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Factors affecting the occurrence and abundance of the Natal Cascade frog, *Hadromophryne natalensis*, at Mariepskop, South Africa

by

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

IN

ENVIRONMENTAL ECOLOGY

FACULTY OF NATURAL AND AGRICULTURAL SCIENCE

UNIVERSITY OF PRETORIA

PRETORIA

SUPERVISOR: PROFESSOR WILLEM FERGUSON

JUNE 2016

Factors affecting the abundance and occurrence of the Natal cascade frog, *Hadromophryne natalensis* along streams at Mariepskop, Limpopo

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Submitted for the degree Master of Science (Environmental Ecology) in the Faculty of Natural and Agricultural Science

SUMMARY

Amphibians have long been recognized as excellent indicators of both biological and ecological health of ecosystems, as they occupy many habitats and environments and have a diverse range of impacts on their immediate environments. It is thus important to investigate the critical habitat requirements, as well as the preferred environmental variables that are associated with different amphibian species in order to provide insight into conservation and management plans, and to predict what effects climate change and disease might have on amphibian populations. The aim of this study was to investigate the abundance and occurrence of the Natal cascade frog, *Hadromophryne natalensis* on Mariepskop Mountain in Limpopo, South Africa. In this regard, we investigated what environmental factors had an effect on the occurrence and temporal variation of *H. natalensis* (i.e. pH, water temperature, stream substrate) and, determined when the optimal breeding period of *H. natalensis* is, and over which seasons they are most active. Taking into account the habitat requirements of these frogs, we also explored in which ways climate change may affect the abundance and distribution of these amphibians. Finally, we investigated if the amphibian disease, chytridiomycosis, is present in the population, and if so what effect this disease is having on the *H. natalensis* population. Our results revealed that forest cover had a significant positive effect on the number of cascade frog observations, rather than low temperatures. Cascade frog distributions seem to be governed by forest distribution and their association with low temperatures is a coincidental result of the distribution of southern African indigenous forest, mostly in the mountains. With reference to climate change and temperature, we predict that *H. natalensis* is unlikely to be directly affected by increased temperatures brought on by climate change as they are able to survive at lower altitudes and warmer temperatures. However, indirect effects of climate change on the distribution of their forest habitat may result in range reductions or range shifts for this species. With reference to disease,

chytridiomycosis incidence was significantly affected by tadpole size, with larger tadpoles more likely to be infected with the disease. We found that 28% of tadpoles sampled at Mariepskop showed visible signs of the disease, suggesting that chytridiomycosis extends much further northwards than reported to date. Overall, the population of *H. natalensis* at Mariepskop appears to be healthy with numerous individuals found when sampling. Interestingly, we found that *H. natalensis* breeds during the winter months, contrary to other studies conducted in South Africa on this species. Breeding appears to be successful as significant numbers of tadpoles, metamorphs and adults were observed during the study period. However, no data are available for previous seasons with regards to population size or disease prevalence, so it is not possible to determine the long term trends for these variables and their effect on this population, thus the importance for long term monitoring.

Keywords- *Hadromophryne natalensis,* Mariepskop, breeding period, climate change, chytridiomycosis

ACKNOWLEDGEMENTS

Thank you to my supervisor, Professor Willem Ferguson for all his support and guidance throughout my Masters. Thanks to Hein du Preez from supplying me with a water meter and to all his staff who took the time to teach me how to use it correctly in the field. Many thanks to Michael Cunningham for sharing his knowledge and expertise with regards to my study species. I would also like to thank Lydia Geldenhuys and Dave Rushworth for the use of their houses during fieldwork. Many thanks to Andrew Davies for the many long hours he spent with me on my statistical analysis. A special thank you to Liezl Taylor, Kimberly Perry and Nadine Coetzer for accompanying, and helping me with my fieldwork on Mariepskop. Finally thank you to all my family, Mac du Toit, Shirley du Toit, Paige du Toit, and to my boyfriend, Mike Mole for all their help with my fieldwork and their continued support and understanding throughout the completion of my Masters.

DISCLAIMER

The work contained in this thesis is the original work of Katrina Heather du Toit (except where stated), done under the supervision of Professor Willem Ferguson. No part of this work has been previously submitted for a degree or for examination.

Katrina du Toit

June 2016

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CHAPTER 1

1 INTRODUCTION

1.1 General

Amphibians represent an extremely diverse taxonomic group (Halliday 2008), with the total number of amphibian species outnumbering that of mammals (Glaw & Kohler 1998). Globally, there are 7229 known amphibian species and new species are still being discovered each year (Frost 2014). However, amphibians are also one of the most threatened taxonomic groups (Stuart *et al.* 2004, Halliday 2008), with approximately 1856 species (32.5%) now listed as globally threatened (Stuart *et al.* 2004), making them 'proportionally the most threatened group of vertebrates globally' (Karssing 2010).

Amphibians have multifaceted lifecycles with both egg and larval stages being confined to water or moist habitats, whilst adults occur in both terrestrial and aquatic environments (Wyman 1990, Blaustein & Wake 1995, Whiles *et al.* 2006). Adult amphibians possess a thin, sensitive skin responsible for respiration and which is extremely susceptible to moisture loss (Wyman 1990, Beebee & Griffiths 2005, Halliday 2008). Thus, both life stages of the amphibian lifecycle require water, making them extremely dependent on mesic environments. This reliance on moist or wet environments for survival makes amphibians particularly susceptible to threats such as habitat loss and fragmentation, introduced species, climate change and disease (Wyman 1990, Collins & Storfer 2003, Beebee & Griffiths 2005, Halliday 2008). However, the most pressing of these threats for amphibians are disease (chytridiomycosis) and climate change.

Chytridiomycosis, a fungal disease, was first described by Berger *et al.* (1998) in frogs from Australia and Central America. They suggested that chytridiomycosis was

responsible for mass frog mortalities in these different regions, which caused global concern amongst scientists and conservationists (Beebee & Griffiths 2005). Since then, this disease has gone global with approximately 500 amphibian species infected with the disease (Tarrant *et al.* 2013). However, despite its threat to amphibian species, chytridiomycosis has been relatively understudied in South Africa with its effect on both native, and in particular Red List species being fairly unknown (Tarrant *et al.* 2013).

Our climate is changing as a result of global warming, with changes in precipitation, temperature and UV radiation having the most pronounced, and often negative, effects on amphibians (Corn 2005, Blaustein *et al*. 2010). Amphibian skin is very porous allowing them to exchange water, ions and respiratory gases (Wyman 1990, Pough 2007), resulting in many amphibian species being highly sensitive to changes in environmental temperatures (Reading 1998). Amphibians are also ectotherms, relying on external sources of heat (i.e. indirect sunlight) to maintain their activity (Wyman 1990, Halliday 2008), growth and development, especially in their larval stages (Alvarez & Nicieza 2002). This, in turn, means that amphibians are exposed to ultraviolet radiation on almost a daily basis, which, in turn, may be one of the many causes contributing to the decline in amphibian numbers (Beebee & Griffiths 2005, Halliday 2008). Scientists have suggested that UV-B radiation can cause sub-lethal effects or interact with other factors such as contaminants, which cause the animal to become more susceptible to disease or tumors (Wyman 1990, Blaustein & Kiesecker 2002, Skerratt *et al.* 2007).

Global temperatures have increased by an average of 0.85°C since the 1880's due to increased greenhouse gas emissions (IPCC climate report 2013). These temperature changes might result in range shifts, as well as, change the abundance (Pounds *et al.* 1999), and behaviour of many amphibian species (Blaustein *et al*. 2010). In nature, air and water temperatures act as vital cues for certain behaviour (i.e. calling, feeding, reproduction) that

amphibians exhibit, however, if these temperatures change as a result of climate change this could result in a shift in the onset of breeding periods and affect the calling behaviour of males (Reading 1998, Carey and Alexander 2003). Changes in annual rainfall or in the timing of rainfall events, could affect the number of eggs deposited (Cadwell 1987, Carey & Alexander 2003). In addition, Pounds *et al.* (2006) suggests that global warming may be promoting favourable temperatures for *Batrachochytrium dendrobatidis* to thrive. Thus, long term studies are essential to see how climate change is affecting temperatures, particularly at high altitude sites, and what these temperature changes mean for amphibian survival.

1.2 The importance of conserving amphibians

The larval stages of most amphibians (i.e. tadpoles) are herbivorous and feed on algae and plants (Blaustein *et al.* 1994, Halliday 2008). Tadpoles play a very important role in ensuring the health of aquatic environments with regard to nutrient cycling, leaf litter decomposition and the prevention of sedimentation (Flecker *et al.* 1999, Whiles *et al.* 2006). Amphibian eggs and larvae are, in turn, prey for a diverse range of predators, particularly other frogs, fish, fishing birds, odonates and aquatic beetles (Blaustein *et al.* 1994, Thumm & Mahony 1999, Karssing 2010). Terrestrial adults are mostly insectivores/carnivores, feeding on insects and worms (Whiles *et al.* 2006, Halliday 2008), whilst conversely, they also represent a very important prey to many semi-aquatic and terrestrial predators such as snakes (Whiles *et al.* 2006). From this, we can infer that amphibian declines could negatively affect ecological processes in both aquatic and terrestrial environments (Whiles *et al.* 2006, Halliday 2008).

As amphibians are sensitive to changes in the environment (Beebee & Griffiths 2005) they have long been recognized as excellent indicators of both biological and ecological health of ecosystems (Blaustein & Wake 1995, Collins & Storfer 2003, Beebee & Griffiths 2005, Karssing *et al.* 2012), as they occur in both aquatic and terrestrial habitats (Wyman

1990, Whiles *et al.* 2006). Amphibian declines in a particular area or ecosystem could therefore be a sign that other species may also be facing a similar fate (Collins & Storfer 2003, Blaustein *et al*. 1994).

1.3 Natal cascade frog *Hadromophryne natalensis*

The Natal cascade frog, *Hadromophryne natalensis*, occurs along the escarpment of South Africa, Lesotho and Swaziland (Boycott 2004), with little known about their biology and ecology due to their elusive nature (Grobler *et al.* 2003). This species inhabits forest steams along the eastern escarpment of southern Africa (Wager 1965, Carruthers 2001, Karssing *et al.* 2012), at altitudes between 580 and 2675m (Boycott 2004, Karssing *et al.* 2012). It occurs in two main vegetation types, namely, forest and grassland, and can be found in habitats such as Afromontane Forest, Afro Mountain Grassland as well Short Mistbelt Grassland, where rainfall is between 800 and 2700 mm a year (Boycott 2004).

The tadpoles feed on algae in cool, clear, fast flowing streams and have a flattened body and broad mouth, equipped with sucking mouthparts in order to adhere to the submersed surfaces of rocks in these streams (Wager 1965, Carruthers 2001, Karssing *et al.* 2012). The tadpoles move and feed on algae covered rocks by means of a ‘walking’ mechanism, by thrusting forward the upper lip and then bringing the lower lip towards the upper lip (Wager 1965). Tadpoles are typically found in sheltered, tree covered areas of streams. However, at higher altitudes they may be found in sunlit patches of streams (Wager 1965). Tadpoles are light to dark brown and often have darker mottled patches (Fig. 1.1c). The tadpoles of the Natal cascade frog have a rare life cycle, growing to between eight and 10 cm long (Fig. 1.1c), and taking up to two years to fully metamorphose (Fig. 1.1b). When the front legs appear in *H. natalensis* tadpoles, the sucking mouthparts disappear and tadpoles

typically become air breathing, often hiding in vegetation in streams or under rocks close to the streams, until the tail is lost completely (Wager 1965).

Adult cascade frogs are nocturnal (Fig. 1.1a), often found in fast-flowing streams, waterfalls and in rock crevasses and occasionally on exposed rocks besides streams (Boycott 2004, Wager 1965). Although the timing of mating and egg deposition sites are unknown, observations of tadpole size distribution's suggest that *H. natalensis* breeds in spring (Rivers-Moore & Karssing 2014), with male calling beginning with the onset of the breeding period (Boycott 2004). However, other observers have suggested that these frogs breed in late summer (March to May), when the water flow in the streams is low. Cascade frog tadpoles are abundant and easily detected, and their long larval period confines them to perennial streams (Wager 1965, Boycott 2004). For these reasons, *H. natalensis* is an excellent candidate for biological monitoring of montane stream health. As this species is primarily limited to forest environments, *H. natalensis* may be particularly at risk to climate change as well as the emerging infectious disease, chytridiomycosis. Thus, it is valuable to have good baseline data relating to amphibian abundance, occurrence and diversity which can then be correlated to ecosystem health and structure.

Figure 1.1. a.) Adult *Hadromophyrne natalensis*, b.) *H. natalensis* metamorph, c.) Larval *H. natalensis.*

1.4 Research Aims

The aim of this study was to investigate the abundance and occurrence of the Natal cascade frog, *Hadromophryne natalensis* on Mariepskop Mountain in Limpopo, South Africa. This dissertation has two main data chapters (Chapter 2 & 3). In Chapter 2, I investigate what environmental factors affect the occurrence and temporal variation in the activity of the cascade frog (i.e. pH, water temperature, stream substrate) as well as, determine when the breeding period of the species is at Mariepskop, and over which seasons they are most abundant. Furthermore, taking into account the habitat requirements of these frogs, we aim to explore in which ways climate change may affect the abundance and distribution of this species. In Chapter 3, I investigate the prevalence of chytridiomycosis in *H. natalensis*

tadpoles at Mariepskop and its relation to altitude, tadpole age, size and the interaction between these factors.

