also recognized and listed sites according to the latest IUCN assessment (IUCN, 2016). The equipment could easily be deployed and monitored during the study period due to the ease of access of the sites (Fig 3-1).

The first site (A) (Fig 3-1) was selected as a long-term monitoring site and is situated on the periphery of the town of George within the grounds of the Nelson Mandela University (NMU). This site (hereafter referred to as the NMU-site) consists of a natural pond of approximately 3000 m^2, filled with water year-round, at the border of the University grounds and flanked by a commercial plantation and an indigenous forest. GPS coordinates, 33.94466º S, 22.52173 7º E.

The second selected site (B) (Fig 3-1) is situated within the De Vasselot Nature Reserve within the Garden Route National Park, Tsitsikamma Forest and Coastal Section. This area is within the Fynbos Biome and more specifically the Tsitsikamma Sandstone Fynbos (SANBI, 2014). GPS Coordinates, 33.96717º S, 23.54098º E, hereafter referred to as the De Vasselot-site. The De Vasselot Nature Reserve is situated between the Sout River and the Bobbejaan River, near the coastal village of Natures Valley. The site of the pond is at the top of the Grootrivier Pass and approximately 15 m from the edge of a tar road. A seasonal pond of approximately 1200m^2, formed in a natural depression within the fynbos vegetation.

Covie was selected as the third site (C) (Fig. 3-1) and is one of the oldest historical sites where *A. knysnae* has been recorded, hereafter referred to as the Covie site. This small hamlet houses mainly forestry workers and is situated within the Bloukrans forestry section of the Garden Route National Park (Tsitsikamma Section). Wetlands and seasonal ponds are interspersed amongst the low impact housing structures. The recording site is situated next to the gravel road leading through the hamlet, approximately 1 km from the main tarred access road leading from Natures Valley. The site is a seasonal wetland of approximately 5000m^2, which fills with water only after good rains and drains fast after the rainy season. GPS coordinates 33.95833º S, 23.60528º E.

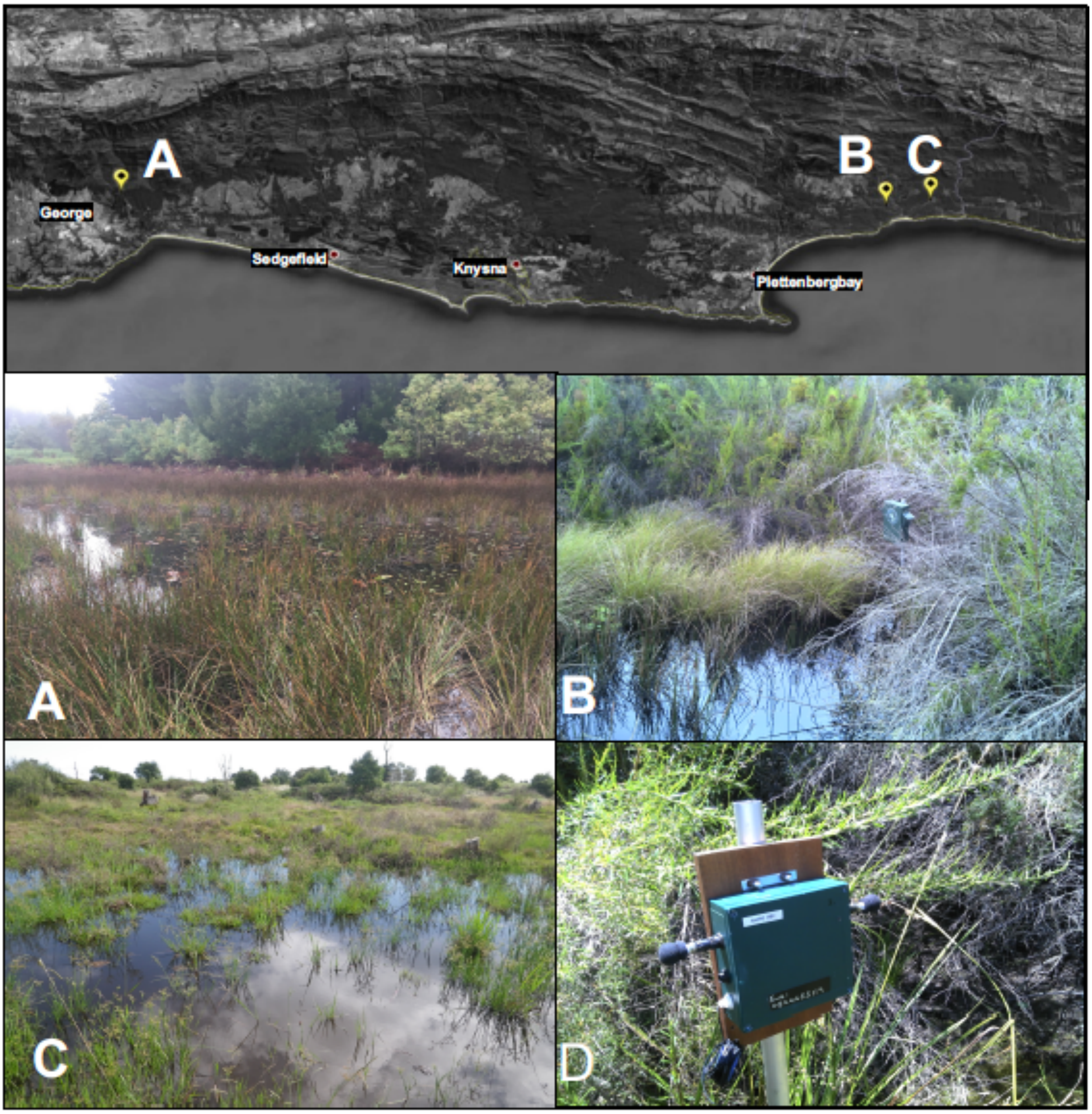


Figure 3-1: The locality map and photographs of sites during the breeding season. NMU-site (A), De Vasselot-site (B) and Covie-site (C). Image (D) indicating a typical Song Meter® installed at locality. (Satellite image: Google Earth®, Photographs: F de Lange)

3.3.2 Bioacoustic surveys

Bio-acoustic surveys by way of Passive Acoustic Methods (PAM) was achieved through the use of Wildlife Acoustics Song Meter SM2+® at the NMU- and De Vasselot sites. A Nagra Ares-ML recorder (Software Ver. 3.24) and a Senheizer directional "shotgun" microphone fitted with an acoustic noise reduction cover was used at the Covie site for directional microphone recordings (DMR). All recordings were captured digitally and were therefore capable of being downloaded onto computers for analysis.

Passive Acoustic Monitoring (PAM):

The specific positioning of the Song Meters® was selected to most effectively capture anuran acoustics at each location. All PAM recordings were made digitally in stereo with 16 kHz sampling frequency and at 16-bit precision. Data was stored on four 32GB memory cards, housed in integrated cardholder slots within the recorders. Prior to installation , each Song Meter® was set to record at 16 kHz, GPS coordinates set and internal microphone settings adjusted to default using Wildlife Acoustics downloaded calibration software and uploaded to the Song Meter via the available internal memory card slot. The Song Meters® were installed near the edge, or within the waterbody and housed in stainless steel containers, specifically manufactured for use with the equipment, in this way preventing any damage to the units from the elements, animal or human interference. At the NMU-site the equipment was fixed against an existing wooden post with the microphones attached to extension cables and deployed approximately 20 meters away from the recorder, closer to the water's edge, on either side of the recorder. Unlike the long-term passive acoustic monitoring at NMU, the meter at the De Vasselot site was installed for a shorter survey period.

Directional microphone recordings (DMR):

Recordings were made at the Covie site during two site visits in February 2018, between 8pm and 11pm. Calling males were located and recordings made from a distance of 1 to 2 m. Metadata including date, time, wind speed, moonlight intensity and air temperature were made during these recordings. Slow approaches on foot were made through the water with as little disturbance as possible, once a calling male was located. The microphone was directed in its direction, with any lighting switched off. Light disturbance was found to cause calling bouts to cease, however, switching off the light source and waiting motionless for a short while normally started calling bouts again. Males usually called from close to the water surface on detritus or flat-leafed floating plants (Fig. 3-2). This behaviour necessitated that recordings were made close to the water, often having to crouch beneath other vegetation and even sit motionless on logs or other substrate to cause the minimum amount of movement during recording sessions.

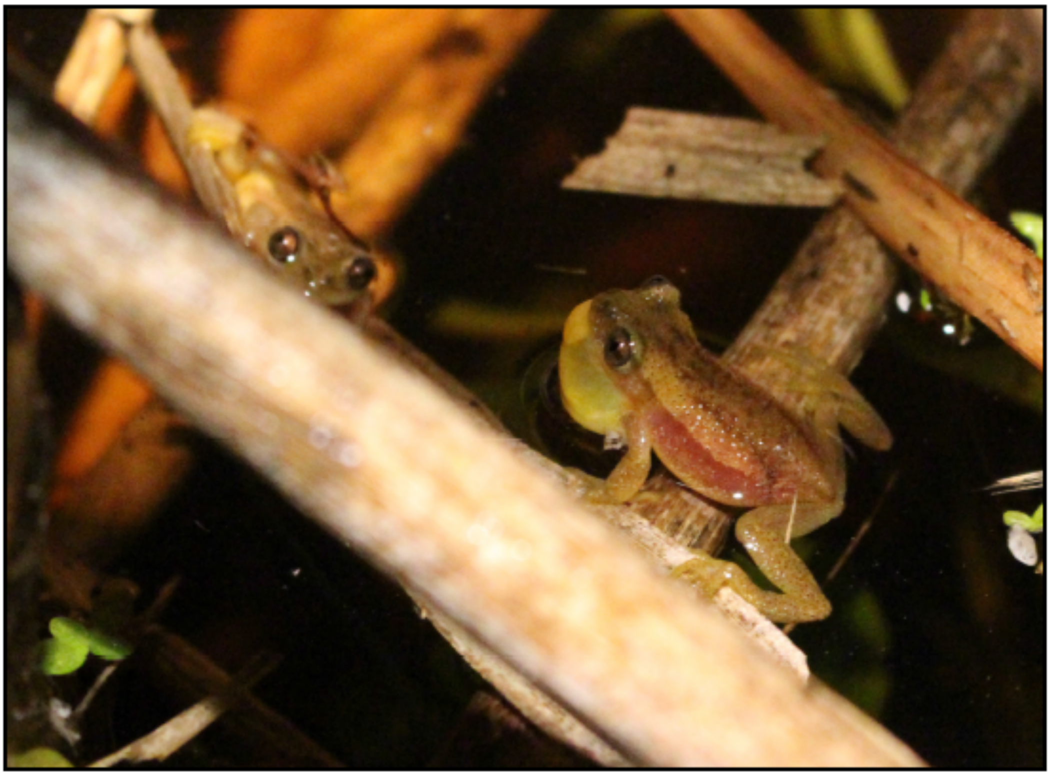


Figure 3-2: Typical courtship behaviour displayed by *A. knysnae* male during approach by female, with inflated vocal sac, communicating his position and intention

3.3.3 Data analysis: acoustic software applications

All digital acoustic recordings were analysed using Raven Pro® Beta ver.1.5 software and Wildlife Acoustics Kaleidoscope® software ver. 4.1.3. Scrutinizing spectrogram patterns created by the Raven® software allows for manually identifying calls of *A. knysnae while t*he Kaleidoscope® application creates clusters to identify the various calls automatically. Both applications use complicated algorithms to produce identifiable spectrogram patterns and frequency modulations of calls.