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CHAPTER 2

Climate change and the environmental requirements of the Natal Cascade frog, *Hadromophryne natalensis*

ABSTRACT

The aim of this study was to investigate the habitat preferences of *Hadromophryne natalensis* at Mariepskop Mountain in Limpopo, and to explore in which ways climate change may affect the abundance and distribution of these amphibians. Secondly, our aim was to investigate whether physical water properties may have an influence on the distribution of *H. natalensis* larvae. Finally, we investigated the breeding period of *H. natalensis* frogs as well as calling activity of this species using automated field-placed acoustic equipment. *Hadromophryne natalensis* are common and breed successfully at Mariepskop. They prefer substrates which offer cover (i.e. loose rocks, sticks), whilst avoid stream areas with high silt/sedimentation load. Considering tadpole abundance and adult calling, cascade frogs breed during the winter coinciding with low rainfall, low stream flow and lower mean temperatures. At a regional scale, this cold-adapted species is mostly found at higher altitudes. However, regional abundance correlates with forest cover and not with cold temperature, suggesting that a cold environment is a consequence of the montane distribution of forest. They are also found at low elevations with higher temperature. Consequently, we predict that *H. natalensis* is unlikely to be directly affected by temperature increases due to climate change; however, indirect effects of climate change (e.g. reduced habitats) could affect their survival.

Key words – Climate change, Critical habitat requirements, *Hadromophryne natalensis*, Mariepskop

1 INTRODUCTION

1.1 General

Globally, increasing temperatures as a result of climate change are likely to have a negative effect on biodiversity, with negative impacts for ecosystems, habitats and species (Corn 2005, Blaustein *et al*. 2010, Minter 2011). There is particular concern for the effect climate change may have on amphibians (Carey & Alexander 2003, Blaustein *et al*. 2010, Minter 2011). Increases in temperature, variability in rainfall, and the frequency of extreme weather events are predicted to have many consequences on amphibians (Blaustein *et al*. 2010). These include reductions in habitat, changes in environmental cues, range shifts (Blaustein *et al*. 2010), changes in behaviour such as calling and feeding (Reading 1998, Carey & Alexander 2003; Blaustein *et al*. 2010), changes in reproduction, such as the onset of breeding periods (Reading 1998, Carey & Alexander 2003) and reproductive output (Cadwell 1987, Carey & Alexander 2003). To conserve amphibian species it is important to investigate the potential effects of climate change on populations, species and ecosystems as a whole.

The Natal cascade frog, *Hadromophryne natalensis*, is a regionally endemic southern African rain forest and montane grassland frog (Boycott 2004, Wager 1965). *Hadromophryne natalensis* can be considered a habitat specialist with a particular affinity for fast flowing, high altitude mountain streams in densely forested areas (Wager 1965, Boycott 2004), and is known to inhabit two main vegetation types, namely, forest and grassland, and can be found in habitats such as Afromontane Forest, Afro Mountain Grassland as well Short Mistbelt Grassland, (Boycott 2004). Minter (2011), suggested that montane frog species may be particularly affected by climate change due to the continuous warming and drying periods, combined with increasing temperatures that may drive montane species further up altitudinal gradients, reducing their habitat ranges. However, the lack of data with regards to cascade frogs precludes predicting its response to climate change. This study addresses the

deficiencies in distributional data, as well as the critical habitat characteristics and environmental requirements of this species, with the ultimate objective of assessing what effect climate change is likely to have on this species.

Existing knowledge about *H. natalensis* derives largely from studies within the central Drakensberg area, namely, Krantzkloof Nature Reserve and Monks Cowl in the uKhahlamba, South Africa (Karssing *et al.* 2012, Rivers-Moore & Karssing 2014). Adult cascade frogs are nocturnal, often found in cascades, waterfalls and in rock crevasses and occasionally on exposed rocks besides fast flowing streams along the eastern escarpment of South Africa, Lesotho and Swaziland (Wager 1965, Carruthers 2001, Karssing *et al.* 2012), at altitudes between 580 and 2675m (Boycott 2004, Karssing *et al* 2012). *Hadromophryne natalensis* avoid substrates without adequate cover, (e.g. with sand, silt and bedrock) and have a strong affinity for fast-flowing riffles (Karssing *et al.* 2012). Indirect evidence suggests that *H. natalensis* frogs breed in spring (Rivers-Moore & Karssing 2014), but other authors have suggested that breeding occurs in late summer to autumn (March-May) when the water flow in the streams is low (Boycott 2004). Tadpoles feed on algae in cool, clear, fast flowing forest streams (Wager 1965, Carruthers 2001, Karssing *et al.* 2012), and have a rare life cycle, growing to between eight and 10 cm long, and taking up to two years to fully metamorphose, thus requiring perennial streams (Wager 1965, Karssing *et al.* 2012). There is little information about this species outside of the central Drakensberg.

This study was performed at Mariepskop, an area with a complex topography (Pretorius & Rautenbach 2012), several vegetation zones and high biodiversity. Mariepskop has some similarities with respect to forests and grasslands compared to sites of previous studies at Kranztkloof, Monks Cowl and Injesuthi (Adie & Lawes 2009, Rivers-Moore & Karssing 2014, Table 2.1), but vastly different from Krantzkloof, which has a warmer climate (Table 2.1, Rivers-Moore & Karssing 2014).

In this study we aimed to: 1) quantify the ecological factors (benthic structure; vegetation; water properties, temperature, flow rate) determining the occurrence of *H. natalensis* in and along streams at Mariepskop; 2) determine the breeding period and the timing of metamorphosis in *H. natalensis*; 3) and, taking into account the previous studies and habitat requirements of these frogs, to explore in which ways climate change may affect the abundance and distribution of this species.

Table 2.1. Table indicating existing studies on *Hadromophryne natalensis* in South Africa focusing on the altitude and water temperatures at which tadpoles were encountered. Mean annual maximum and minimum air temperatures were obtained from the South African Weather Service database for stations in close proximity to those of studies listed. Annual water temperatures were based on mean monthly temperatures taken during the sampling season of Karssing *et al.* 2012 and Rivers-Moore & Karssing 2014. Air temperatures obtained from the data set of the South African Weather Service were based on daily average temperatures (°C).

Location	Site	Altitude (m.a.s.l)	Annual water temperature (°C) range (approximately)	Mean annual maximum air temperature (°C) from 2004 to 2014	Mean annual minimum air temperature (°C) from 2004 to 2014	Author/Source
Kwa-Zulu Natal	Monks Cowl	1292-1727	6.5 - 17	21.1*	8.7*	Karssing *et al.* 2012
Kwa-Zulu Natal	Injesthi	1615-1653	7- 16.5	21.1*	8.7*	Karssing *et al.* 2012
Kwa-Zulu Natal	Monks Cowl	1280-1440	7.5- 16	21.1*	8.7*	Rivers-Moore & Karssing 2014
Kwa-Zulu Natal	Injesthi	1594-1727	7.5 -15	21.1*	8.7*	Rivers-Moore & Karssing 2014
Kwa-Zulu Natal	Kranskloof	426	13 -20	25.6**	16.6**	Rivers-Moore & Karssing 2014

* Based on meteorological data from the Giant Castle weather station (station number: 0240808A2)

** Based on meteorological data from the Durban South weather station (station number: 0268016AX)

2 METHODS

2.1 Study Site

Mariepskop Mountain, part of the northern Drakensberg escarpment, South Africa, has an altitudinal gradient from 500m to 1946m. The natural vegetation on Mariepskop is diverse, and varies with altitude and aspect, ranging from various forms of savanna woodland at the lower altitudes to evergreen Afromontane forests at mid-altitudes and heath and high mountain grasslands on top of the mountain (van der Schijff & Schoonraad 1971).

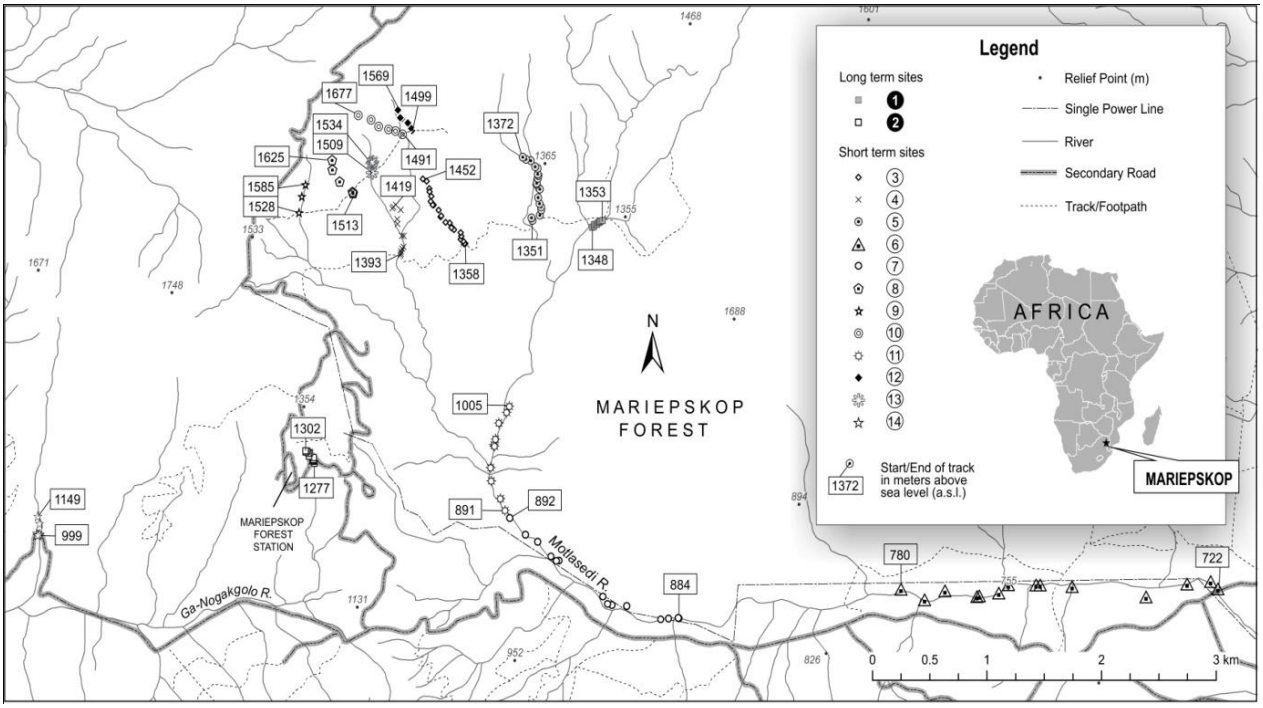


Figure 2.1. Sites on Mariepskop Mountain where cascade frog tadpoles were sampled. Predatory fish were encountered only at site six.

2.2 Study design and protocol

Abundance data for tadpoles and adult frogs were not pooled as these amphibian life stages inhabit different ecological niches (Dupuis & Friele 2006, Hunter 1998). Two approaches were used when selecting streams for sampling. Two long term sites were chosen, which were repeatedly sampled (i.e. two samples per long term site per trip), as well as 12 additional

streams each only sampled once (Fig. 2.1). All streams were perennial and tadpole sampling took place during the day while adults were sampled at night, starting approximately one hour after sunset. Due to restricted accessibility, night sampling of post-metamorphic individuals only took place at the two long term study sites. Tadpoles were classified according to their different lifestages, which could be easily identified in the field (Adapted from Gosner 1960; Table 2.2).

2.2.1 Long term sites

Two streams were selected as representatives of the range of perennial stream conditions at Mariepskop. The Klaserie River site (Long term site 1; 1352 m.a.s.l.) on the southern side of the mountain (Fig. 2.1) includes the largest watercourse on Mariepskop (approximately 0.5-1.0 m deep and 5-10 m wide), and is a fast flowing, clear water river with numerous small cascades and some larger drops below the study transect. It is minimally disturbed, with low silt load, and runs over a substrate of sand, bedrock and large boulders. The stream is surrounded by a broad gallery of Northern Mistbelt Forest (Mucina & Rutherford 2011) with *Pinus* and *Eucalyptus* plantations beyond the Forest. Decaying tree-trunks are sometimes found in the stream with some shrubby vegetation along the banks. The stream banks are covered by a mixture of trees (approx. 8-10 m in height) and medium shrubs (approx. 4m in height) offering cover, with gaps in the overhead canopy exposing parts of the stream to periodic sunlight.

The Ga-nogakgolo River site (Long term site 2; 1292 m.a.s.l.) was situated in the headwaters of the Ga-nogakgolo River on the western side of the mountain (Fig. 2.1), flowing into the Blyde River to the north, representing a different water catchment. This stream is fast flowing, dropping steeply over several cascades, with larger waterfalls below. In contrast with the Klaserie site, however, this is a small forest stream (approximately 1-3 m wide and 0.05-0.20 m deep) on a substrate of largely silt, sand and small boulders. It is somewhat turbid,

with a high silt load due to disturbances associated with the upstream forestry activities. Much of the stream is covered by overhanging vines and shrubs, with some shrubs and fallen wood in the stream itself. The stream flows under a closed canopy of the Northern Mistbelt Forest, which in this site is confined to a narrow strip within the surrounding *Pinus* and *Eucalyptus* plantations.

Transects of 100-120 m in length at each of these two sites were sampled every 6 to 8 weeks during the period March 2013-Febuary 2014. Each transect was divided into 10 m segments.

The Klaserie River was divided into ten sections, each 10 m in length (totalling 100 m) whilst the Ga-nogakgolo River, was divided into twelve sections, each 10 m in length (totalling 120 m). These between-site differences were due to accessibility restrictions. Both sites were then divided into an upper and a lower half, each with either five or six 10 m segments. Sampling always took place moving in an upstream direction to avoid sampling disturbed substrate.

During each field visit, two 10 m segments (one in the upper half and one in the lower half i.e. 20 m per stream) were randomly selected and intensively sampled. Relative abundance of tadpoles was expressed in terms of number of tadpoles encountered per segment. Tadpoles were sampled using dip-netting. The observer (KdT) walked methodically upstream (in a pre-determined transect section), rolling rocks with a net placed on the substrate slightly downstream to catch any dislodged tadpoles. A field assistant took notes. Tadpole were measured, assigned a life history stage (Table 2.2), and returned to the stream. All sample locations were recorded using a GPS.

2.2.2 Short term sites

During the winter months (May-July) of 2013, rivers on Mariepskop were examined to see which streams had dried up and which were perennial. All perennial streams were geo-

referenced and systematically sampled. Observers walked upstream, actively searching for 50 minutes, taking GPS waypoints of any evidence of *H. natalensis*, such as the distinctive tadpole feeding marks and any frogs or tadpoles observed. After 50 minutes, a 10 m stream segment was sampled for tadpoles using the dip-netting technique, following the protocol described above for the long term sites. Observers repeated this protocol (observations along the stream for 50 minutes; 10 m tadpole sampling) until unable to ascend further (e.g. steep waterfall or impenetrable vegetation).

2.3 Night sampling

Sampling of adult frogs was performed at the long term sites at night as this is when adult cascade frogs are active (Wager 1965). Sampling started one hour after sunset and both long term study sites were sampled during a night. Due to travel time between study sites only the lower half or the upper half (i.e. around 50m) was sampled on any single night. Lower and upper transects were interchanged on successive nights. Night sampling took place using active searching methodology combined with spotlighting. Once frogs were located, they were caught, counted, measured and their developmental stage and sex (if sex could be determined, see Table 2.2) was recorded along with their geographical position after which they were then returned to the stream.

2.4 Environmental data

For both long and short term sites qualitative and quantitative environmental data were recorded at stream level, the sampling segment level, or the sampling session level which is two sampling events per long term sites (1 & 2) and three to five sampling events at the short term sites (3-14) depending on the terrain.

2.4.1 Aquatic characteristics

Temperature loggers (Thermochron iButtons -DS1922L, Maxim Inc) were placed at both long term study sites for continuous monitoring of water temperature, air temperature and humidity. These loggers took readings every 30 minutes during the study period from April 2013-February 2014, and were collected and replaced each trip; however, some data were lost due to faulty mechanisms or data loggers being washed away after heavy rain.

A Yellow Spring Instrumentation (YSI) 6600 Multi Parameter System (MPS) probe was used to determine physical and chemical characteristics of the water. Temperature, pH, dissolved oxygen and conductivity were measured at the long term sites and at nine short term study sites in the months of August 2013, November 2013 and January 2014 to measure water characteristics just after the winter season (August), early summer (before the rainy season, November), and late summer (after/during the rainy season, January). Measurements were done in accordance with the Rand Water Method Numbers 1.1.2.14.1; 1.1.2.16.1 and 1.1.2.15.1 (Rand Water 2006a, b, c).

2.4.2 Benthic structure and surrounding streamside vegetation

Before each sampling event, twelve qualitative environmental variables were measured in the 10 m segment chosen to be sampled to determine which variables have an effect on the number of tadpoles. These variables were divided into four main categories: substrate (loose rock, bedrock, sand, silt); rock size (small rocks, hand-sized rocks, large rocks-too large to lift); vegetation (undercut banks, vegetation on the banks, dead wood on banks, canopy gap) and fish (present/absent).

All streams were covered by forest canopy, with some open sunlit stretches. In all streams rock, sand and vegetation or a mixture of these substrates covered the bottom. Due to geographic barriers such as waterfalls, most streams (except short term site 6) harboured no predatory fish, although Odonata larvae, water beetles and spiders where observed.

2.4.3 Water flow and water speed

Water flow rate were estimated during the months of August 2013, November 2013 and January 2014 at sites which had water pipelines (cylinders) which directed water beneath a road (i.e. Site 1, 3, 4 & 5). Flow rate was measured in cubic meters per second, whilst water speed was measured in meters per second.

These pipes were used to calculate water speed by using the following equation:

The central angle that subtends the width of the water in the pipe (θ).

$$\theta = arccos\left(1\text{-}\frac{h}{r}\right) \times 2 \quad \text{.....(1)}$$

$h =$ Height of water in cylinder

$r =$ Radius of cylinder

$\theta =$ central angle (radians)

Surface of segment (A) subtended by the above angle:

$$A = \frac{r^2}{2}\left(\frac{\theta \times \pi}{180}\right) - \sin(\theta) \quad \text{.....(2)}$$

$\theta =$ central angle (radians)

$\pi = 3.14$

$r =$ Radius of cylinder

$A =$ Cross sectional area of water in cylinder (m^2, if r and h are in meters)

Water flow (m^3/s) = water speed (m/s) x A (m^2).....(3)

Table 2.2. Life history stages assigned to captured *Hadromophryne natalensis* (Adapted from Gosner 1960). Stages in brackets correspond to Gosner lifestage classification.

	Lifestage	**Description**
1	Eggs (Stage 1-16)	
2	Hatchling (Stage 17-24)	Newly hatched tadpoles, still possessing external gills
3	Tadpole (Stage 25)	Distinct body and tail associated with a tadpole
4	Tadpole with limb buds or limbs present (Stage 26-36)	Distinct tadpole morphology as well as the presence of limb buds and/or small limbs which are beginning to form
5	Tadpole with distinct hind limbs (Stage 37-41)	Development of distinct toes on hind limbs (forelimbs not yet developed)
6	Young adult frog, still inhabiting water (Stage 42-43)	Fully developed fore and hind limbs, tail still present, tadpole still inhabiting water
7	Young metamorph, air breathing (Stage 44-45)	Air-breathing metamorph, fully developed fore and hind limbs, tail resorbing, fully formed mouth
8	Juvenile frog (Stage 46)	Tail stump resorbed (+-20-30mm)
9	Sub adult	Near adult size (+-40mm), sex indeterminate
10M	Adult male	Spines on thumb and chest, as well as black spots present (+-40-50mm)
10F	Adult female	Spines and spots absent (+-40-50mm)

2.5 Acoustic recorder: Calling activity

In order to investigate at which times and months *H. natalensis* males were the most active in calling, a SongMeter (SM2$^+$, Wildlife Acoustics, Inc., Concord, MA, USA) was placed at the Ga-nogakgolo River (site 2) from May 2013-February 2014. The recorder was placed in a metal box attached to a tree, 2.5m above ground and 5m from the stream. As *H. natalensis* is nocturnal, the device was set to record for five minutes every hour from 18:00-00:00. Any cascade frog calling during these five minute periods was documented as 'present', else wise as 'absent'. However, some data were lost due to leaking/flat batteries. Sound recordings were analysed using the Raven Lite 1.0 sound analysis software (Cornel Laboratory of Ornithology, Ithaca, NY, USA). The dates and times of all calling were recorded.

2.6 Statistical Analyses

All analyses were performed in R statistical package, version 3.01 (R Development Core Team 2008). Environmental variables were analysed using a generalized linear model to determine whether individual environmental variables had an influence on tadpole numbers. Linear regressions were used to investigate the effect of altitude on water chemistry, physical water properties, tadpole size and tadpole abundance. Additionally, a Fishers Exact Test was used to test whether there was a significant difference in tadpole numbers between seasons and at different altitudes. A one-way analysis of variance (ANOVA) was performed on log transformed water flow (m/s^3) and water speed (m/s) data to determine if water flow and water speed differed significantly between months. With regards to cascade frog regional distribution, a linear regression was performed to see if forest cover had a significant effect on *H. natalensis* density/observations. A second linear regression was performed to determine if air temperature had a significant effect on forest cover as well as cascade frog observations.

2.7 Air Temperature and Altitude

Air temperature data, derived from WorldClim data (Hijmans *et al.* 2005), were downscaled to a quarter-degree (QDS) resolution. Air temperature data were calculated using daily mean temperatures taken over 24 hours for each QDS where Natal cascade frogs were found. These data were then used to find the corresponding air temperatures for the QDS's associated with Natal cascade frog geographical distribution. Mean altitude above sea level for each QDS was calculated using ASTER (Advanced Spaceborne Thermal Emission and Reflection) Global Digital Elevation Map with a spatial resolution of 30 m (NASA and Japan ASTER Program 2011). Air temperature data were then compared to the mean altitude for the points within each QDS.

2.8 Distribution

Distributional data (frog count data) of *H. natalensis* was obtained from the South African Frog Atlas Project (Animal Demography Unit, university of Cape Town). These QDS-based data were divided into five categories reflecting the number of observed individuals within each QDS (see Fig 2.6). It is important to note that there may be other factors affecting the frog count data and intensity of sampling per QDS, which may affect the number of records we obtained from the South African Frog Atlas Project. Forest cover was obtained from VegMap 2012 (beta version; South African National Biodiversity Institute 2016). The forest biome was selected, which consisted of 11 different forest types and from which we calculated the total forest cover per QDS (km^2). This number was then divided by the total area of the QDS (km^2), and converted into percentage forest cover per QDS. A linear regression was then performed to see if forest cover had a significant effect on *H. natalensis* density/observations.

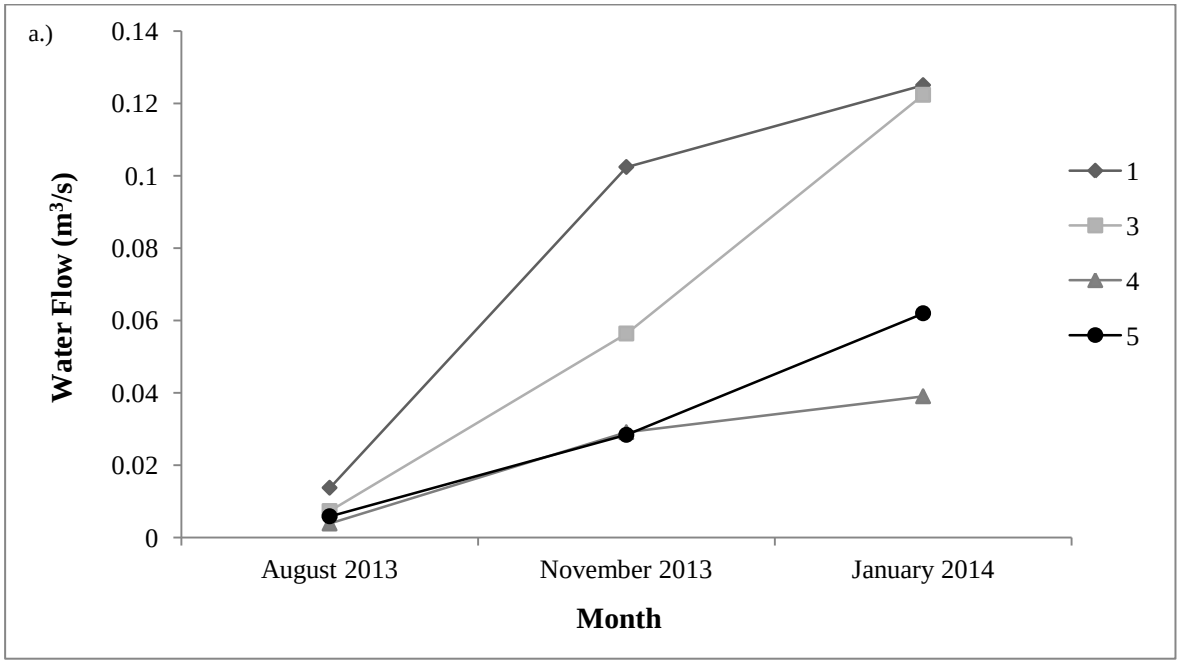
3 RESULTS

3.1 Environmental variables

3.1.1 Physical water characteristics

Water flow and water speed

For all four study sites, measured water flow (m/s^3) increased from low values during the dry winter month of August to the start of the rains in November to the late rainy season in January (Fig. 2.2a). At all study sites water speed increased from August to November (Fig. 2.2b). After November, site 3 and 5 showed an increase in water speed (m/s) from November to January whilst site 1 and 4 showed a decrease in water speed between these months (Fig. 2.2b). There was a significant effect of month on water flow (m/s^3) recorded between sites [ANOVA $F(2, 9) = 6.41$, $p<0.05$].