Kaleidoscope®

This application functions by producing cluster files of similar spectrograms representing calls. These clusters are then grouped with reference to the best representative sound sample of any specific automatically identified sound portion or call. The produced clusters were manually examined to identify the presence of *A. knysnae* calls within the clustered sections. Kaleidoscope® (www.wildlifeacoustics.com) produces a Comma Separated Value (.csv) file describing these clusters by way of rows and columns indicating amongst others, date, time, duration, similarity offset from each other and various other user selectable variables of these identified calls. Data are then imported into MS Office Excel and produce spreadsheets for analysis.

Raven Pro

As a powerful sound analysis application, Raven Pro® software has the ability to automatically recognize calls, but recognizers must be constructed manually, and this proved to be complicated for *A. knysnae* (www.ravensoundsoftware.com). As the *A. knysnae* calls are overpowered by most other frog species, manual detection and analysis of calls turned out to be more effective and accurate, although a tedious and time-consuming exercise. Various tools within the application are then available to analyse frequencies, amplitudes, temporal measurements and other variables regarding the call or calling bouts. Data tables produced manually from this analysis, can also be downloaded and manipulated in MS Office Excel.

3.3.4 Data analysis: Call structure

Raven Pro® Software was used to produce spectrograms and waveform oscillograms from the directional call recordings at Covie. These sound images and waveform oscillograms were produced using a Hamming window with an FFT-length of 256 points and a 50% overlap. The calls were then analysed to ascertain individual spectral and temporal properties during calling bouts, temporal alignment with each other and finer scale call structure. The distinct spectrogram structure portions of the calls were examined using a number of temporal parameters for these call types. Various call syllables, notes and pulses produced during the digital recordings were measured with parameters relating to frequencies, amplitude, duration and energy of the various call components.

Calls from *Afrixalus spinifrons* were also obtained from recordings made by other investigators during field surveys. These calls were similarly examined and analysed in Raven Pro® using the same parameters as those during analysis of *A. knysnae.* Fewer calls of *A. spinifrons* were however available for analysis. The recording quality of these calls was of a high standard and all achieved with directional recording equipment. No information regarding environmental conditions during these recording were however available, and therefor only structural, temporal and spectral comparisons could be made.

3.4 Results

3.4.1 Call structure analysis – *Afrixalus knysnae*

Spectrograms of *A. knysnae* calls indicate two distinct patterns, a multiple note call of varied length and a single note call, also of variable length. The multiple note portion, or note grouping is identified as the A-call (Fig 3-3) and the single note portion as the B-call (Fig 3-4). Both call types' notes however consist of a variable number of pulses.

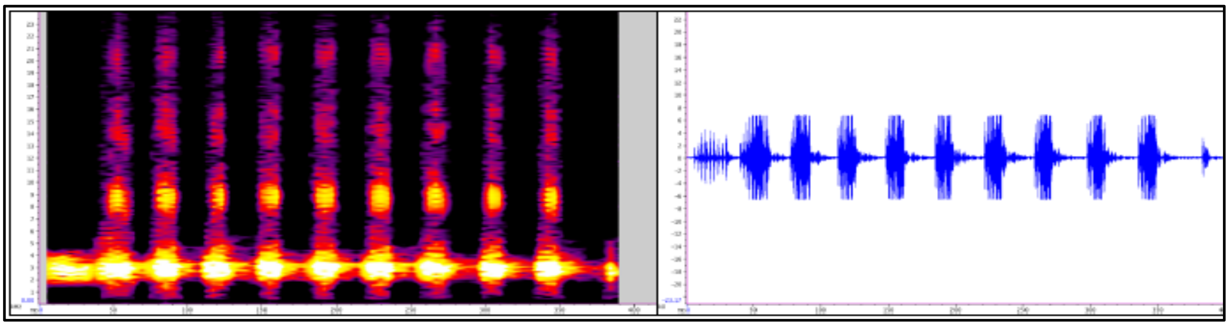


Figure 3-4: Spectrogram of the B-call with corresponding Waveform graphic.

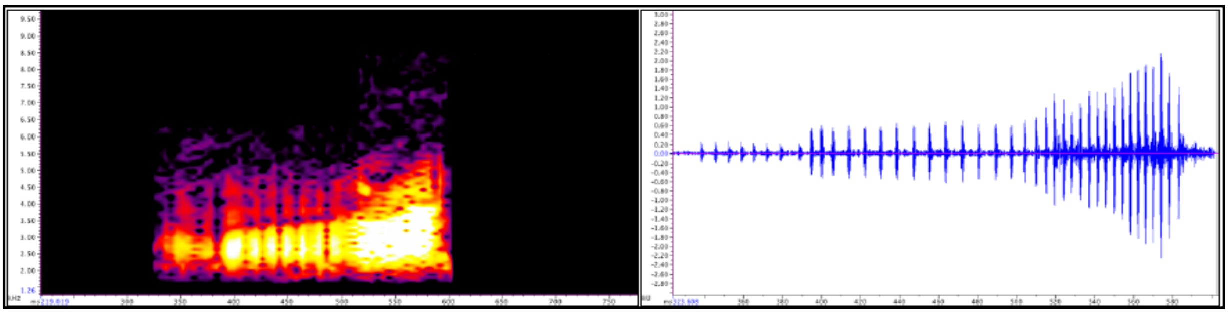


Figure 3-3: Spectrogram of the A-call (left) with corresponding Waveform graphic (right).

Each note of the A-call consists of a variable number of pulses (Fig 3-5) while the pulses in the one-note B-call are very rapidly produced and likewise vary in number (Fig 3-4). *Afrixalus knysnae* produces both calls either independently, or often starts its repertoire with the B-call transitioning into the A-call immediately thereafter. The latter mode creates a combined call, the spectrogram and waveform diagram thereof depicted in Figure 3-6.

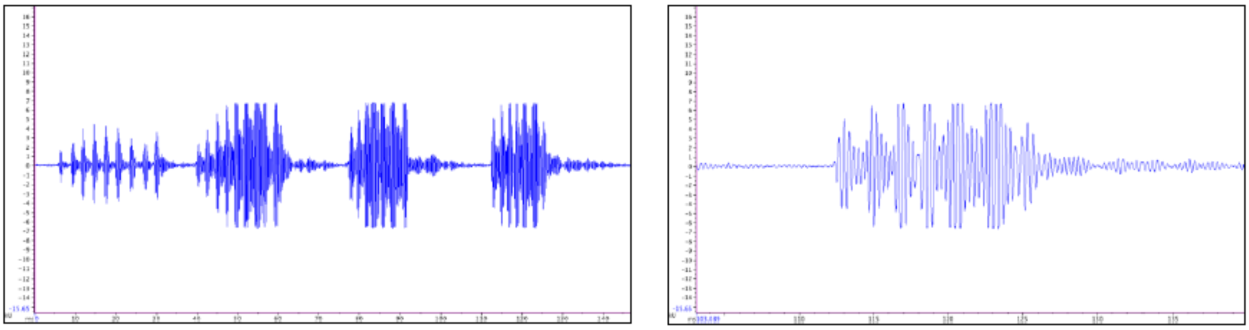


Figure 3-5: **Waveform images of a portion of the notes produce in the A-call, with one note's wave pattern expanded to show the pulses within a typical note.**

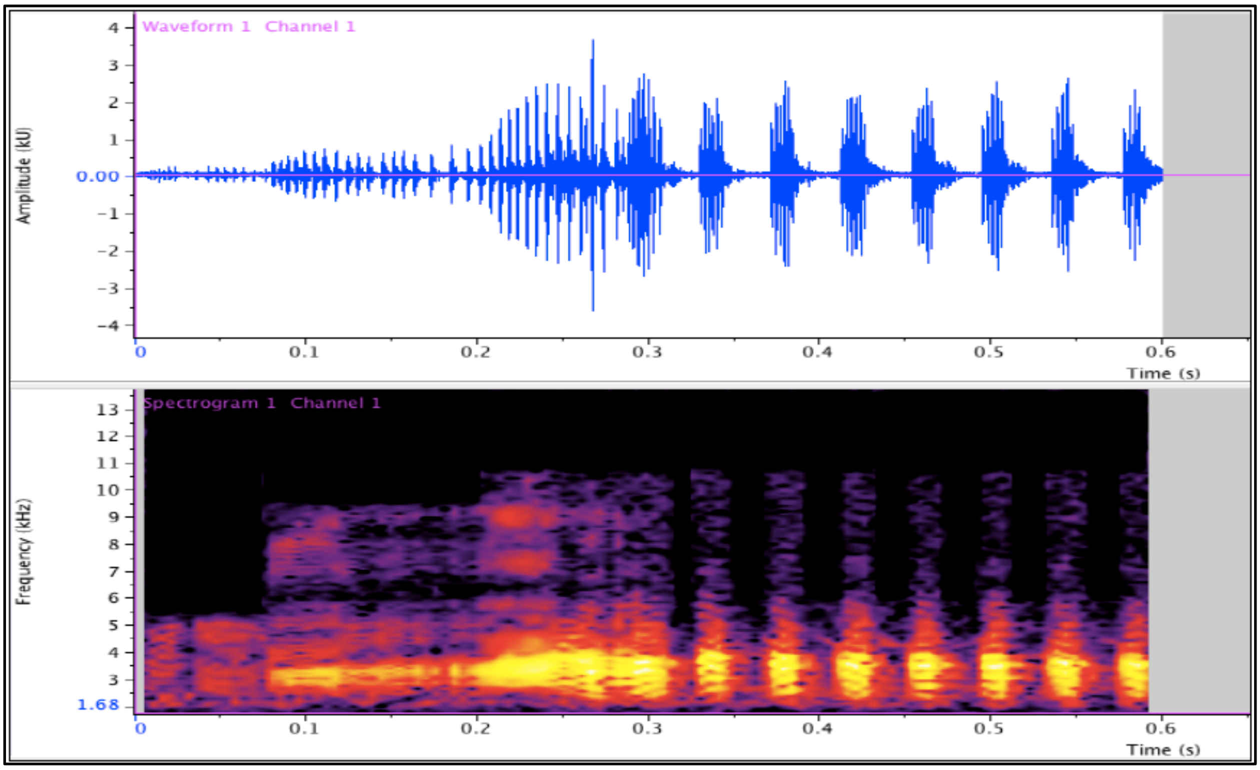


Figure 3-6: **Spectrogram and waveform graphic of a typical Combined call, starting with the B-call transitioning into the A-call immediately.**

A total of 68 A-calls, 27 B-calls and 28 Combined Calls were analysed. Within the A-calls, 65 notes and 134 pulses were measured. These measurements were made from the most prominent calls containing the least amount of background noise. Pulses of only one B-call was measured to determine its typical characteristic, while a count of the number of pulses was done for each of the 27 B- calls.