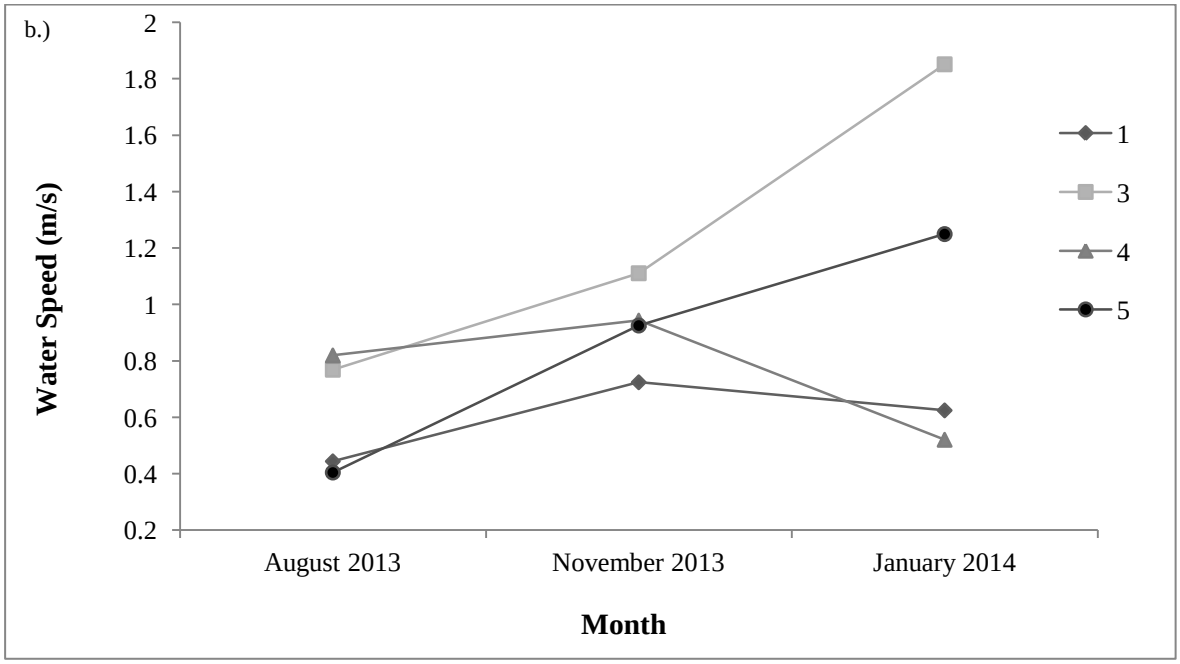


Figure 2.2. a.) Water flow (m^3/s) for four of the 13 sample sites where stream flow estimates were possible. b.) Water speed (m/s) for four of the 13 sample sites where water speed estimates were possible. Measurements were taken in August, November 2013, and January 2014. Sites are numbered according to Figure 2.1. There was a significant effect of month on water flow (m/s^3) recorded between sites [ANOVA $F(2, 9) = 6.41$, $p<0.05$].

Temperature

Water temperature measured during August, November and January differed some 5 ºC between the single low-lying site (site 6: 722 m.a.s.l) and the higher lying sites (1292-1528 m.a.s.l, Fig. 2.3a). Temperatures ranged from 9.5 ºC (site 8-1513 m.a.s.l) to 18.8 ºC (site 6-722 m.a.s.l) during August and from 15.9 ºC (site 3-1358 m.a.s.l) to 21.4 ºC (site 6-722 m.a.s.l) in January (Fig. 2.3a). *H. natalensis* tadpoles were only found at sites with water temperatures below 18ºC. Altitude had a significant ($p<0.05$) negative correlation with water temperature in the months of August and November (Fig. 2.3a). Rainfall was high from October 2013-February 2014 (Fig. 2.5). Short term floods (less than a day in duration, not presented here) were evident immediately after heavy rainfall, therefore maximum flow rates are expected to be substantially higher than the mean values calculated (Fig. 2.2).

Dissolved Oxygen

Dissolved oxygen (DO) in the single low-lying site (site 6: 722 m.a.s.l) was some 7% higher than the equivalent figures for high-lying sites (1292-1528 m.a.s.l, Fig. 2.3c). DO fluctuated between 54% (site 12-1499 m.a.s.l) to 78.2% (site 6-722 m.a.s.l) during August (the cold, low-flow season), while in January (the hot, high-flow season) the dissolved oxygen concentration ranged from 67.4% (site 5-1357 m.a.s.l) to 78.3% (site 6-722 m.a.s.l). Altitude had a significant ($p<0.05$) negative correlation with dissolved oxygen in the month of January (Fig. 2.3c) when water flow rate was the highest.

3.1.2 Water chemistry

Conductivity

High-lying sites (1292-1528 m.a.s.l.) had water conductivity values some 20 units lower than the single low-lying site (site 6: 722 m.a.s.l., Fig. 2.3b). Overall, the highest conductivity

values were encountered during November (early summer), while the lowest readings were recorded in early January (mid-summer) (Fig. 2.3b). Altitude had a significant ($p<0.05$) negative correlation with conductivity in the months of August and November (Fig. 2.3b).

pH

pH ranged from 5.22 (site 12: 1499 m.a.s.l.) to 7.71 (site 6: 722 m.a.s.l.) during August, whilst in January the pH ranged from 6.19 (site 13: 1508 m.a.s.l.) to 7.6 (site 6: 722 m.a.s.l.; Fig. 2.3d). Altitude had a significant ($p<0.05$) negative correlation with pH in the months of August and November (Fig. 2.3d).

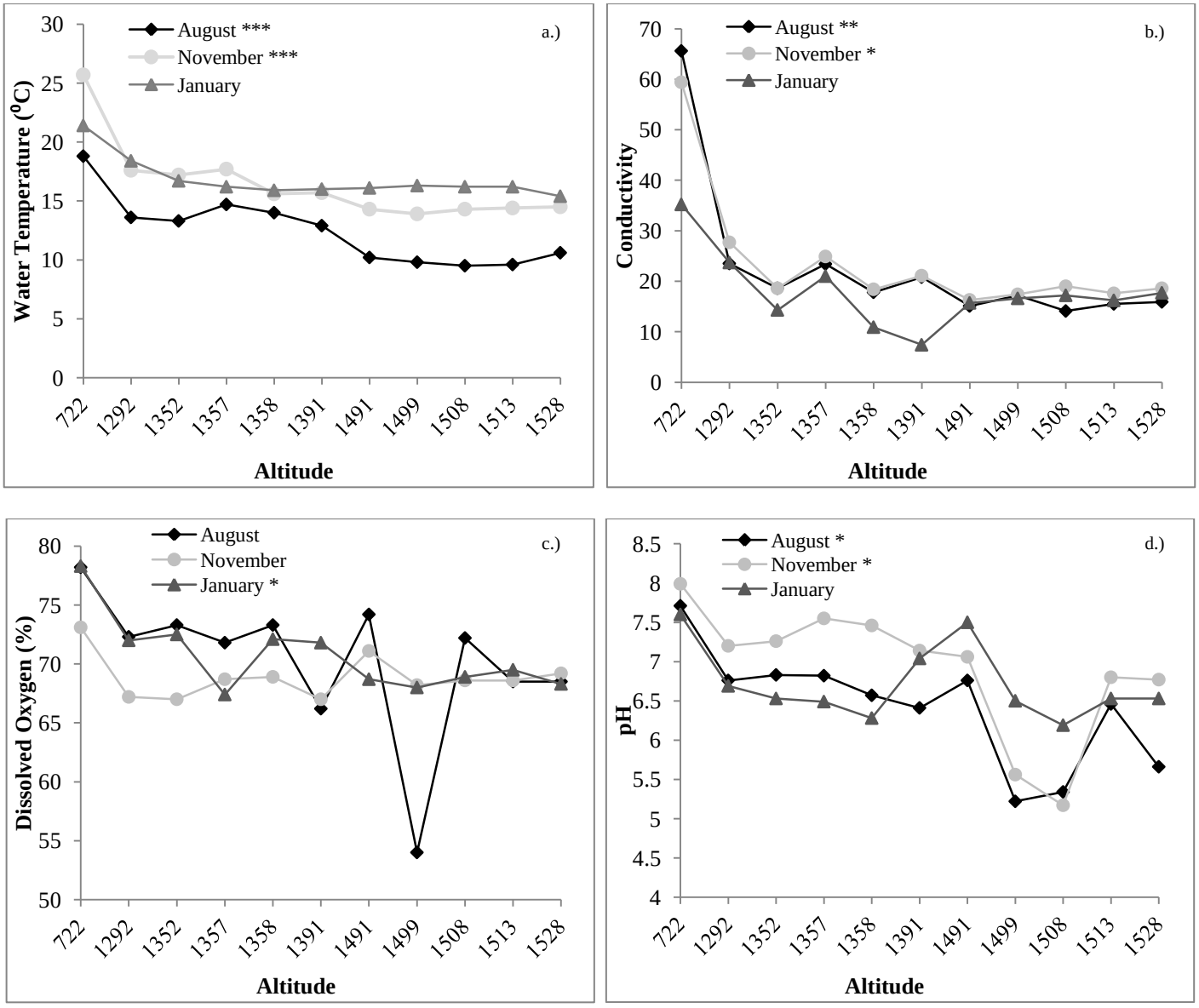


Figure 2.3. Changes in stream water characteristics over an altitudinal gradient at Mariepskop for three sampling periods, each indicated by a different line: a.) Temperature (^{0}C), b.) Conductivity (C), c.) Dissolved oxygen (%) and d.) pH. Asterisks indicate statistically significant altitudinal trends (linear regression: * p=0.05; ** p=0.01; *** p=0.005). Values on X-axes not according to scale.

3.1.3 Environmental variables

Of the 12 environmental variables measured, loose rock ($p<0.001$), vegetation on the banks of the stream ($p<0.001$), and sticks ($p<0.05$), had a significant positive correlation with the

number of tadpoles found per sampling session (Table 2.4), whilst silt ($p<0.001$) showed a significant negative correlation with the number of tadpoles found per sampling session (Table 2.4). When looking at the effect of altitude on tadpole size and abundance, our data revealed evenly distributed abundances across high and low sites, with exception to the winter period of lifestage 3 where abundance values were markedly different between high (above 1300 m.a.s.l) and low altitudes (below 1300 m.a.s.l, Table 2.3). With regards to tadpole size, small tadpoles (<35 mm) were more abundant at higher altitudes, however there was little seasonal variation in tadpole size within different lifestages at low (below 1300m), and high altitudes (above 1300m, Table 2.3).

The standard error of the mean (SEM) for lifestage 3 and lifestage 4 were low in comparison to our mean tadpole sizes, indicating that our mean values are a good representation of the actual population mean (Table 2.3). The linear regression revealed a significant inverse relationship between tadpole size and altitude ($p<0.0001$, $r^2= 0.119$), whilst altitude had no significant effect on tadpole abundance ($p>0.05$, $r^2= 0.0310$).

Lifestage 3 abundance values indicate a sharp increase in tadpole abundance at low altitudes and a sharp decline in tadpole abundance at high altitudes from the winter to summer season (Table 2.3, Fishers Exact Test $p<0.01$).

Table 2.3. Mean *Hadromophryne natalensis* tadpole size variation, abundance (tadpoles per sampling session) and Standard error of the mean (SEM) in winter (April-September) and summer (October-March), below 1300m (low altitude) and above 1300m (high altitude). Data range and SEM are indicated in brackets, 'n' represents the sample size. The '*' indicates a significant difference (Fishers Exact Test) in tadpoles numbers between seasons and at different altitudes.

Lifestage 3	**Winter**	**Summer**
Mean tadpole size (mm) below 1300m	38.94 (range=31-55mm, SEM= 1.371)	43.35 (range=25- 60mm, SEM= 1.368)
Mean tadpole size (mm) above 1300m	37.42 (range=25-56mm, SEM= 0.766)	34.97778 (range= 25-57mm, SEM= 1.242)
Total tadpole count below 1300m	17 (n=35)*	47 (n=32)*
Total tadpole count above 1300m	85 (n=46)*	45 (n=32)*
Abundance below 1300m	0.49	1.47
Abundance above 1300m	1.85	1.4
Lifestage 4	**Winter**	**Summer**
Mean tadpole size (mm) below 1300m	61.75 (range=50-82mm, SEM= 7.620)	65.6 (range=55-75mm, SEM= 4.261)
Mean tadpole size (mm) above 1300m	56.8 (range=52-65mm, SEM= 3.453)	62.85 (range= 50-78mm, SEM= 2.517)
Total tadpole count below 1300m	4 (n=4)	5 (n=5)
Total tadpole count above 1300m	5 (n=4)	7 (n=6)
Abundance below 1300m	1	1
Abundance above 1300m	1.25	1.16

Table 2.4. Analysis of variance table from a generalized linear model, indicating the effects of environmental variables on the number of tadpoles found per sampling session. The '*' indicates a significant relationship between the environmental variable and the number of tadpoles found per sampling session.

Coefficients:	Estimate	Standard Error	Z value	Pr (>|z|)
Loose rock	0.889	0.2451	3.668	0.000244***
Banks vegetated	1.0563	0.3205	3.296	0.000982***
Sticks	0.4228	0.1704	2.481	0.013113*
Silt	-0.7457	0.2125	-3.509	0.000449***

3.2 Calling Activity

3.2.1 Advertisement Activity

Hadromophryne natalensis males called most actively from May to July, with much lower calling activity recorded in September and October and no calling detected during the summer months. As the SongMeter was only assembled in May, we cannot provide insight into the calling activity during March/April 2013. The frogs had a higher calling frequency during the early winter when compared to the spring and summer. Calling was consistent after 18:00 with no clear temporal trends evident from 18:00 to 00:00 (Fig. 2.4b). Calling activity (i.e. primary breeding period), coincided with months of low rainfall (ml) and lower mean temperatures (°C) (May to July 2013) (Fig. 2.5).

3.2.2 Time of year for breeding

Breeding was successful at Mariepskop since many pre-metamorphic individuals were found over a year-long period, and post-metamorphic individuals (n=9) were found during the

summer months. Many small individuals (<35mm) were found in late winter (July-September), representing a new cohort of tadpoles hatched in late winter (Fig. 2.4a).

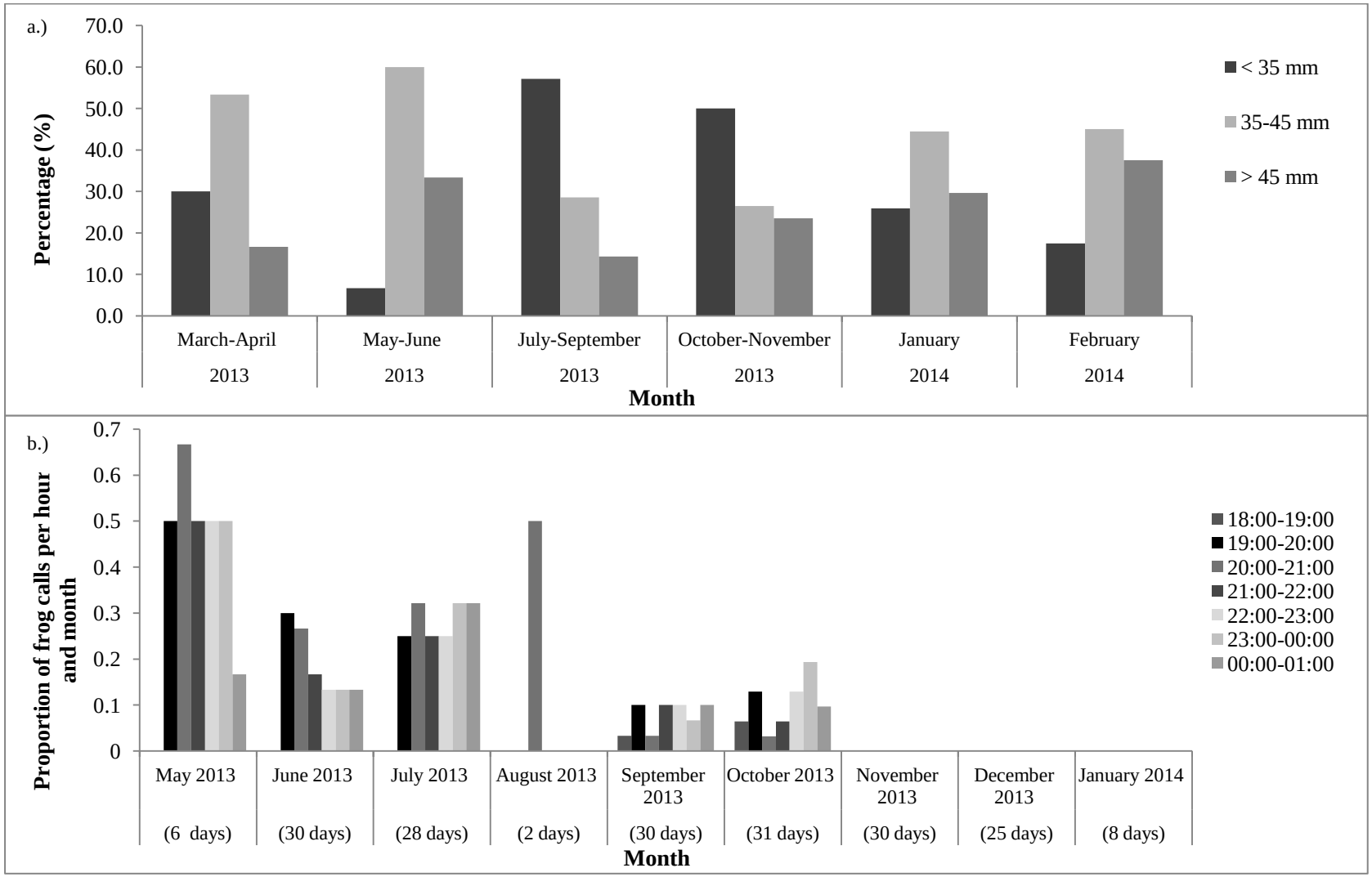


Figure 2.4**.** a.) Tadpole size distribution (mm) during the 2013-2014 sampling season for lifestage 3 & 4 b.) Proportion of recordings with frog calls for each time of night and each month**.** If cascade frog calling was heard during the five minutes of recording time, it was documented as 'present', or otherwise 'absent'. For example, in May during the 6 days the SongMeter was recording, the cascade frogs called for three out of six days during 19:00-19:05, thus the proportion is 0.5 or 50%.

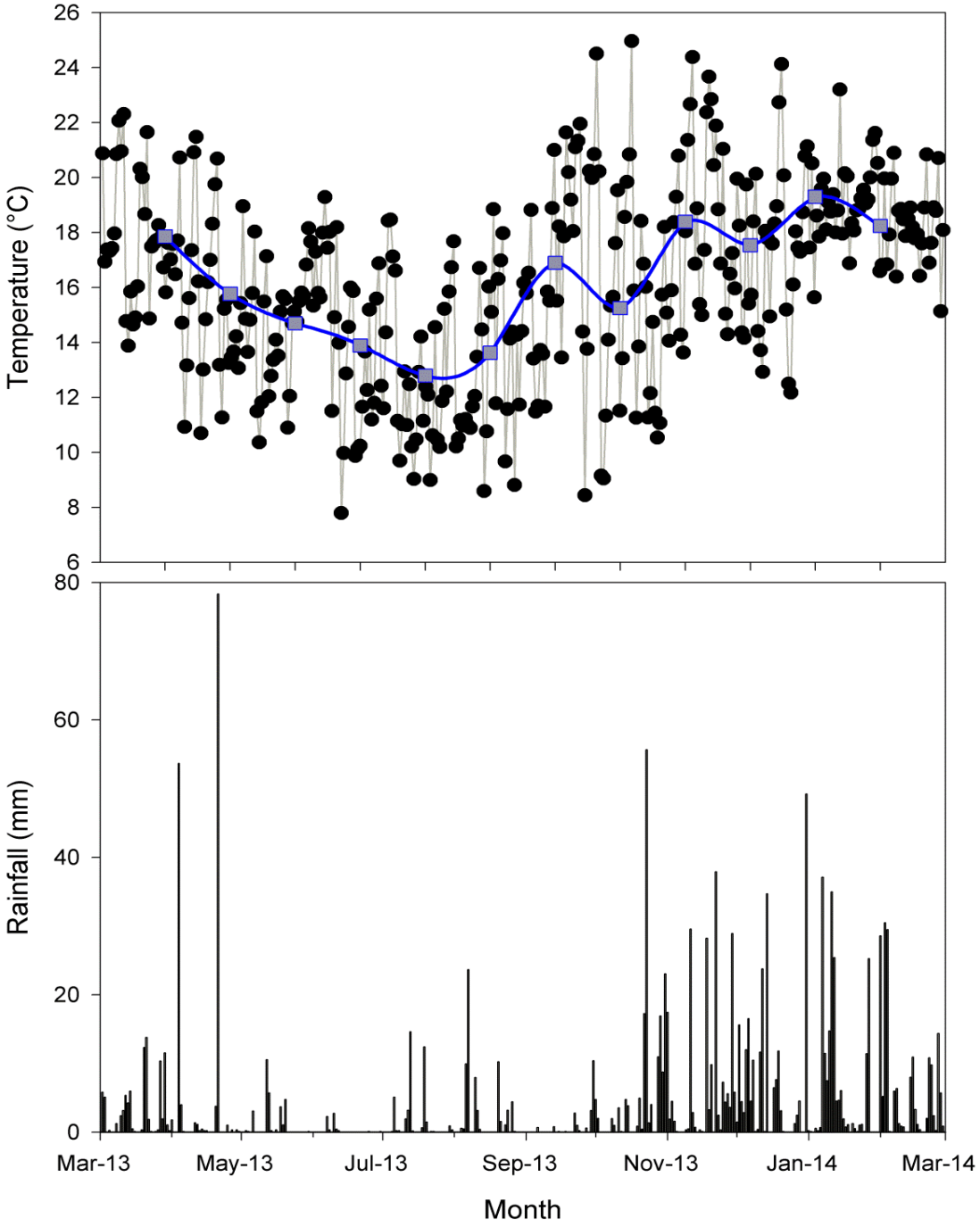


Figure 2.5. Daily air temperature (ºC) and rainfall (ml) showing the mean values from two permanent automated weather stations on the southern slope of Mariepskop at 1300m and 1700m. The blue line represents the mean monthly temperatures during the study period.

3.3 *Hadromophryne natalensis* observations in South Africa

Hadromophryne natalensis occurs widely in Kwa-Zulu Natal, with a distribution following the Drakensburg escarpment into Swaziland, Mpumalanga and Limpopo (Fig. 2.6, Boycott 2004). Observations of post-metamorphic *H. natalensis* ranged from 1-20 individuals per QDS unit. Thus, even though there have been many single sightings around southern Africa, population numbers of this species remain unknown. In South Africa, Frog Atlas data illustrates that cascade frog observations occur at altitudes ranging from 100 m.a.s.l. to 2700 m.a.s.l. and occur over a wide range of temperatures (Fig. 2.7). However, the number of observations peak at altitudes of 1100 and 1400 m.a.s.l, corresponding to the dark QDS regions in Fig. 2.6. Cascade frog observations increased significantly with forest cover (linear regression, $p<0.05$). Mean annual temperature had a significant positive correlation with forest cover as well as cascade frog observations (linear regression, $p<0.05$).

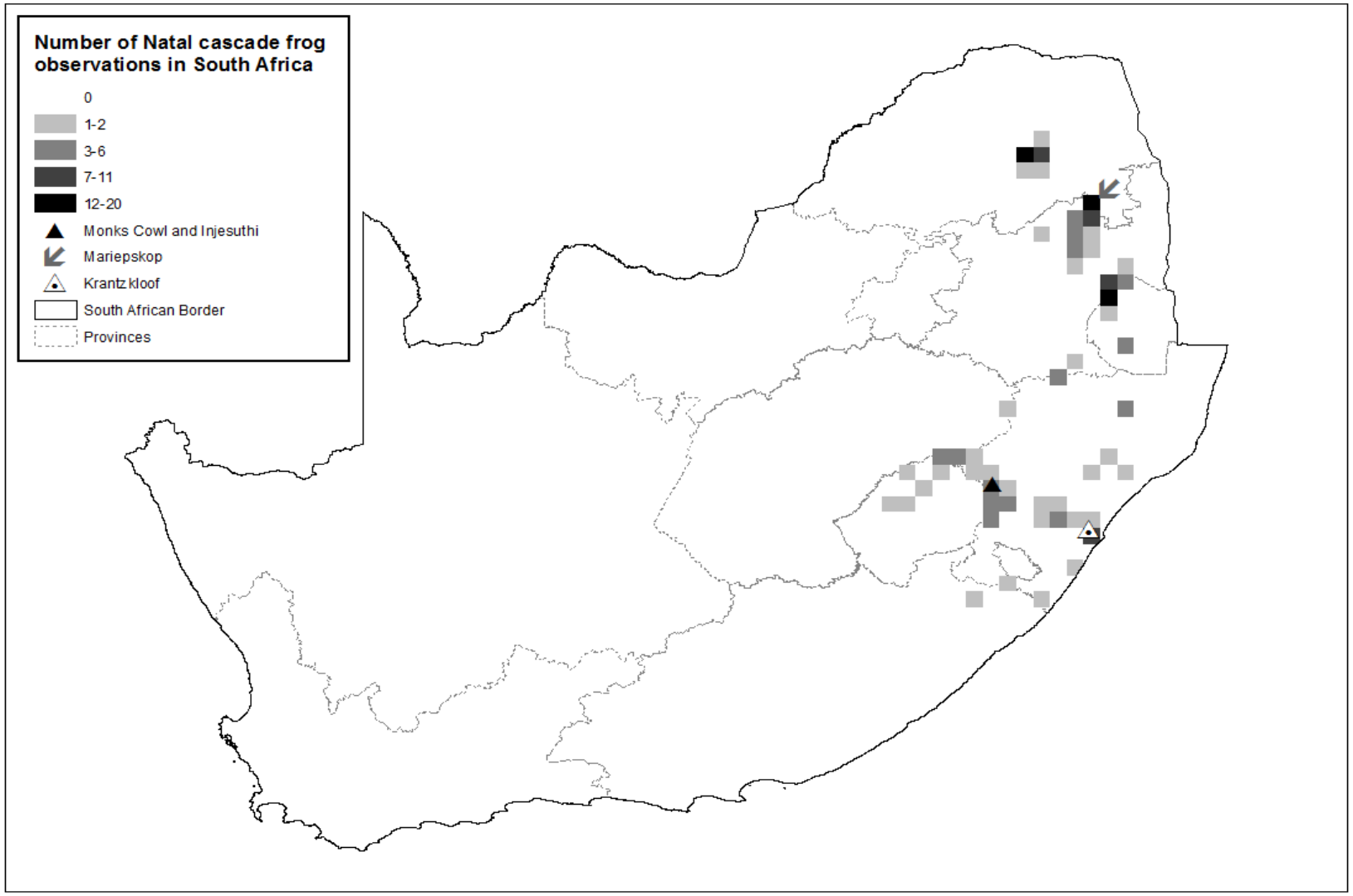


Figure 2.6. Natal cascade frog, *Hadromophryne natalensis* observations in South Africa, represented at a quarter degree resolution (QDS). Darker squares correspond to areas with higher numbers of recorded individuals.