Measurements of the length of the calls were made from the start of the first note to the end of the last note of each call. Notes were measured in each call from the start of each note to the end of that note as well as the inter-note period, being from the end of such note to the start of the next. This allowed for the measurement of the total note period in every call analysed. Every note was further expanded in the waveform view in order for the oscillogram to reveal the various pulses it contained and to perform the measurements. Similarly, to the measurement methodology of the notes and note period, the pulses and inter-pulse periods were measured, resulting in the analysis of the pulse period (Fig. 3-7).

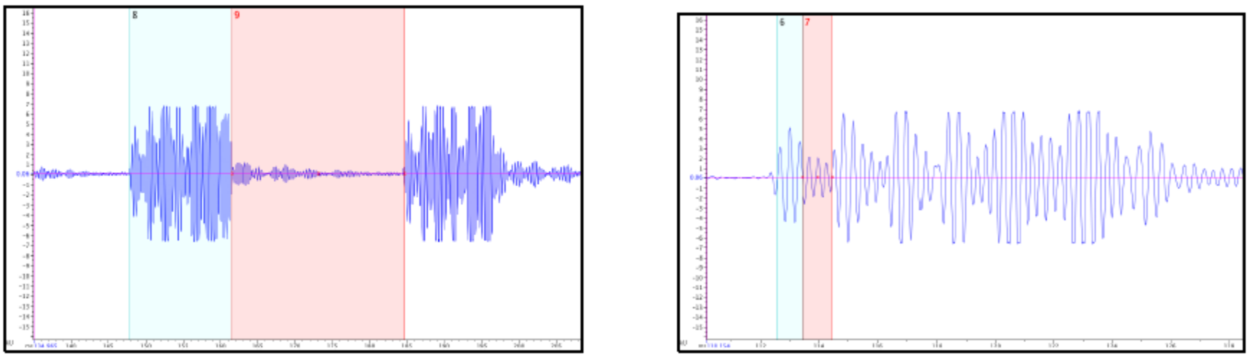


Figure 3-7: Waveform graphics of notes (left) and pulses within a note (right) with indications of the note, pulse and period measurements.

Measurements of these parameters enabled the calculation of the note repetition rates and the pulse repetition rates within and across the analysed calls. Repetition rate (Rr) is a function of the note or pulse periods occurring over the span of a specific time period. The resultant measurements of the calls of *A. knysnae* is given in rate per second (s^{-1}). The note repetition rate (nRr) as well as the pulse repetition rate (pRr) was calculated for all the sampled A and B portion calls. These calculations are recorded in Table 3-1 indicating the number of sampled calls, the number of call-components measured from each call type and the calculated repetition rates.

Table 3-1: Results obtained from measurements of the various call types, notes and pulses

Call Type	Call Component	(n)	($Rr\ s^{-1}$)	Mean (s)	StdDev (s)
A-call	Entire call	68		0,75	0,68
	A - Notes	56	25,6	0,01	0,003
	A - Pulses	134	300,71	0,00013	0,0006
B-call	Entire call	27		0,2	0,1
	B - Notes	1	-	-	-
	B - Pulses	41	160	0,00013	0,0005
Combined	Entire call	28		0,6	0,42

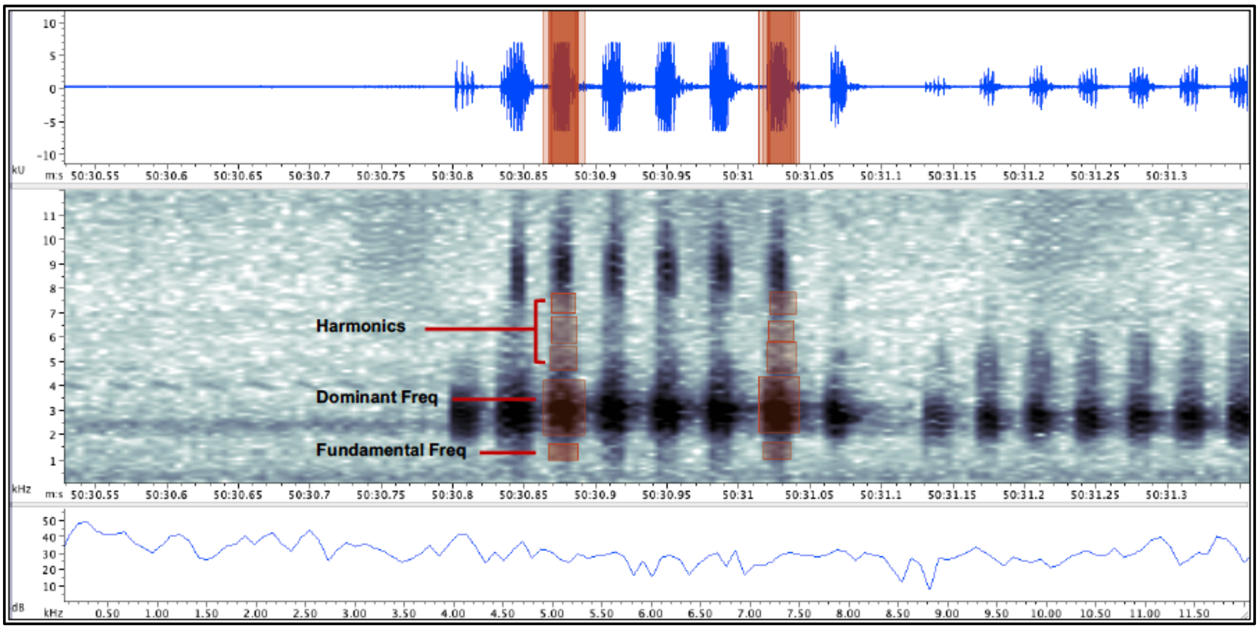
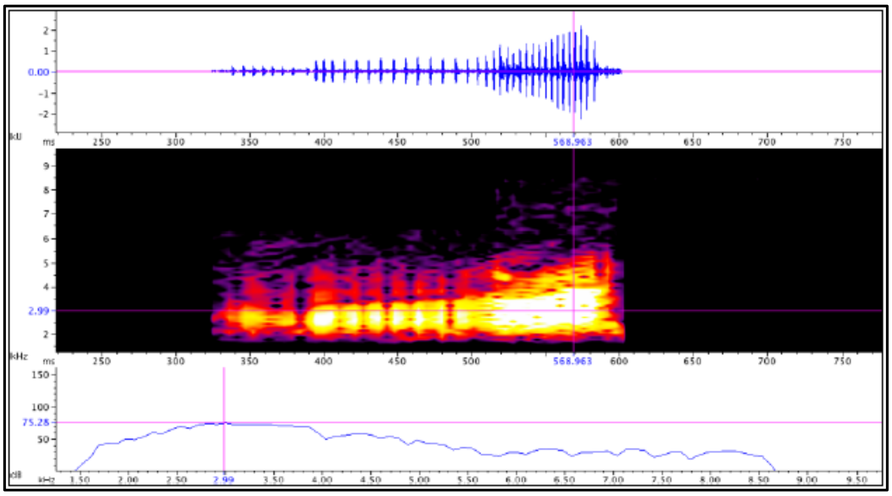


Figure 3-8: Illustration produced by Raven Pro® indicating the three screen components during calculations: waveform-, spectrogram- and power spectrum views. The three main frequency components of each note is indicated in the spectrum view.

Spectral analysis was performed on the A-call with measurements made of the fundamental frequency, the dominant frequency and harmonics (Fig. 3-8). A-calls were sampled randomly from the recordings and ten random notes from these calls were measured. The dominant frequency in the sampled notes had a mean *($\bar{x}$)* = 3056,27 Hz with a standard deviation *(s)* = 166,54 Hz. Power spectra were simultaneously calculated in Raven Pro® thereby indicating maximum amplitude (known as "F peak") of the dominant -, fundamental - and harmonic frequencies.

Table 3-2: Measurements of various frequency components as automatically measured by Raven Pro® upon selection of the specific attribute

Call Note	Fundemental frequency		Dominant frequency		Harmonic1		Harmonic2		Harmonic3	
#	Max Power (dB)	MaxFreq (kHz)	Max Power (dB)	MaxFreq (kHz)	Max Power (dB)	MaxFreq (kHz)	Max Power (dB)	MaxFreq (kHz)	Max Power (dB)	MaxFreq (kHz)
1	62,40	1781,20	95,20	3656,20	74,40	5531,20	58,80	6281,20	54,10	7593,80
2	59,60	1218,80	95,80	2437,50	68,30	3750,00	58,70	5156,20	47,80	6656,20
3	62,50	1312,50	92,00	2625,00	66,50	3937,50	56,80	5250,00	45,50	6656,20
4	66,70	1687,50	95,00	3187,50	73,30	4781,20	56,20	5437,50	53,20	6187,50
5	63,70	1406,20	95,20	2906,20	72,50	3468,80	54,80	4687,50	55,10	6187,50
6	65,50	1687,50	95,20	3093,80	74,40	4500,00	58,80	6000,00	54,10	7875,00
7	63,30	1500,00	96,10	3000,00	72,80	4593,80	55,60	6281,20	55,70	7875,00
8	60,50	1593,80	94,70	3093,80	74,10	4968,80	58,40	5906,20	54,70	7687,50
9	62,60	1500,00	97,40	3000,00	75,30	4875,00	61,70	6187,50	56,70	7593,80
10	62,20	1406,20	94,10	2812,50	68,10	4406,20	57,50	6093,80	47,60	7312,50
Mean ($\bar{x}$)	62,90	1509,37	95,07	2981,25	71,97	4481,25	57,73	5728,11	52,45	7162,50
StDev(s)	2,10	179,24	1,40	330,12	3,14	619,96	2,00	556,30	3,95	674,90



Call#	Max Freq (Hz)
1	2625
2	2812,5
3	2906,2
4	2812,5
5	2812,5
6	3281,2
7	2437,5
8	3375
9	3468,8
10	3750
Mean	3028,12
StDev	417,05

Figure 3-9: Screen views of B-call characteristics with frequencies of the 10 sampled calls (right).