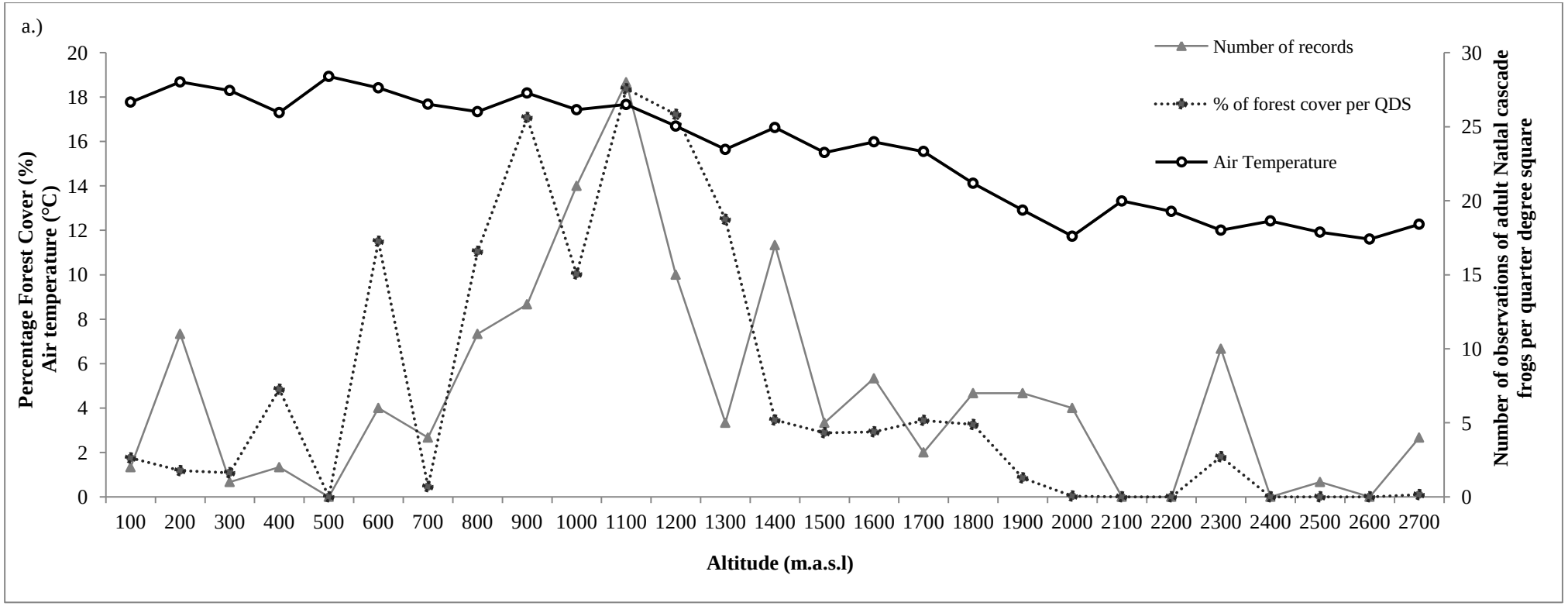


Figure 2.7. Daily mean air temperature (°C), Percentage (%) forest cover of each Quarter Degree Square (QDS), and number of cascade frog records over an altitudinal gradient. Each altitudinal point represents the mean altitude of a specific quarter degree square where *H. natalensis* was encountered. The peaks in the 'number of records' corresponds to the dark squares in figure 2.6, which represent higher numbers of recorded individuals. Forest cover per QDS had a significant positive effect (p=0.008) on the number of cascade frog observations.

4 DISCUSSION

Aquatic environment and habitat requirement

In aquatic environments, water properties are an important determinant in shaping species compositions, such that survival of certain species may depend on the stability of water properties such as pH and dissolved oxygen (Moore & Townsend 1998, de Almeida *et al.* 2014). Dissolved oxygen concentrations increase with decreasing temperature, with the exception of extreme altitudes where pressure may also play a role (Jacobsen *et al.* 2003). Additionally, high flow rates will also lead to increased dissolved oxygen concentrations (Likens *et al.* 1970). We expect that certain conditions may have a positive effect on the abundance of tadpoles. It has been suggested that low water temperatures promote larger tadpole sizes at metamorphosis (Morrison & Hero 2003, Liao & Lu 2012), which may result in larger post-metamorphic individuals (Morrison & Hero 2003).

Sites with tadpoles (i.e. all sites except site 6, 722 m.a.s.l) showed seasonal variation of physical water properties such as temperature (°C), conductivity, pH and dissolved oxygen (%). However, although seasonal variation was apparent in our data, tadpoles were generally found in colder water temperatures (<18°C), and between a pH level of 5 and 7.5. Tadpoles were also more abundant at higher altitudes. *Hadromophryne natalensis* had a particular affinity for fast flowing streams where water speed ranged from 0.44 to 1.85m/s, more than double that of Karssing *et al.* (2012), who found *H. natalensis* tadpoles in streams with water speeds from 0.15 to 0.85 m/s. Additionally, water flow measurements recorded during this study are not indicative of maximum water flow throughout the year as rainfall events are likely to result in short term flooding, and increased water flow rates, not represented in our dataset. As a result, our water flow rates are likely an underestimate of the short term water speed at which these tadpoles can survive. Overall, tadpoles were encountered in greater

numbers at higher altitudes and in streams that were fast flowing and had low temperatures and high dissolved oxygen concentrations.

Site 6, was an anomaly as this was the only site where no tadpoles were encountered. It was located within a similar habitat structure to the other sample sites (forested area with fast water flow), however, it had the lowest altitude and showed higher water properties (water temperature, pH, conductivity), compared to all our other sample sites. A possible reason for this anomaly was the presence of predators (i.e. fish and dragonfly nymphs) at this site. In perennial aquatic habitats, fish are the apex predators (Petranka *et al.* 1987, Hecnar & M'Closkey 1997), playing a key role in shaping the structural dynamics of certain amphibian populations (Morin 1983, Hero *et al.* 1998), even affecting species distributions through the elimination of anuran species in certain areas due to the presence of predators (Bradford *et al.* 1993, Hecnar & M'Closkey 1997). Thus, predation is likely to shape the geographical distribution of tadpoles at Mariepskop.

Tadpole abundance was positively correlated with the presence of loose rock, sticks and vegetated banks, suggesting that they have a strong preference for stream structure which provides shelter consistent with Karssing *et al.* (2012), who suggest that tadpoles avoided substrates which did not offer a degree of cover (i.e. sand silt and bedrock). Eterovick (2003), showed a positive correlation between canopy cover, often associated with cooler water temperatures and more shaded streams, and tadpole habitat preference in three amphibian species. As *H. natalensis* prefers cool, clear, fast flowing streams (Wager 1965), one would expect that they would prefer shaded areas of streams i.e. areas with vegetated banks, that promote cooler water temperatures (Eterovick 2003, Afonso & Eterovick 2007), and food availability (Afonso & Eterovick 2007). Silt was the only variable that had a negative effect on the number of tadpoles. Gillespie (2002) argued that increased sedimentation might cause tadpole foraging efficiency to decrease, which has negative effects on their growth and

development and ultimately survival. We suggest that this could be a possible explanation for why fewer tadpoles were found in silted streams. Thus, physical characteristics of the environment (plants and rocks) appear to be important in determining the habitat suitability for *H. natalensis*

Altitudinal effects on tadpole size and abundance

Water temperature is a likely factor controlling pre-metamorphic growth rates of tadpole species at high altitudes (Liao & Lu 2012). Cooler temperatures, associated with high altitude sites, cause slower growth rates (i.e. longer larval periods), and larger body sizes upon metamorphosis (Morrison & Hero 2003, Liao & Lu 2012, Rivers-Moore & Karssing 2014). Table 2.3 revealed that tadpoles were smaller in size and developmental stage (i.e. lifestages 3 and 4) at higher altitudes during both winter and summer sampling seasons. Furthermore, altitude had a significant effect on tadpole abundance between seasons within lifestage 3. Table 2.3 indicates that large tadpoles make up a greater percentage of those at lower altitude, especially during the summer season. Tadpole abundance increases significantly at low altitudes from winter to summer. There are two possible hypotheses to explain this seasonal effect: 1) increased predation or mortality at high altitudes, 2) small tadpoles from higher altitudes get washed down to lower altitudes where they mature, resulting in greater numbers of large tadpoles downstream.

We suspect that seasonal floods could be having an effect on abundance, causing tadpoles from high altitudes to be washed down to lower altitude sites during summer, causing significant seasonal attitudinal variation in our data (Table 2.3). Thus, seasonal rainfall rather than altitude may be affecting the abundance and distribution of *H. natalensis* tadpoles over an attitudinal gradient.

Breeding period

Amphibians rely on two fundamental environmental factors in order to achieve breeding success, namely temperature and rainfall (Carey & Alexander 2003, Vaira 2005). It has been suggested that amphibian species are more likely to time their breeding seasons with the onset of the rainy season (Prado *et al.* 2005, Vaira 2005), especially in seasonal environments (Prado *et al.* 2005). Forested areas, often associated with high moisture, high rainfall and high humidity (Prado *et al.* 2005), would not be considered seasonal habitats but rather considered relatively stable in terms of moisture availability. *Hadromophryne natalensis* is a forest specialist, inhabiting predominantly forest streams (Boycott 2004). This was supported by our results, which indicate that forest cover per QDS had a significant positive effect on the number of cascade frog observations (Fig 2.6, 2.7).

With regard to breeding season, there are some differences in opinion about the breeding season of *H. natalensis*. Current literature suggests that *H. natalensis* in the central Drakensberg breed in spring (Rivers-Moore & Karssing 2014), but other authors suggested that breeding occurs in late summer to autumn (March-May) when the water flow in the streams is low (Boycott 2004).

Our results suggest that the main breeding period for *H. natalensis* is early (May) to late (July) winter, coinciding with low rainfall and low mean ambient temperatures. Many small tadpoles (<35mm) were found in late winter (July-September) representing a new cohort of tadpoles that hatched in late winter to early spring. Calling activity of *H. natalensis* at Mariepskop also supported a winter breeding period with calling activity peaking from May to July, with much lower calling activity recorded in September and October, with no calling activity from November to January. However, size measurements of tadpoles suggest that breeding is not a single clear pulse over a short period, but a protracted activity with a peak in winter. We suggest that because of the forested environments which *H. natalensis*

inhabits, this species may not be limited to breeding during seasons when rainfall and temperatures are high, but rather, may breed when environmental conditions for eggs and hatchlings are favourable (low stream flow rates).

How climate change and changing temperatures could affect *Hadromophryne natalensis*

Temperatures in southern Africa are expected to increase between 0.7 and 1.6 °C between the 2020's and 2050's (Hulme *et al.* 2001). Changing temperature and moisture regimes can have detrimental effects on certain behaviour such as the breeding and the reproduction period of amphibians (Reading 1998, Carey & Alexander 2003, Blaustein *et al*. 2010). It is important to consider what effects this will have on *Hadromophryne natalensis*. This species has been studied extensively in Kwa-Zulu Natal from the interior Drakensburg area to the low-lying area (420 m.a.s.l) of Krantzkloof.

Hadromophryne natalensis does not appear to be limited in terms of water or air temperatures as this species has been found in water temperatures ranging from 6.5 to 20°C, and air temperatures ranging from 8-25°C (Table 2.1), and across a wide range of altitudes (Fig. 2.7). Fewer frogs were observed at cold air temperatures (corresponding to altitudes above 1800 m) and this is a fortuitous consequence of the montane distribution of forests rather than the inability of *H. natalensis* to survive at cooler temperatures. It is clear that temperature is not a limiting factor for this species, as they are very temperature tolerant. Thus, this species is not necessarily limited to high altitudes and specific temperatures but rather forested habitats.

Rivers-Moore & Karssing (2014) suggest that warmer water temperatures may cause a reduction in the length of the larval period from two years to one year. Furthermore, warmer water temperatures may allow individuals to grow faster and in turn, reach sexual

maturity earlier, allowing them to begin breeding at a younger age and may allow them to produce more than one clutch a year (Morrison & Hero 2003). Morrison & Hero (2003), compared the highland and lowland populations of *Rana pretiosa* and suggested that the lowland population of *R. pretiosa* matured much faster, and were able to breed annually, when compared to the highland population who took longer to mature and only bred every 2-3 years. Colder water temperatures associated with high altitudes result in larger larva at metamorphosis, and in turn larger post-metamorphic individuals who are able to produce larger clutch sizes and larger offspring (Morrison & Hero 2003). Thus, although *H. natalensis* can survive in warmer water temperatures, which could induce a faster rate of first reproduction, it is likely that offspring may be smaller (Morrison & Hero 2003). However, as the tadpoles are smaller and have a faster generation time this could make them more resilient to environmental change allowing them to recover faster after a disturbance (Morrison & Hero 2003).

My study showed water temperatures ranging between 9-25 °C, however tadpoles were only encountered between a temperature range of 9-18 °C. If we compare this water temperature range to studies conducted in Kwa-Zulu Natal (Table 2.1), my study has slightly higher minimum water temperature value when compared to the Kwa-Zulu Natal region. This study provides insight into habitat preferences of *H. natalensis* outside of the well studied Kwa-Zulu Natal region, and could represent an intermediate environment, in terms of temperature, between the high altitude Drakensberg site and low lying Kranskloof areas.

Thus, increasing water temperatures caused by climate change may not have a direct effect on the distribution of *H. natalensis* as it has a wide thermal tolerance. While warmer water temperatures may result in shortened life cycles (Rivers-Moore & Karssing 2014) and smaller offspring (Morrison & Hero 2003), *Hadromophryne natalensis* is not primarily a high-altitude specialist (Fig. 2.7). Rather, it is a forest specialist (Fig. 2.6) requiring fast-

flowing water with forests, coincidentally occurring along mountain slopes at mid-level elevations. Present evidence shows that the cascade frog occurs in streams over a wide range of temperatures and that it is unlikely that temperature change in the order of 1-4 ºC will, in itself, strongly affect their survival.

Additionally, climate change could have an effect on the spread of disease in amphibian populations (Pounds *et al*. 2006, Bosch *et al*. 2007). According to Piotrowski *et al.* (2004), *Batrachochytrium dendrobatidis* (resulting in chytridiomycosis) grows optimally at intermediate temperatures of between 17 and 25 ºC. *Batrachochytrium dendrobatidis* can also survive at very low temperatures, whilst temperatures above 29 ºC appear to be lethal for this fungus (Piotrowski *et al.* 2004, Bosch *et al*. 2007). Chytridiomycosis outbreaks in temperate regions will be more common in montane areas in the warmer months of the year, and at lower altitudes in the colder months of the year (Piotrowski *et al.* 2004). Tadpoles on Mariepskop were generally found in colder water temperatures (<18°C) and tadpoles and adult frogs at air temperatures between 7.9°C and 19.5°C. These temperatures are on the lower end of the optimal temperature range for *Batrachochytrium dendrobatidis* growth. Chytridiomycosis is present and common in the Mariepskop *H. natalensis* population, however, despite the presence of this disease, the population appears to be healthy and reproducing (discussed in the chapter three). Thus, it is hard to predict what the long term effects of climate change will be on this population as a small increase in temperature could bring the temperature firmly within the optimum temperatures for *Batrachochytrium dendrobatidis* growth, or conversely, if the temperature decreases this could result in temperatures at Mariepskop which are out of the optimum range for this disease to flourish.

Overall, secondary effects of climate change are likely. If climate change causes forest ranges to shrink, *H. natalensis* distribution in South Africa is likely to become threatened. Thus, this species is unlikely to be directly affected by long term temperature change.

However, increasing temperatures may have indirect consequences for the survival of this species, in terms of forest cover and disease.

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CHAPTER 3

Factors affecting the incidence of Chytridiomycosis infection in Natal Cascade frog (*Hadromophryne natalensis*) tadpoles across an altitudinal gradient

ABSTRACT

Chytridiomycosis, a fungal disease caused by *Batrachochytrium dendrobatidis*, affects the keratinized epidermis of adult amphibians and is of particular concern to conservationists as it has been implicated as a leading cause of mass amphibian mortalities worldwide. It has been argued that climate change increases the prevalence of chytrid infections of amphibia, especially in mountainous habitats. This study investigates how chytrid infection incidence, tadpole size and developmental stage vary across an altitudinal gradient for the forest-living Natal cascade frog *Hadromophryne natalensis* on the slopes of the Mariepskop Mountain, South Africa. An inverse relationship was found between tadpole size and altitude with chytridiomycosis incidence being significantly correlated with tadpole size but not with altitude. With 32 of 114 photographed tadpoles [=28%] showing symptoms of chytridiomycosis, our study indicates that the geographic range of this disease (area with high incidence) extends much further northwards in South Africa than reported to date. However, the population of *H. natalensis* at Mariepskop appears to be healthy; the adults are relatively common and showed no signs of disease, and successful breeding was taking place since many tadpoles, metamorphs and adults were observed during the study period. However, strong evidence for good survival in the face of chytridiomycosis can only be obtained via long term monitoring to determine whether amphibian populations are indeed healthy and

reproducing despite the presence of the disease. This would require repeatable and rapid protocols for assessing amphibian abundance and survival.

Key words - Chytridiomycosis, *Batrachochytrium dendrobatidis, Hadromophryne natalensis*, Mariepskop, long-term monitoring

1 INTRODUCTION

1.1 General

Many amphibians travel repeatedly between their aquatic and terrestrial habitats depending on their foraging and breeding needs (Wyman 1990). They have a sensitive skin, through which gaseous exchange takes place and that loses moisture rapidly. They also produce eggs that must be kept moist. These factors often limit them to mesic environments (Halliday 2008) and make them particularly susceptible to certain threats, such as habitat destruction, introduced species, climate change and disease (Beebee & Griffiths 2005, Halliday 2008). Of these threats, the disease chytridiomycosis is of particular concern.

Chytridiomycosis is caused by a chytrid fungus, *Batrachochytrium dendrobatidis* (Longcore *et al.* 1999, Briggs *et al.* 2005), hereafter referred to as *Bd.* It was first described by Berger *et al.* (1998) in frogs from Australia and Central America and has been linked to mass frog mortalities in these regions. Chytrid fungi are cosmopolitan and omnipresent, occurring in many moist habitats such as bodies of water (i.e. streams, lakes and ponds), and moist soil (Powell 1993, Berger *et al.* 1998, Daszak *et al*. 1999). They are saprobes, often associated with the decomposition of cellulose, keratin and chitin (Powell 1993, Berger *et al.* 1998, Daszak *et al*. 1999). McMahon *et al.* (2013) determined that non-amphibian species such as mosquitofish (*Gambusia holbrooki*) and crayfish (*Procambarus spp.* and *Orconectes virilis*) are successful hosts and transmitters.

Chytridiomycosis is classified as an emerging infectious disease (Daszak *et al.* 1999, 2001, 2003, Rosenblum *et al*. 2013) with (a) increases in geographic range; (b) increased incidence and effects on organisms (Lips 1998) and (c) infection of new host populations (Daszak *et al*. 2000, 2001, 2003). Even though it is a fast spreading and harmful disease with negative effects on infected individuals (Daszak *et al*. 1999, Olson *et al.* 2013), these effects are often difficult to quantify since deceased adult frogs are often scavenged or decay rapidly,

leading to unobserved mass mortalities (Skerratt *et al.* 2007). This disease is most apparent in the post-metamorphic stage of amphibians (Berger *et al.* 1998, Beebee & Griffiths 2005), with symptoms including uncharacteristic and awkward posture, lethargy (Daszak *et al.* 1999), and shedding of the epidermal layer of skin resulting in lesions on the skin, muscles and eyes (Daszak *et al.* 1999). This has detrimental effects on important processes, such as gaseous exchange and moisture retention, both of which rely on an intact and functioning epidermal layer (Rosenblum *et al.* 2013). Although it is unlikely to result in death (Berger *et al.* 1998), *Bd* may also have negative effects on tadpoles, causing deformities of the mouthparts (Smith *et al.* 2007). These deformities may lead to a decline in their ability to feed, causing slower growth rates (Smith *et al.* 2007, Parris & Cornelius 2004)

Batrachochytrium dendrobatidis grows optimally at an intermediate temperature between 17 and 25 ºC, (Piotrowski *et al.* 2004, Rowley & Alford 2013) and at a pH of between 6 and 7 (Piotrowski *et al.* 2004). However, it can survive at temperatures as low as 4 ºC, which means it can survive cold winter climates (Piotrowski *et al.* 2004). Consequently, the disease may be more infective at the intermediate temperatures associated with high altitudes habitats such as mountains and plateaus (Lips *et al.* 2003, Stuart *et al.* 2004, Pounds *et al.* 2006). Chytrid infection rates increase with increasing altitudes (Stuart *et al.* 2004, Rowley & Alford 2013). This trend may be due to the disease favouring intermediate temperatures for optimum growth (Woodhams *et al.* 2003). Endemic amphibian populations at high altitude sites are generally characterized by low abundances and small, restricted geographic distributions. Therefore, the negative effects of chytridiomycosis may be more apparent for these populations, especially when compared to species which have greater abundances and larger geographic distributions (Lips *et al.* 2003).

Global warming is expected to promote the prevalence of favourable temperatures for *Bd* in mountains (Pounds *et al.* 2006, Bosch *et al.* 2007), since climate change may cause an

increase in cloud cover (Pounds *et al*. 2006) that will favour the chytrid fungus by keeping environmental temperatures within their optimum temperature of 17 – 25 ºC (Pounds *et al*. 2006).

This study focuses on the Natal cascade frog, *Hadromophryne natalensis*. Adult frogs are nocturnal and often found in cascades, waterfalls, rock crevasses and occasionally on exposed rocks alongside fast flowing streams along the eastern escarpment of Southern Africa, Lesotho and Swaziland (Carruthers 2001). They occur at altitudes between 480 and 2675m (Boycott 2004, Karssing *et al.* 2012), and in two main vegetation types, namely forest and montane grassland (Wager 1965, Boycott 2004). Daszak *et al*. (1999) suggested that chytridiomycosis is especially prevalent in 'regionally endemic rain forest specialists with low fecundity that reproduce in streams and live at high altitudes'. *Hadromophryne natalensis* fits all these characteristics and may therefore be particularly at risk. The breeding period for *H. natalensis* may vary from population to the next, some authors have suggested that breeding occurs in spring (Rivers-Moore & Karssing 2014), whilst others believe they breed in late summer to autumn (March-May) when the water flow in the streams is low (Boycott 2004). The tadpoles feed on algae in cool, clear, fast flowing forest streams (Wager 1965, Carruthers 2001). They have a rare life cycle, growing to between 8 and 10 cm in length and taking up to two years to fully metamorphose and therefore require perennial streams (Wager 1965, Karssing *et al* .2012). Since *H. natalensis* tadpoles have large sucking mouthparts it is possible to easily identify the chytrid fungus by the brown pigmentation marks on their mouthparts (Smith *et al.* 2007).

In this study, we investigated the prevalence of chytridiomycosis in *H. natalensis* tadpoles at Mariepskop, South Africa and its relation to the effects of altitude, tadpole age, size and the interaction between these factors.

2 METHODS

2.1 Study Site

Mariepskop Mountain, part of the northern Drakensberg escarpment, has an altitudinal gradient from 500m to 1946m. The natural vegetation on Mariepskop is diverse, and varies with aspect, ranging from various forms of savanna woodland at the lower altitudes to evergreen montane forests at mid-altitudes and heath and high mountain grasslands on top of the mountain (Fig. 3.1; van der Schijff & Schoonraad 1971).

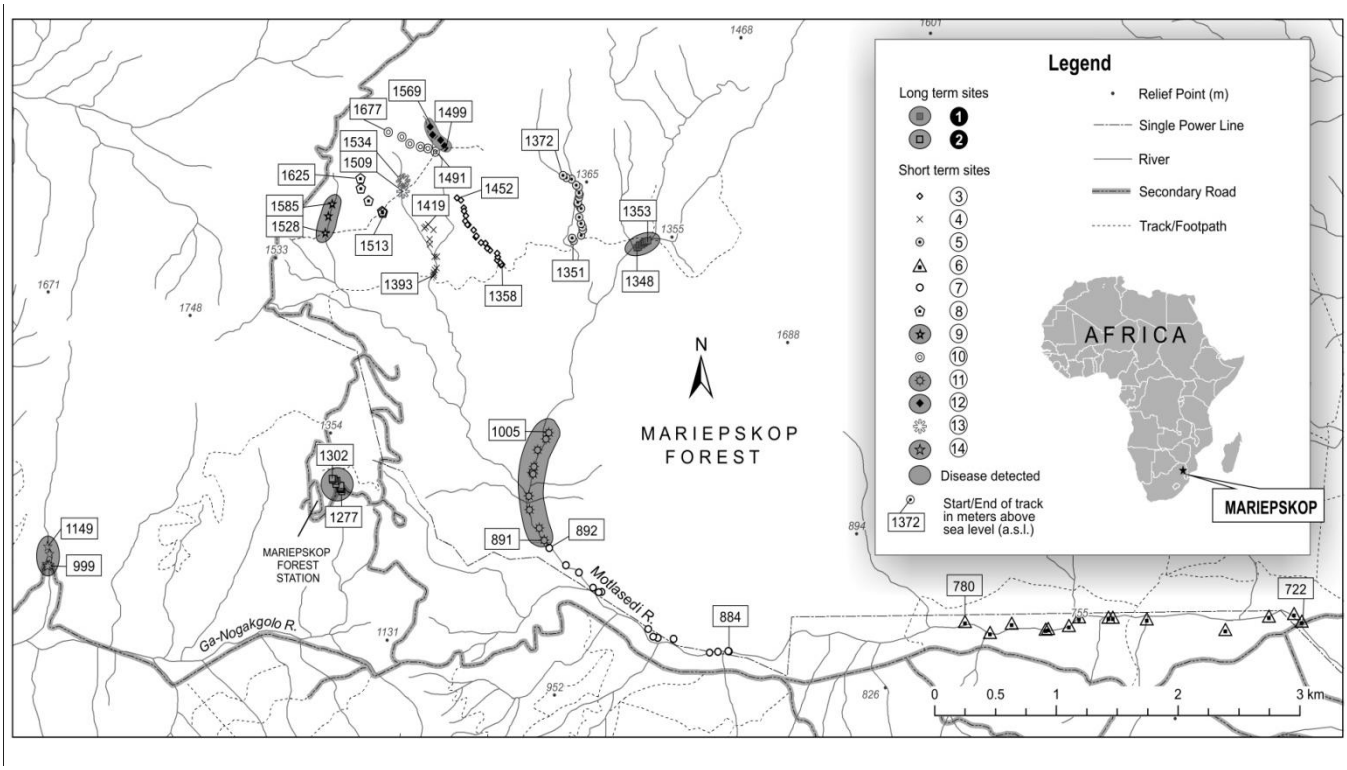

Figure 3.1. Sites on Mariepskop Mountain where cascade frog tadpoles were sampled. Sites circled in grey indicate where chytridiomycosis disease was found.