Ten randomly selected B-calls were analysed with regard to their maximum dominant frequencies. The sample of B-portion calls had a dominant mean frequency of 3028,12 Hz and a standard deviation of 417,05 Hz (Fig 3-9)

3.4.2 Call structure analysis: *Afrixalus spinifrons*

Recordings of calls from *A. spinifrons* obtained from other investigators[1] were analysed using the Raven Pro® Software in a similar method to those of *A. knysnae*. This closely related species has the same two-part call structure with a varied number of notes and pulses as described for *A. knysnae*. Spectral and waveform analysis reveal very similar structures (Fig. 3-10). The call consists of rapidly repeated notes, while each note in turn contain a varied number of pulses (Fig. 3-11).

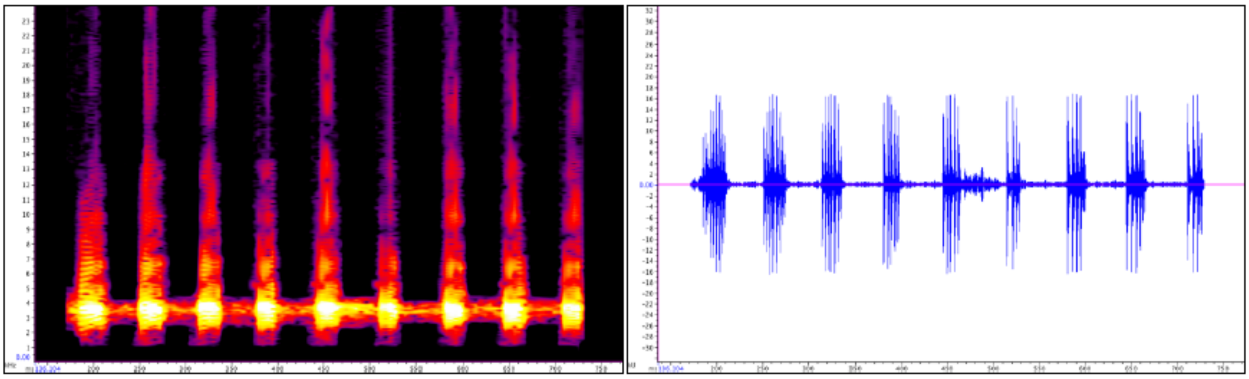


Figure 3-10: Calls from *A. spinifrons* show the same multiple note patterns to those of *A. knysnae* (A-call).

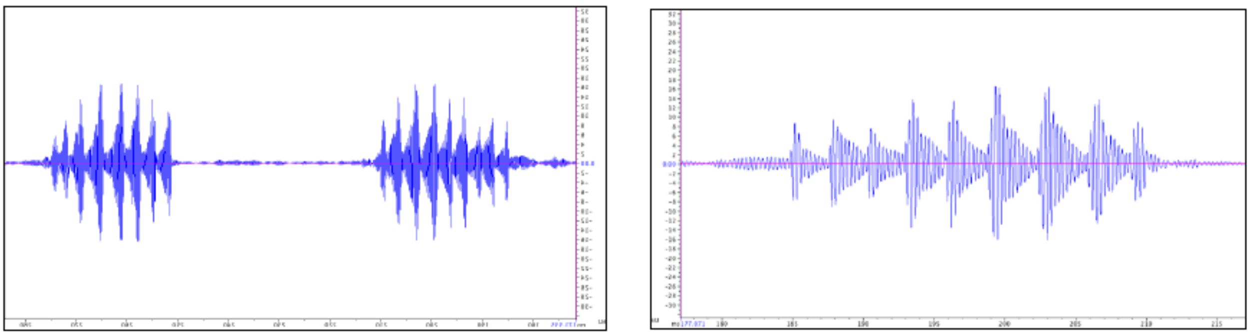


Figure 3-11: Waveform views of some notes from the *A. spinifrons* calls, again indicating the rapid pulse structure contained in each note.

[1] Recording obtained from Werner Conradie, Herpetologist Port Elizabeth Museum. Recordings from Silaka Nature reserve, Eastern Cape Province, South Africa during 2014.

A short recording obtained of *A. spinifrons*, containing a very limited number of clear calls was used for analysis. Calculation of the call lengths, the note and pulse lengths as well as note- and pulse repetition rates were performed on these calls (Table 3-3).

Table 3-3: Calculations from measurements of *A. spinifrons* call components.

	Calls (n)	Notes (n)	Pulses (n)
Qty (n)	4	36	181
Rr (s^{-1}) Mean ($\bar{x}$)		14,9	293,41
Rr (s^{-1}) StdDev (s)		1,34	4,67

Note Repetition Rate in *A. knysnae* was found to be nRr = 25,60 s^{-1} while the Pulse Repetition Rate is pRr = 300,71 s^{-1} (Table 3-1). Analysis of *Afrixalus spinifrons* calls (n=4) show nRr = 14,90 s^{-1} and pRr = 293,41 s^{-1}. This indicates a large discrepancy of the Note Repetition Rate (nRr) between the two species, (*A. knysnae)* 25,60 s^{-1} > *(A. spinifrons)* 14,90 s^{-1}, (±10,7 s^{-1})

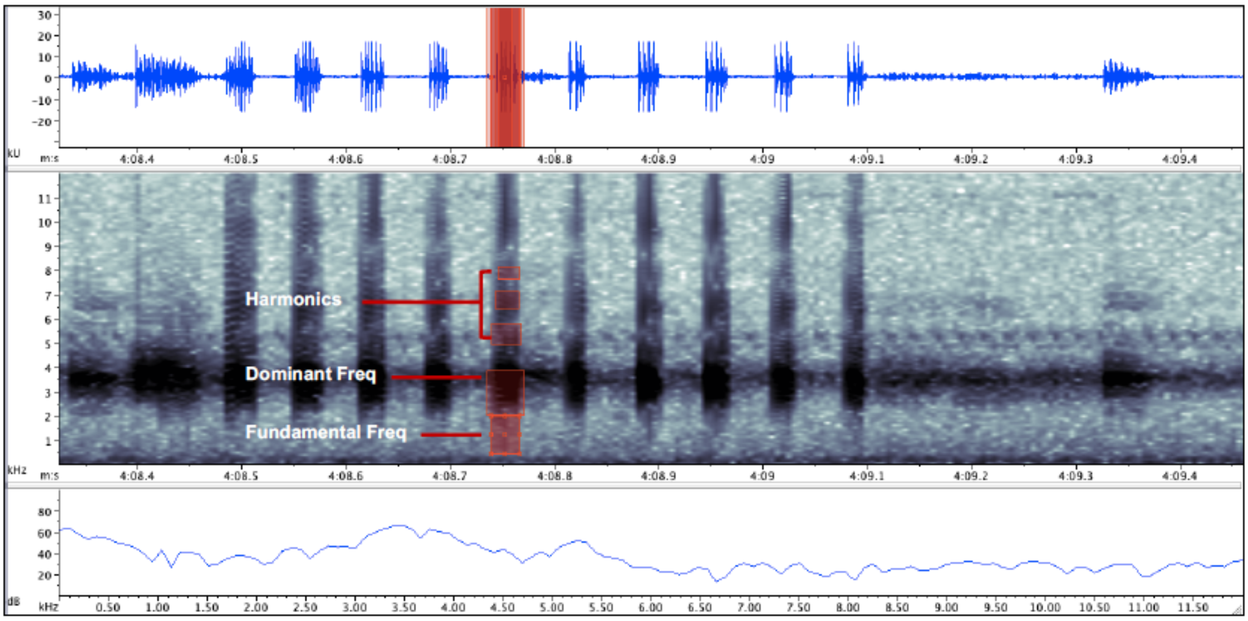


Figure 3-12: Waveform-, spectral and power spectrum views of *A. spinifrons* call with frequency components indicated.

Figure 3-12 depicts the spectral analysis conducted on the *A. spinifrons* calls, indicating the maximum dominant frequency to be 3750 Hz. The maximum fundamental frequency was at 1875 Hz, while harmonic one at 5156,2 Hz, harmonic two at 6656,2 Hz and harmonic three at 7781,2 Hz.

Table 3-4: Comparisons between the call mean ($\bar{x}$) frequencies of the spectral components of *A. knysnae* and *A. spinifrons.*

Frequency (Hz)	*A. spinifrons*	*A. knysnae*	Diff. Δ
Fundemental	1875,00	1509,37	-365,63
Dominant	3750,00	2981,25	-768,75
Harmonic 1	5156,20	4481,25	-674,95
Harmonic 2	6937,50	5728,11	-1209,39
Harmonic 3	7781,20	7162,50	-618,70

Spectral measurement comparisons of the two species reveal frequency differences at all the note components. This confirms the almost indistinguishable audible perceptions between the species when manual aural surveys are done, yet *A. knysnae* does call at marginally lesser frequencies. (Table 3-4).

3.5 Discussion

3.5.1 Basic Call description and measurements

The call of *Afrixalus knysnae* consists of two distinct call structures, being a longer "trill" sound (described herein as A-call) and a short “zip” sound (B-call). The A-call consists of a variable number of notes, produced in rapid succession, while the B-call only has one note, starting at a lower frequency and ending abruptly at a high frequency. Both these components are of variable length and the A-call is more conspicuous during calling bouts than the B-call. Both sounds are made frequently, but no consistent repetition rate or pattern in the producing thereof was observed. Both sounds are either produced separately, the one before the other or in sequence with each other or itself. The results from the analysis of the directional recordings indicate the complexity of both portions.

Measurements of the structures of both type of calls, revealed large variability in the duration of each call. Köhler (2017) suggests that calls are described with reference to call duration, notes and pulse characteristics. These parameters allow the calls to attain a certain “signature” or identifiable characters and intrinsic patterns that standardise the call types. As a result of the large variability in call duration and silent periods between calls, measurements of the call repetition rate of *A. knysnae* cannot be determined. The variable lengths are purely a function of the individual male’s ability to produce extended repertoires, but the structure of those variable calls remain essentially the same. The note and pulse characteristics within each separate call therefor distinguish the vocalisation of the species. *Afrixalus knysnae* does not show any call repetition pattern but calls rather seem to be induced in relation to approaching females or males encroaching on the caller’s territory. Both call patterns are produced in this way, with no specific repetition pattern.

3.5.2 Call comparisons with congeneric species

Measurements of these fine attributes of anuran calls, greatly assist in distinguishing species from one another, especially when acoustic signals are similar between congeneric species leading to investigations of speciation (Hoskin *et al.*, 2005). Taxonomic classification based on acoustic evidence have been investigated in avian species (Alstrom and Ranft, 2003) and bioacoustic studies have been instrumental in description of various cryptic frog species since the end of the 20th century (Glaw and Köhler, 1998; Vences and Köhler, 2008). This implies species comparisons can be based on acoustic attributes, specifically the spectral analysis thereof as investigated herein. Intraspecific as well as interspecific approaches have been studied to comprehensively understand species delineation based on acoustic characters (Forti *et al.*, 2012).

The current study does not deal with taxonomical delineation of *A. knysnae* based on acoustic evidence, but the uniqueness of the calls of *Afrixalus* in general makes this topic worthwhile to revisit. In this study a quick comparison between *A. knysnae* and its closest conspecific relative *A. spinifrons* gives a glance into this phenomenon. Comparing the attributes of the A-call of both species clearly shows that both species employ this call mode, both consisting of a number of individual notes and at varied lengths. The similarities are striking when the spectrograms are compared and from this comparison alone, finer spectral differences are still concealed. Analysing the notes individually however, differentiates more clearly between the attributes of the call characteristics.

Although the length of the calls in both species differs almost every time during calling bouts, the note repetition and pulse repetition rates are a fixed function of the duration of each call. Comparison of these components are therefore based on the same measurements and calculations and expressed as rate per second. These differences may however be negligible to the human ear, but may have profound influences on attracting mates within a chorus where inter- or even intra competition for resources and reproductive successes are vital (Schwartz *et al.*, 2001).

The variability of the two sounds produced by *A. knysnae* males probably have separate signalling intentions. This call structure also occurs in other *Afrixalus* species and thus seems to be a call characteristic of the genus (Blackwell, 1988). Duration of the two different components itself is a measurement of some kind of intention being signalled, either as advertisement, aggression, or territorial behaviour. The prolonged A-portion is used much more frequently, while the shorter B-sound seems to be produced more frequently during the inception of a calling bout yet not exclusively. Observations of calling activity could however not distinguish the exact purpose of the two portions of the call. The same male would produce both sounds at various times during the calling bout and often without any other male or female in close proximity. Studies suggests that the two-part call in *A. spinifrons* fulfil specific functions however, either as courtship calls, territorial delineating calls or aggressive signals (Blackwell and Passmore, 1990)

Spectral and aural comparisons in general between the various southern African *Afrixalus* species indicates the similarities in their calls. Congeneric species other than *A. spinifrons spinifrons* and *A. spinifrons intermedius* occur further up the East coast of South Africa in the Provinces of the Eastern Cape and KwaZulu-Natal and into Mozambique. *Afrixalus delicatus* (Pickersgill, 1984), *A. aureus* (Pickersgill, 1984) and *A. crotalus* (Pickersgill, 1984) in this instance all have similar A-call structures. Basic call structures diagrams of these species reveal the similarities which follows that aural observation should also confirm these similarities (Fig 3-13 (A) - (D)).

Afrixalus fornasini (Bianconi,1849) which occurs sympatricaly with the latter two species, does however show a difference (Fig 3-13 (D)). The frequency of *A. fornasini* calls seem much lower while it lacks the two-part structure. Various other clearly distinguishable morphological differences are also present in this species (i.e. size and colouration) and together with acoustics can assist investigators in making clear taxonomical findings in this regard.

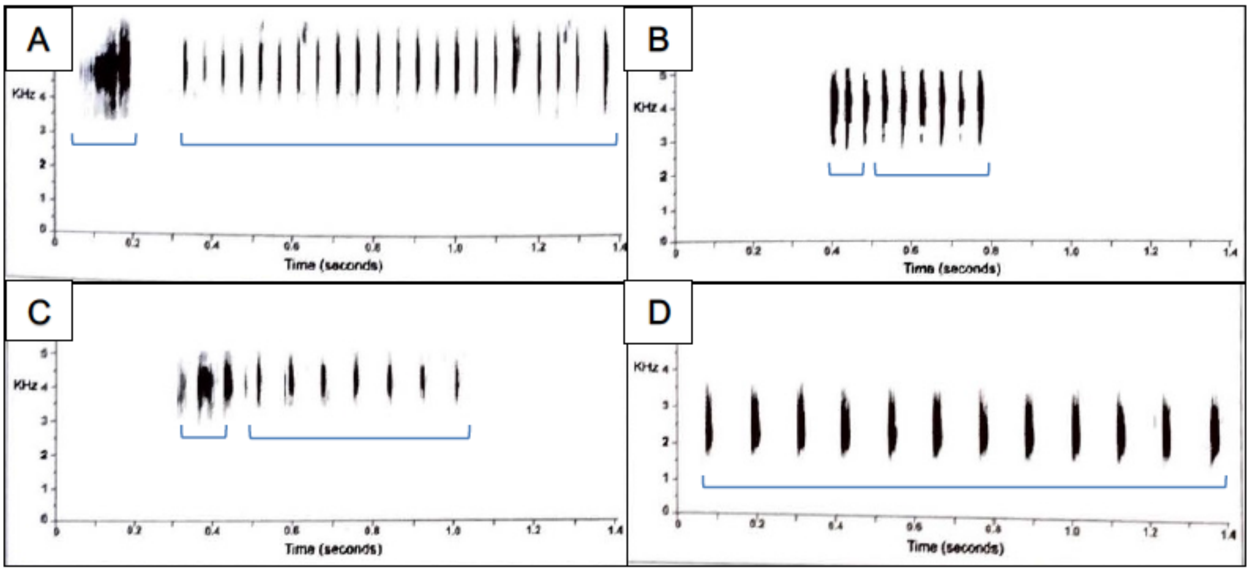


Figure 3-13: Spectrograms of congeneric species to *A. knysnae*: (A) *A. delicatus*, (B) *A. aureus*, (C) *A. crotalus* and (D) *A. fornasini*. (Du Preez and Carruthers, 2017)

3.5.3 Call comparison with other sympatric species

Afrixalus knysnae occurs sympatricaly with *Hyperolius marmoratus marmoratus, Strongylopus grayii, Strongylopus faciatus, Semnodactylus weallii, Cacosternum nanum, Hyperolius horstockii* and *Sclerophrys delalandi*, all of which have been recorded at the sites surveyed using PAM. At most of the sites, calls from *H. m. marmoratus, S. grayii, S. faciatus, C. nanum* and *S. weallii* are so prolific that calls of *A. knysnae* are tremendously masked from PAM recordings. All of these species call at different frequency bandwidths and at varied amplitudes. Call signatures as depicted in graphics produced with Raven Pro® clearly indicated the spectral difference in comparisons with *A. knysnae* (Fig. 3-14). The vast numbers of individuals at the sites increases the dominance of their calling bouts and in most instances, when a chorus is in full song, the calls seem to dominate the soundscape across the spectrogram lasting up to 30 or 40 minutes at a time. Spectral analysis of calling periods however greatly assist in identifying these spectral “signatures’ and indicates the ecological significance of using PAM as a monitoring and analysis tool in anuran assemblage studies.

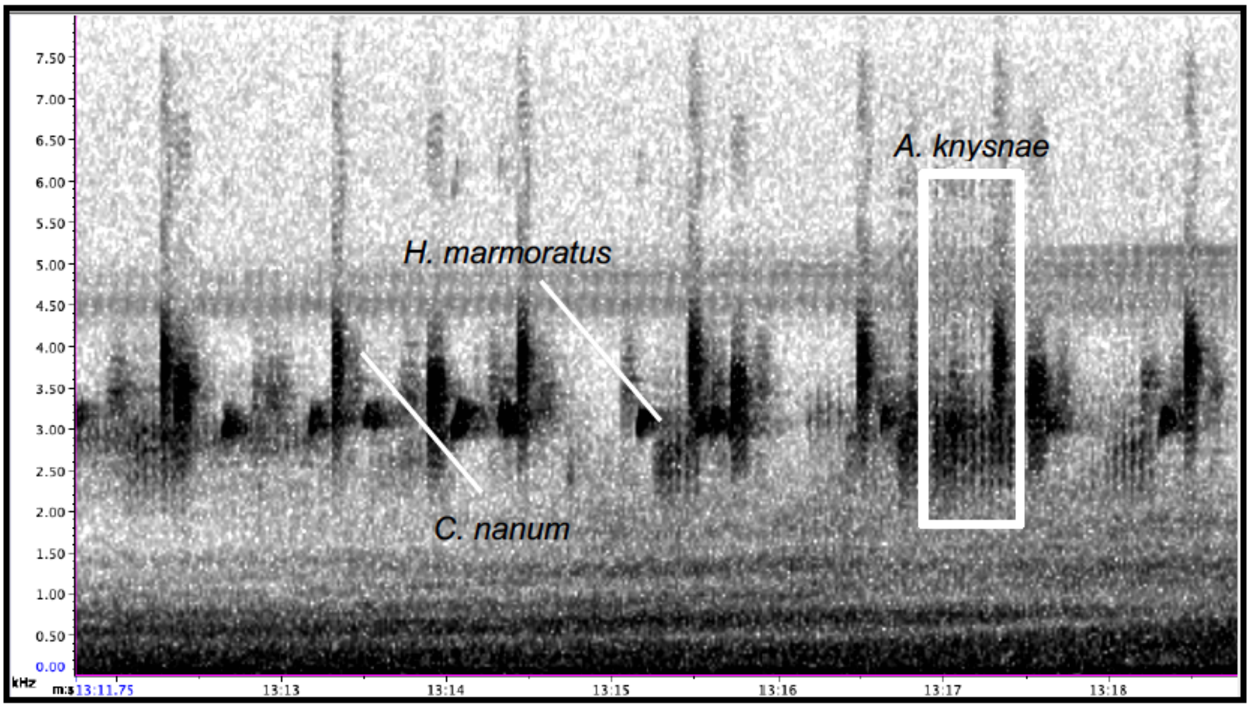


Figure 3-14: Image from Raven Pro® indicating the calls of certain sympatric species during a chorus of various species at a common habitat.

3.6 Conclusion

Passive Acoustic Monitoring as a tool for surveying not only individual species or species assemblages, but also to collect data on whole ecosystem functioning, is gaining huge momentum in ecological studies (Pijanowski *et al.*, 2011; Gasc *et al.*, 2013). One of the main advantages of PAM is its low- to non-intrusion factor during monitoring periods. Collecting continuous data throughout a monitoring period maximises the effort required to achieve significant outcomes. Vast amounts of data can also be collected in this manner with a fraction of the resources required to obtain the same results with conventional fieldwork methods. Species or environments that are difficult to survey either due to temporal constraints (i.e. nocturnal), aquatic or even subterranean habitats or seasonal difficulties can be surveyed in this manner. Ecoacoustics as a discipline of ecology studies are leaping into the future with equipment become technologically better and costs being driven down through sheer use and applicability thereof (Gibb et al. 2018).

Basic studies such as acquiring absence and presence data of A. *knysnae* herein was tremendously simplified and empowering to a sole investigator having to do fieldwork over a vast geographic range. Results was achieved with only two recorders available, yet the data collected is much more than the scope of this study allowed. Data from these recordings regarding many more aspects of the species, its environment, ecology and conservation are still being processed and will be produced in follow-up publications, reports and presentations.

Spectral analysis with purpose designed software applications enable fine scale measurements and calculations of frog calls. *Afrixalus knysnae* is a species with a number of anomalies and uncertainties that needs to be clarified. Data regarding its taxonomical relationship within its genus and family have not completely been resolved, but with a tool such as digital analysis of bioacoustics, information in this regard may well assist future taxonomic studies. The use of bioacoustics in behavioural studies such as those studied in other species of *Afrixalus* (Blackwell, 1988) has already proven extremely successful.

CHAPTER 4 TADPOLE MORPHOLOGY AND REPRODUCTIVE BEHAVIOUR

Man is an artefact designed for space travel. He is not designed to remain in his present biologic state any more than a tadpole is designed to remain a tadpole.

William S. Burroughs
1993

4.1 Introduction

Afrixalus knysnae is by nature a cryptic species with a subdued voice and is thus difficult to locate. Collecting tadpoles has also proven difficult by many investigators over the years with only a few specimens available at the Port Elizabeth Museum, Herpetological Department and three specimens in very good condition housed in the South African Institute for Biodiversity (SAIAB) collection for Amphibians situated at the North West University, Potchefstroom.

In terms of the research agreement with SANPARKS following ethics clearance and the conservation status of *A. knysnae*, no specimens were able to be collected and fixed for further investigation from the natural habitats during this study. The specimens from the SAIAB collection at Potchefstroom were used for a paper presented to and accepted by ZOOTAXA on the morphology of the tadpoles of the species (De Lange and Du Preez, 2018).

This paper also included description of the egg deposition after oviposition and early larval development of the tadpoles. Investigation of the aspects of egg laying took place within natural habitat at various sites identified in the study while early development of larvae was observed under controlled aquarium conditions. Results and discussion of this portion of the study is incorporated in this document by presenting the ZOOTAXA article herein (*Verbatim* and as formatted for publication).

4.2 The tadpole of *Afrixalus knysnae* (Loveridge) (Anura: Hyperoliidae), with comments on reproductive biology

Leaf–folding frogs of the genus *Afrixalus* comprise 33 species across South-East, Central and West Africa (Frost *et al*. 2018) with tadpoles being described for *Afrixalus aureus* (Pickersgill, 1984~~)~~, *A. delicatus* (Pickersgill, 1984~~)~~, *A. dorsalis* (Peters, 1875), *A. fornasini* (Bianconi, 1849), *A. fulvovittatus* (Cope, 1861), *A. laevis* (Ahl, 1930), *A. morerei* Dubois,1986, *A. nigeriensis* Schiøtz, 1963, *A. spinifrons* (Cope, 1862), *A. stuhlmanni* (Pfeffer, 1893), *A. uluguruensis* (Barbour & Loveridge, 1982), *A. vibekensis* Schiøtz, 1967, *A. vittiger* Peters, 1876, and *A. weidholzi* (Mertens, 1983) (see Pickersgill 2005; Channing *et al*. 2012). Members of the genus have a unique approach to egg deposition, laying their eggs on soft-leaved hydrophytes. The male folds or rolls the leaf with his hind legs into a sheath following oviposition and fertilisation (Rose 1950; Wager 1954; Du Preez & Carruthers 2017), excreting an adhesive substance and enclosing the eggs within while the “glue” cures (Rose 1950, Wager 1954, authors pers. obs.). *Afrixalus knysnae* (Loveridge) is the only leaf-folding frog species present in the Southern Cape Fynbos biome in Southern Africa (FitzSimons 1946; Loveridge 1954; Poynton 1964) and is currently listed by the IUCN as Endangered (EN) (IUCN 2016). This species breeds in rain-filled temporary pools and depressions. Wager (1954) anecdotally described the tadpoles of this species as belonging to *A. spinifrons* (as *Megalixalus spinifrons*). Wager (1965) reported that the tadpole of *Afrixalus brachycnemis* was morphologically the same as that of *A. knysnae*. Channing *et al*. (2012) describes aspects of morphology of various tadpoles across *Afrixalus* with basic descriptions of *A. knysnae* tadpole relating to its size, small nostrils and sharp tail tip. Recently, Du Preez & Carruthers (2017) updated their field guide with basic descriptions of body size and shape, almost transparent tail fin and acute tail tip. Description of the mouth parts from both sources describes a single row of laterally and posteriorly positioned papillae, while Du Preez and Carruthers (2017) refers to a second aboral row of poorly developed papillae. Pickersgill (2005) treats *A. knysnae* as subspecies of *A. spinifrons* and refers to the mouthparts as having no labial teeth and a rudimentary second row of papillae.

In the current study we provide a detailed description of the tadpole of *Afrixalus knysnae*, emphasizing differences from Wager’s description and illustrations and as well as supplementing the descriptions of the mouth parts by Pickersgill (2005), Channing *et al*

(2012) and Du Preez and Carruthers (2017). We furthermore provide information regarding the egg deposition, general utilisation of vegetation resources during this process and some observations regarding initial tadpole development.

Tadpoles were collected on 23 December 2010 at a site near Covie (33.95833º S, 23.60528º E) in the southern Cape region at a temporary endorheic wetland, one of only seven recorded localities. *Afrixalus knysnae* tadpole morphology differs significantly from all other species in the region. Due to its conservation status, only three tadpoles were collected. Tadpoles were photographed and subsequently fixed and preserved in 10% neutral buffered formalin and accessioned in the South African Institute for Biodiversity (SAIAB) collection for Amphibians at the North West University, Potchefstroom, South Africa (SAIAB Catalogue Number: 205811). Measurements include: total length (TL); body length (BL); body width (BW); body height (BH); tail length (TAL); maximum tail height (TAH); tail musculature width (TMW); tail musculature height (TMH); dorsal fin height (DFH); ventral fin height (VFH); internarial distance (IND); interorbital distance (IOD); eye diameter (ED); narial diameter (ND); eye-spiracle distance (ESD); snout-eye distance (SED) and oral disc width (ODW). Measurements and terminology follow Lambiris (1998), Altig and McDiarmid (1999) and Altig (2007) Measurements and photographs of preserved specimens were obtained using a Nikon AZ100 dissecting microscope using NIS Elements-D software. Photographs of egg clutches and live tadpoles were taken with a DSLR camera fitted with a 100mm macro lens and twin flashlight.

For observations on reproductive biology, site surveys were conducted at three breeding localities to determine the nature of vegetation used by the species. These sites were at George (Nelson Mandela University -George Campus) (33.94466º S, 22.52173 7º E: WGS84, 223 m), Covie (33.95833º S, 23.60528º E; WGS84, 207 m) and Groenvlei (34.02596º S, 22.83424º E: WGS84, 14 m). The Covie site was at a temporary endorheic wetland that holds water after heavy rain over several days, whereas Groenvlei is a permanent freshwater lake (approximately 360 ha), completely isolated from any river or the ocean and entirely fed by rainfall and underground water (Parsons, 2014). Three folded *Hydrocotyl bonariensis* leaves containing newly deposited egg clutches were collected and housed in an open glass aquarium in order to determine egg development and clutch size. Water was taken from the habitat where the leaves were collected, divided into three compartments and filled to a depth of 200 mm (similar to natural conditions). Tadpoles were allowed to hatch naturally in the compartments.

Tadpole morphology. The description of the *A. knysnae* tadpole is based on the three specimens, Stages 25–28 (Gosner, 1960). Tadpole long and slender with the tail three times the body length (Fig. 4-1 A–C). In dorsal and ventral view, the body is ovoid and triangular, in lateral view a streamlined head from above and below tapering to the terminal mouth. Snout is rounded in dorsal view and more pointed than round in lateral view. Eyes laterally spaced and prominent, pupil round. Nostrils small, situated close to tip of the snout and widely spaced. Spiracle is sinistral and originates at level of gill chamber, with spiracle opening just below the body axis and facing in a posterior direction; visible from dorsal, ventral and lateral view. Vent is small, dextral and supramarginal. Tail long (73% of TL) and 18% deeper than the body. Caudal muscle shaft height, equal distance from the dorsal and ventral fin margins for first third (1/3) and then tapering sharply to an acute tip. Dorsal fin starting off low rising gradually to reach deepest point one-third down the tail; gradually tapering down in a curve with the last 25% diminishing sharply into an acute tip. Ventral fin starting at base gradually increases in depth to reach the deepest point one-third down the tail; ventral fin tapering gradually towards the tip with the last 25% of the fin diminishing sharply into an acute tip. At the deepest part of the tail, the dorsal fin makes up 28.4%, tail shaft 45.2% and ventral fin 26.4%. Oral disc is subterminal, small and only 2.3 mm wide, this being 53% of BW (Fig. 1D). A single row of short fat finger-like papillae, that are as long as wide, at the mouth corners and along the lower lip. The medial third of the mental papillae are directed forward while the remainder of the papillae are directed laterally. Labial tooth rows absent (LTRF 0/0). Jaw sheaths delicate to moderate; margin darkly coloured; edges deeply serrated along the entire length of both supra- and infrarostodont; suprarostodont curved to form an arch extending past the infrarostodont at corners. Mouth aperture is wide and shallow.

Measurements [in mm (range)]. TL 24.8 (22.4–29.3); BL 6.7 (6.1–7.7); BW 4.3 (4.2–4.4); BH 3.6 (3.4–3.8); TAL 18.0 (16.2–21.5); TAH 4.4 (4.7–4.0); TMW 2.1 (1.6–2.4); TMH 2.2 (2.0–2.7); DFH 1.4 (1.4–1.4); VFH 1.3 (1.3–1.3); IND 1.0 (0.9–1.2); IOD 2.7 (2.5–3.0); ED 1.3 (1.2–1.5); ND 0.3 (0.3–0.4); ESD 2.0 (1.9–2.1); SED 2.7 (2.5–2.9); ODW 2.3 (1.9–2.7).

Colouration. In life (Stage 28), dorsum yellow olive-brown with dark stippling concentrated around the nostrils (Fig. 4-1 A,B). A band of dark stippling extends from behind the nostril at 45° towards the midline, then backwards and out in the direction of the eye, and back again to the midline. A diamond shaped pineal ring is thus formed on the centre of the head, tapering to a narrow band towards the tail. Orbitonasal line is absent. Two dark oval shaped fields present above abdominal region. The eyes are prominent, golden with peripheral dark stippling. A faint band of dark stippling extend over the upper half of the body from the mouth, over the eye to the tail. Lower part of the body is semi-transparent in lateral view with reddish gills and yellow gut visible. Developing hindlimb is white. Ventral and dorsal fins semi-transparent and delicate with very fine white flecks present along the fin margins. V-shaped myomeres visible along full length of tail shaft. In ventral view the skin is transparent with abdominal area covered by a silver membrane, jaw sheaths are black, red heart in the middle of the body, and pinkish gill arches are visible. Spiracle transparent. In preservative (Fig. 4-1C), tadpoles appeared slightly lighter, stippling brown and more pronounced but shape and position similar. Red and yellow of heart, gills and gut faded to a pale white; eyes black with grey pupil. Dorsal and ventral fins almost completely transparent. Myomeres prominent only in anterior third of tail shaft.

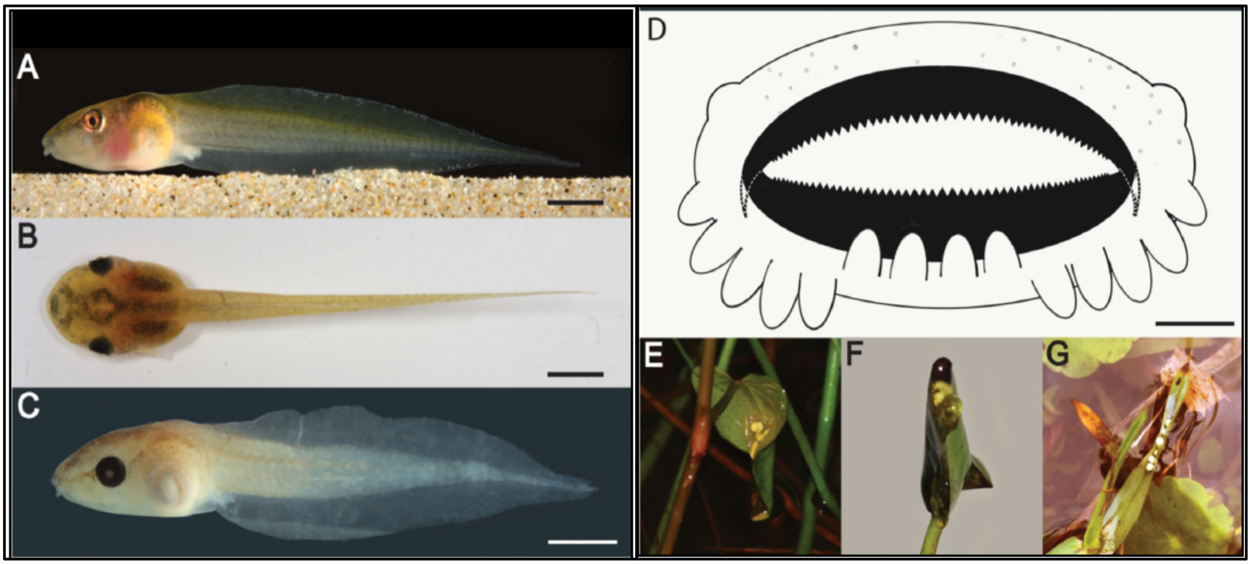


Figure 4-1: **Graphic depictions of live and fixed tadpole larvae of *A. knysnae* (A-C), drawing of mouth (D) and egg deposits on various leaf-types (E-G).**

Reproductive biology. At the George site, large stands of *Polygonum acuminatum* (Fig. 4-1E) are found within the water body and are utilised by *A. knysnae* for egg deposition. These plants have small soft acuminate leaves ending in a sharp tip, no longer than 50 mm. Oviposition was at approximately one-third of the leaf length from the node, deposited in a clutch, longitudinal towards the tip. The leaves were folded in a cylindrical or funnel-like shape. Leaves of these plants used in this method were positioned close to the edge of the water body and partially shaded by overgrowing vegetation with the leaves containing egg-clutches situated just above or just below the water surface.

At the Covie site, a low growing creeper-like plant, *Hydrocotyl bonariensis* (Fig. 4-1F), with a disc-like leaf on a single stalk and attached by rhizomes to the ground was recorded as dominant plant. *Afrixalus knysnae* uses the *H. bonariensis* leaves extensively for egg deposition at this site. During flooding, the leaves grow through the water surface and float. The leaves of *H. bonariensis* are folded and “glued” downwards onto the stem or upwards from the stem resembling a half moon. The folded leaves sink back into the water just beneath the water surface. *Polygonum acuminatum* was not observed at this site at all.

Finally, at Groenvlei the preferred breeding sites of *A. knysnae* are located within a wide sedge marsh and reed marsh collar dominated by *Typha capensis* and *Phragmites australis*. Human development around the lake caused the bank to be planted with *Pennisetum clandestinum* (Kikuyu grass) (Fig. 4-1G). This fast-growing grass creates elongated strands during growth spurts with long thin, lanceolate leaves which then extends into the water body at the breeding sites. *Polygonum acuminatum* and *H. bonariensis* are also present at these water edges. The young leaves of any one of *P. acuminatum*, *H. bonariensis* and those of *P. clandestinum* are used at this site by *A. knysnae* during egg deposition. Leaves of *P. clandestinum* are however very narrow and as a result only folded in half to cover egg masses, often resulting in more exposed eggs than those in *P. acuminatum* and *H. bonariensis*. No data was recorded regarding the number of different plants used or total preferences of plants but rather the occurrence of different resources available to aid in reproduction activity.

Larvae from eggs within the *H. bonariensis* leaves housed in the aquarium appeared within 12 to 15 days from being collected. After hatching, larvae remained attached to the leaf substrate for approximately 2–3 days using a pair of adhesive glands situated posterior and on either side of the forming mouth opening. All larvae observed at this stage were at Gosner Stages 20–22, large external gills visible, cornea transparent, and mouth visible. Characteristic dark markings and patterns on the dorsal side were visible. These characteristics seem to indicate that larvae undergo further development within the leaf sheath after initial hatching and this seem to confirm Wager's observations (Wager 1954). Following detachment from the leaf, the tadpoles dropped to the bottom of the container and for the first three days remained mostly motionless during the day. Clutches of 13, 18 and 21 larvae hatched from the three egg masses, while one leaf contained one undeveloped egg. The 52 detached larvae had an average length of 6.9 mm ($\pm$0.9 mm).

Afrixalus knysnae tadpoles do however have some morphological similarities to other Southern African species, *A. aureus*, *A. delicatus* and *A. spinifrons* (Pickersgill 2005). Basic descriptions of these congeneric species indicate that it reaches maximum lengths 29 mm in *A. aureus* and 34 mm in both *A. delicatus* and *A. spinifrons*, slightly less than the 38 mm of *A. knysnae.* Furthermore, the tail of the tadpole of *A. knysnae* is of similar length as *A. spinifrons* and *A. delicatus* at between 2.3 to 3 times its body length. The dorsal portion of the body is pigmented while the ventral side is pale with the tail and fins ending in an acute tip for all four species (Wager 1954; Pickersgill 2005; Channing *et al.* 2012). Tadpoles of *A. knysnae* differ from all other southern African species on a combination of characteristics. *Afrixalus fornasini* tadpoles are double the length of all other *Afrixalus* tadpoles in the region and one mental labial tooth row is present (Wager, 1965). Tadpoles of *A. aureus, A. delicatus, A. spinifrons and A knysnae* completely lack labial tooth rows and have only a single row or oral papillae. Pickersgill (2005) refers to a second row of poorly developed mental papillae in *A. spinifrons*, which were not observed by the authors. Oral papillae of *A. knysnae* are short and fat while the papillae of all other southern African species are longer and more slender. The medial third of the mental papillae that are directed forward appears as an immediate contrast to the rest of the row unlike all other *Afrixalus* species from the region.

CHAPTER 5 GENERAL DISCUSSION AND REMARKS

“If you want a happy ending, that depends, of course, on where you stop your story.”

Orson Welles
1982

5.1 Introduction

Little research has been done and limited information is available regarding the distribution, biology, conservation status or taxonomy of *A. knysnae* in the Southern Cape. The species is however one of the priority species according to the South African National Biodiversity Institute’s Conservation Research Strategy document (2011). In this instance, further research was highlighted as a requirement in order to determine the actual current threats (if any) on the populations and the biology of the species (Measey, 2011). Similarly, the IUCN lists the species as endangered (EN) with notes on the lack of data on the species and the need for further research (IUCN, 2018). Resulting from this study, the first paper regarding a detailed description of the tadpole morphology and resource use during breeding activity was published and reproduced herein (Chapter 4), with notes on early larval development (De Lange and Du Preez, 2018). Data collected over the last three years and still being processed should assist in updating records and serve conservation authorities in their tasks regarding this priority species.

5.2 Ecological assessments for *Afrixalus knysnae*

Complete ecological assessment of this species was not the main aim of this study, but rather the attempt to update information on the species, understanding distribution of populations and status of the habitats where populations seem to persist. More detailed information regarding breeding behaviour, call structure and habitat is presented herein, but these are still basic with much more data still available to continue these studies. In closing therefor, some general remarks regarding the current study with some comments on the conservation efforts for the species in the future.

5.2.1 Investigative methods

Modern day technological advancements in methods of information collection and data acquisition has impacted on the methods employed to observe species with less intrusion.

These lower-impact observational methods allow animal behaviour to be displayed or continue with little to no disturbance yet recording every portion thereof by the investigator (Digby *et al.*, 2013; Wimmer *et al.*, 2013). Ecological studies using remote sensing equipment, static and movie cameras as well as acoustic recorders all achieve these results today, with the technology improving constantly (Acevedo *et al.*, 2009; Köhler *et al.*, 2017)

In this study Ecological Niche Modelling was performed using MaxEnt and readily available computer hardware and software to determine distribution probabilities. This is a cost-effective tool that enables the use of desktop data, online sources and other recorded data to delimit an area to be surveyed, almost without leaving the office or desk. Investigators working alone, as I was during this study, having vast areas to cover during limited breeding seasons, can use ENM with great success. It functions as a virtual group of fieldworkers covering a vast geographic area to ascertain suitable habitats to study. Together with GIS mapping software, preferable study areas or specific localities can thus be identified to do further finer scale observations.

The automated survey methodology followed in this study was continued by inspecting the individual identified sites by way of Passive Acoustic Monitoring (PAM) methods. Frogs extensively make use of vocalisation which has significant impact on their evolutionary, biology and reproductive fitness traits (Pijanowski *et al.*, 2011). Bioacoustic surveys using PAM during this study, enabled findings regarding absence or presence at localities. Analysis of this data with the use of software applications such as Wildlife Acoustics', Kaleidoscope® and Cornell University's Raven Pro® make disseminating large volumes of data far simpler with a higher degree of accuracy.

Presence and absence data together with temporal calling behaviour learned from the PAM process, then enabled investigations at probable localities. Ground truthing could then confirm the populations at each identified site. Directional Microphone Recordings (DMR) was used at very specific sites to collect data and further analyse spectral attributes of specific vocalisations. Visual Encounter Surveys at these sites further confirmed other behavioural traits pertaining to breeding biology and habitat use.

The main advantage of autonomous survey methods as used during the study, was the assistance it gave a lone researcher. Minimal resources were used to survey and study in excess of 100 km^2 of mountainous terrain. The methods employed thus ultimately resulted in the discovery of a new locality for *A. knysnae* within the area of interest for the study. Vast amounts of long-term data pertaining to seasonal activity, acoustic niche, micro-climatic influences on site selection, taxonomy and biology of the species have been collected during this study. This data volume is beyond the scope of this work and further analysis and reporting thereof will take place in future.

5.2.2 Historical and current site condition and changes

Information obtained from the surveys of the historical sites as reported in literature and IUCN Red List of Threatened Species Reports, highlighted the need for more continuous species censuses and habitat scrutiny to ensure species protection. Absence of *A. knysnae* at these localities resulted in searches to establish where viable populations may have been established in light of the degradation of the original recorded sites. The vast time lapse between information recording and studies since the 1940's has also caused data to degrade with records not being fully detailed during modern day data capturing. These factors make location of populations difficult with obvious negative conservation efforts.

Location of populations however revealed certain general aspects of areas where *A. knysnae* was recorded and where future populations may be located. Common attributes of these sites were *inter alia* the soft-leaved macrophytes used for oviposition, clarity and minimal depth of water bodies, temperature of the water and size of breeding areas within the water bodies. Many historical sites have been transformed either by agricultural activities or housing developments. Development can in no way merely be halted, yet the pressure from anthropogenic activities seems to be all encompassing with little regard towards the natural environment. Critical habitat areas for species such as *A. knysnae* often disappear as result of land use changes, mainly because of its proximity to human settlements, unspecific characteristics, and often being completely overlooked in drier seasons (Fig 5-1).

Authorities need to employ much stricter controls on wetland conservation during development application approvals. The seasonality of the wetlands used by *A. knysnae* for breeding purposes can easily cause breeding habitat areas to be overlooked during the dry season or drier periods. Historical data of localities of wetlands must be scrutinised to ensure that developments aren't merely approved because no wetland exist during the application period. Provision must be made to protect possible wetland habitat in the future, thereby ensuring breeding success for *A. knysnae* at viable habitat.

Figure 5-1: Location of the thriving population of *A. knysnae* at the Covie site. During the breeding season, the habitat is lush with vegetation and wetland characteristics are obvious (left); however, during dry periods, it almost looks degraded and incapable of housing viable populations (right).

A cryptic species such as *Afrixalus knysnae*, does however seem to have a relatively large Extent of Occurrence (EOO) given its low vagility. The question therefore arises whether the species recently populated new locations or were the populations not recorded at these sites as a result of the lack of research in the area. Populations at new sites are always encouraging for endangered species and the new recorded sites must be preserved to ensure population sustainability. Research need to focus on these sites, yet always ensure that historical sites are investigated to ensure correctness of distribution data, and ultimately inform decision makers when development or even conservation effort is undertaken.

5.3 Distribution Shift

Updated records supplied to the IUCN, through the AASG and SA-FRoG (expert-led IUCN assessments that have been held since 2009) regarding distribution localities of *A. knysnae* is the first step towards ensuring ongoing research into this species. The data indicate that the AOO is extremely small on a geographic scale while the initially reported EOO shows a decline. The revision of the locality records is extremely important for species of special concern. This is true even if the new recorded sites are only sites where previous investigations lacked to ascertain presences and not new in the literal form of the expression. Updating findings of this nature ensures the correct IUCN status of species at all times, informs conservation authorities of the task at hand and assists authorities in planning development.

Inconclusive information regarding taxonomy on *A. knysnae* needs to be updated. Geographic distributions are an important factor in this regard, together with habitat preferences, acoustic studies, morphology and DNA sequencing. The current study clearly indicates changes in the AOO and distribution further west from historical records. Comparisons with congeneric species further east, then becomes more important to ensure taxonomic certainty. Results from at least the geographic factors on distribution of this species is a starting point in questioning the place of *A. knysnae* within the *Afrixalus* genus.

The new locality recorded during this study also indicated amendment of the reported altitude of occurrence of *A. knysnae*. The locality at Farleigh Ranger Station is of significance in the light of climate change regimes, indicating that this species is capable of occupying habitats up to a 500 m difference in altitude. Population dynamics at these varied altitudinal gradients may be able to inform on climatological tolerance of *A. knysnae*. Similarly, this new site needs to be further investigated to ascertain if it is a manifested shift in population distribution, or incompleteness of earlier studies regarding the actual distribution of the species.

5.4 Temporal and Spectral Acoustic positioning

Various environmental and behavioural triggers cause calling bouts in frogs, amongst these are temperature, precipitation, inter-male competition and female responses to male proximity. At the same time, calls are at specific frequency bandwidths and amplitudes thereby distinguishing it from sympatric species or conspecific males (Minter, 1995). Use of sophisticated sound analysis software, requiring minimal training and no software coding knowledge, was able to clearly analyse fine scale spectral detail in the different call types of *A. knysnae*. Raven Pro® is a powerful tool in the hands of ecologists making use of bioacoustics in survey processes (Köhler *et al.*, 2017). Detailed measurements and calculations can be made, using this software application in the description of call structure. These analyses created identifiable characters of species-specific sounds and the behavioural use thereof. Comparison with other species within communities or the same genus are possible as result of these analysis.

Afrixalus knysnae calls were compared to basic call recordings of *A. s. spinifrons* and showed certain structural difference, indicating that analysis like this can assist in possible taxonomic interpretations. The scope of this study did not allow for complete taxonomic investigation or description but with very little data, clear results were obtained and future work in this regard may prove valuable for species conservation efforts.

5.5 Preliminary findings on breeding biology

Afrixalus knysnae has a very unique way of attempting to ensure that maximum progeny survive to at least tadpole stage by enclosing its eggs in a leaf sheath to develop hidden from predators. The diminutive size of the species, forces them to use small-leaved vegetation and as result only small numbers of eggs are deposited in this way during oviposition. Vegetation choice during breeding activity is however more intuitive than specific. Resources available at any given habitat is used to suit the species' immediate requirements without being biased towards any specific leaf type or discriminating between indigenous or exotic plants. Only size and pliability are a pre-requisite together with the proximity to the waterbody and shelter.

Enclosed egg-clutches further seem to assist tadpoles to develop in its early stages within the protection of the leaf-sheaths. Early development within these protecting folded leaves seem to be one strategy in which the species are ensuring breeding successes. The extremely small size of the tadpoles when it hatches may require them to initially remain in the leaf-sheath to fully develop in free-swimming larvae capable of surviving in the chosen habitats. Predator pressure on the egg-clutches and larvae of *A. knysnae* have not been studied herein, but future work on data collected during this investigation may assist in understanding the evolutionary significance of this breeding strategy.

5.6 Closing remarks

Amphibians worldwide are under tremendous extinction pressure with it being the vertebrate class of animals with the highest rate of species loss. South African species overall seem better off than the global trend, but still a number of species are classified as Critically Endangered, Endangered and Threatened. *Afrixalus knysnae* is the only endangered species occurring in the Southern Cape Region of South Africa and all efforts should be made to ensure the status does not change for the worse. Current research on the species, and even frogs in general within this region is lacking with almost no programmes to focus on these animals. Specialist investigators, literature, internal resources and a lack of basic knowledge are some of the factors hampering conservation plans.

Public awareness campaigns regarding the plight of frogs worldwide are however on the increase (www.savethefrogs.com). Initiatives such as Leap Day for Frogs and the Threatened Amphibian Programme of the EWT further enhance the awareness towards the plight of frogs within a South African context. These programmes give great impetus which drive communities to engage with authorities regarding conservation action plans. Education is still lacking amongst rural communities and children, although initiatives in this regard are usually eagerly embraced once introduced to these demographic sectors (Phaka *et al.*, 2017). Industry and business sectors often see conservation or environmental planning as a threat to economic and developmental advancements. Programmes engaging business leaders to understand how conservation and environmental planning is vital for economic sustainability needs to be more prevalent.

Although amphibians do not make up the bulk of the global biomass, mainly as a result of their size and distribution, their impact as ecoservice providers is immense (Gibbons *et al.*, 2006). Understanding reasons and biological processes leading to evolutionary divergence of the species across geographic boundaries can be studied through species such as *A. knysnae*. Protecting every species is important, but as *A. knysnae* is the only one of its genus in the area, its conservation in this context seems vital.

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