2.2 Study design and protocol

When selecting streams for sampling, two different protocols were used. Two long term sites were chosen, which were repeatedly sampled (i.e. two samples per long term site per trip), as well as 12 additional streams each only sampled once (Fig. 3.1). Streams were chosen during

the winter months (low stream flow) in order to make sure that they were all perennial. Tadpole sampling took place during the day whist adults were sampled at night, starting approximately one hour after sunset. Night sampling only took place at the two long term study sites due to restricted accessibility. Tadpoles were classified according to their different lifestages which could be easily identified in the field (Adapted from Gosner 1960; Table 3.1). When analysing the abundance data, tadpoles and adult frogs were not pooled as these amphibian life stages inhabit different ecological niches (Dupuis & Friele 2006, Hunter 1998).

2.2.1 Long term sites

Two streams were selected to be representative of the range of perennial stream conditions at Mariepskop. The Klaserie River site (Long term site 1; 1352 m.a.s.l.) on the southern side of the mountain (Fig. 3.1) includes the largest watercourse on Mariepskop (approximately 0.5-1.0 m deep and 5-10 m wide), and is a fast flowing, clear water river with numerous small cascades and some larger drops below the study transect. It is minimally disturbed, with low silt load, and runs over sand, bedrock and large boulder substrates. The stream is surrounded by a broad gallery of Northern Mistbelt Forest (Mucina & Rutherford 2011) with *Pinus* and *Eucalyptus* plantations beyond this Mistbelt Forest. Decaying tree-trunks are sometimes found in the stream with some shrubby vegetation along the banks. The stream banks are covered by a mixture of trees (approx. 8-10 m in height) and medium shrubs (approx. 4m in height) offering cover, with gaps in the overhead canopy exposing parts of the stream to periodic sunlight.

The Ga-nogakgolo River site (Long term site 2; 1292 m.a.s.l.) was situated in the headwaters of the Ga-nogakgolo River on the western side of the mountain (Fig. 3.1), flowing into the Blyde River to the north. This stream is fast flowing, dropping steeply over

several cascades, with larger waterfalls below. In contrast with the first site, this is a small forest stream (approximately 1-3 m wide and 0.05-0.20 m deep) on a substrate of largely silt, sand and small boulders. It is somewhat turbid, with a high silt load due to disturbances associated with the forestry activities upstream. Much of the stream is covered by overhanging vines and shrubs, with some shrubs and fallen wood in the stream itself. The stream flows under a closed canopy of the Northern Mistbelt Forest, which in this site is confined to a narrow strip within the surrounding *Pinus* and *Eucalyptus* plantations.

Transects of 100-120 m in length at each of these two sites were repeatedly sampled every 6 to 8 weeks during the period March 2013-Febuary 2014. Each transect was divided into 10 m segments.

The Klaserie River (site 1) was divided into ten sections, each 10 m in length (totalling 100 m) whilst the Ga-nogakgolo River (site 2), was divided into twelve sections, each 10 m in length (totalling 120 m). The permanent study sites differed slightly in length due to accessibility restrictions, but as sampling was random this was not expected to affect the analyses. Both sites were then divided into an upper and a lower half, each with five or six 10 m segments. Sampling always took place moving in an upstream direction to avoid disturbing the substrate.

During each field visit, two 10 m segments (one in the upper half and one in the lower half i.e. 20 m per stream) were randomly selected and intensively sampled. Relative abundance of tadpoles was expressed in terms of number of tadpoles encountered per segment. Tadpoles were sampled using dip-netting. The observer (KdT) walked methodically upstream (in a pre-determined transect section), rolling rocks with a net placed on the substrate slightly downstream to catch any dislodged tadpoles. A field assistant was present during all sampling sessions in order to take accurate notes. Tadpoles were measured, assigned a life history stage (Table 3.1), and returned to the stream. In addition, photographs

were taken of the mouthparts (Fig. 3.2a & 3.2b) to detect chytridiomycosis (Smith *et al.* 2007). All sample locations were recorded using a GPS.

2.2.2 Short term sites

During the winter months (May-July) of 2013, rivers on Mariepskop were examined to see which streams had dried up and which were perennial. All perennial streams were geo-referenced and systematically sampled. Observers walked upstream, actively searching for 50 minutes, taking GPS waypoints of any evidence of *H. natalensis*, such as the distinctive tadpole feeding marks and any frogs or tadpoles observed. After 50 minutes, a 10 m stream segment was sampled for tadpoles using the dip-netting technique, following the protocol described above for the long term sites. Observers repeated this protocol (observations along the stream for 50 minutes; 10 m tadpole sampling) until unable to ascend further (e.g. steep waterfall or impenetrable vegetation). Photographs were taken of the mouthparts (Fig. 3.2a & 3.2b) of all tadpoles captured to diagnose chytridiomycosis.

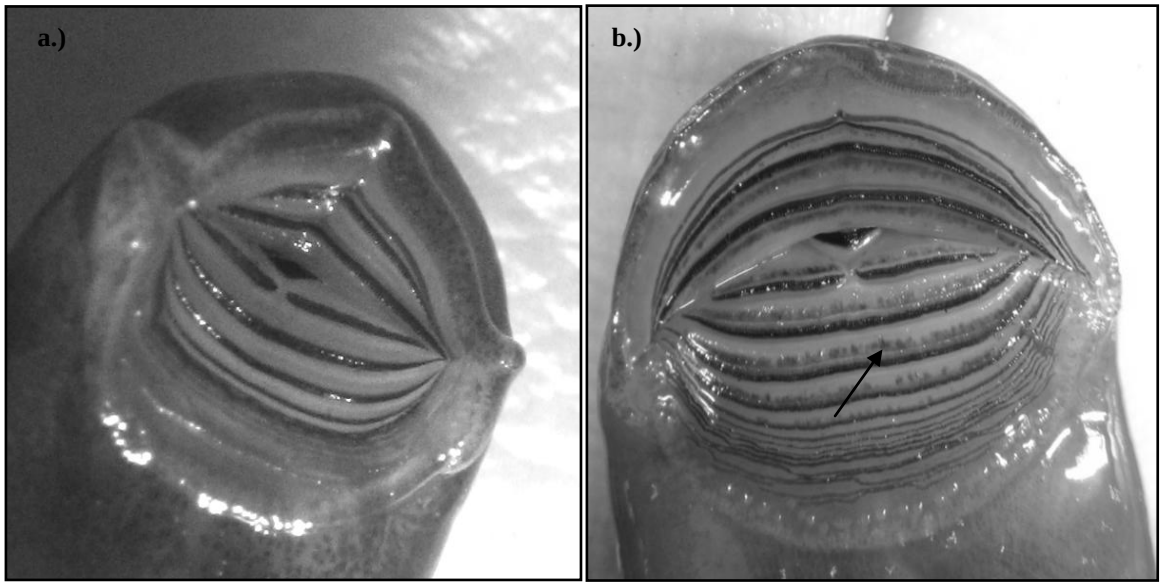

Figure 3.2. Photographs of *Hadromophryne natalensis* tadpole mouthparts to detect chytridiomycosis at Mariepskop. a.) Mouthparts with no signs of chytridiomycosis. b.) Brown pigmentation marks on the rows of papillae (as shown in the picture-two rows above and four below) indicate a tadpole infected with *Batrachochytrium dendrobatidis.* Also see Smith *et al.* (2007).

2.3 Night sampling

As *H. natalensis* are most predominately nocturnal (Wager 1965), sampling started one hour after sunset, after which both long term study sites were sampled in a single evening. Only the lower half or the upper half (i.e. around 50 m) was sampled on any single night due to the travel time between study sites. Lower and upper parts of the study sites were interchanged on successive nights. Night sampling took place through active searching with spotlights. Once a frog was located, its posture was recorded, after which it was slowly approached and captured. Adult frogs infected with chytridiomycosis are often associated with an abnormal posture and the inability to correct this (Daszak *et al.* 1999). Its skin was then examined for the presence of chytridiomycosis i.e. epidermal sloughing and ulceration (Daszak *et al.* 1999), its morphological features were measured, sexed (see Table 3.1) and documented with its geographical position, after which it was released.

2.4 Statistical Analyses

All analyses were performed in R statistical package, version 3.01 (R Development Core Team 2008). For pooled analysis of all sites, sampling bias occurred due to more intensive sampling at the long term sites. To avoid this, a random sample was used from each of the long term sites and pooled with the data for the short term sites. Chytridiomycosis occurrence in tadpoles was expressed as a binary variable, presence of disease=1 or absence of disease=0. A generalised linear model (GLM) was used to investigate whether tadpole size and lifestage had a significant effect on the incidence of disease. Furthermore, a linear regression was performed to see whether altitude had an effect on tadpole size and disease incidence.

To compare the abundances of tadpoles with and without visible disease in the different streams, we used a chi-squared test with a correction for continuity. The numbers of

tadpoles with and without visible disease symptoms were compared using only the data from the two long term sites (with larger sample sizes). For this, a Fisher's Exact Test was used.

Additionally, to investigate whether there was any significant difference in overall mean tadpole size (mm) of diseased and healthy tadpoles within each lifestage, we also used a Fisher's Exact Test.

Table 3.1. Life history stages assigned to *Hadromophryne natalensis* (Adapted from Gosner 1960). Stages in brackets correspond to Gosner lifestage classification.

	Lifestage	**Description**
1	Eggs (Stage 1-16)	
2	Hatchling (Stage 17-24)	Newly hatched tadpoles, still possessing external gills
3	Tadpole (Stage 25)	Distinct body and tail associated with a tadpole
4	Tadpole with limb buds or limbs present (Stage 26-36)	Distinct tadpole morphology as well as the presence of limb buds and/or small limbs which are beginning to form
5	Tadpole with distinct hind limbs (Stage 37-41)	Development of distinct toes on hind limbs (forelimbs not yet developed)
6	Young adult frog, still inhabiting water (Stage 42-43)	Fully developed fore and hind limbs, tail still present, tadpole still inhabiting water
7	Young metamorph, air breathing (Stage 44-45)	Air-breathing metamorph, fully developed fore and hind limbs, tail resorbing, fully formed mouth
8	Juvenile frog (Stage 46)	Tail stump resorbed (+-20-30mm)
9	Sub adult	Near adult size (+-40mm), sex indeterminate
10M	Adult male	Spines on thumb and chest, as well as black spots present (+-40-50mm)
10F	Adult female	Spines and spots absent (+-40-50mm)

3 RESULTS

3.1 Altitudinal effects on tadpole size and chytridiomycosis

Cascade frog (*H. natalensis*) tadpoles were sampled at 14 locations, ranging from an altitude of 884 to 1570 m.a.s.l. Tadpole body size ranged from 25 to 82 mm in length, measured from snout to tail tip (Table 3.2). The linear regression revealed a significant inverse relationship between tadpole size and altitude ($p<0.0001$, $r^2 = 0.119$). Low altitude and high altitude sites were sampled in both summer and winter (Table 3.2), thus eliminating the possibility that the relationship between altitude and tadpole size may be influenced by growth across seasons. There was no statistically significant relationship between altitude and the incidence of chytridiomycosis in tadpoles (Table 3.3).

Table 3.2. Mean *Hadromophryne natalensis* tadpole size variation (lifestage three and four) and sample size in winter (April-September) and summer (October-March), below 1300m (low altitude) and above 1300m (high altitude). Sample size (n) and range of tadpole size indicated in brackets.

	Winter	Summer
Mean tadpole size (mm) below 1300m	43.29 (n=21; range=31-82)	45.53 (n=51, range=25-75)
Mean tadpole size (mm) above 1300m	38.5 (n=90; range=25-65)	38.73 (n=52, range= 25-78)

3.2 The effect of tadpole lifestage and size variation on the incidence of chytridiomycosis

Chytridiomycosis incidence was significantly affected by tadpole size (Generalised Linear Model (GLM), $p< 0.0002$, Table 3.3). When examining our lifestage data there was a greater proportional incidence of chytridiomycosis at lifestage four (Table 3.4), compared to lifestage

three. However, the effect of lifestage on chytridiomycosis (Table 3.3), was not significant when a GLM and Fisher's Exact Test were performed. Furthermore, there was no significant difference ($p>0.05$) in size between diseased and healthy individuals within each of lifestages three and four (Table 3.4).

Table 3.3. Analysis of variance table from a GLM, indicating the effects of altitude, tadpole size and lifestage on the incidence of chytridiomycosis in *Hadromophryne natalensis*. The order of significance is ranked with an asterix, thus *** indicates a high significance.

The effect of altitude on tadpole size				
Coefficients:				
	Estimate	Standard Error	Z value	Pr (>|z|)
Intercept	1.881	0.067	28.057	<0.00001
log (Altitude)	-0.0002268	0.00005027	-4.512	0.0000134***
The effect of altitude on the incidence of disease				
Coefficients:				
	Estimate	Standard Error	Z value	Pr (>|z|)
Intercept	-11.942	11.222	-1.064	0.287
log (Altitude)	1.533	1.563	0.98	0.327
The effect of tadpole size and lifestage on the incidence of disease				
Coefficients:				
	Estimate	Standard Error	Z value	Pr (>|z|)
Intercept	-1.45913	1.707	-0.855	0.392667
Tadpole length (mm)	0.10116	0.02627	3.851	0.000117 ***
Lifestage	-1.22145	0.69804	-1.75	0.08015

Table 3.4. Abundance and size of diseased (chytridiomycosis) and healthy *Hadromophryne natalensis* tadpoles in different lifestages.

Number of healthy and diseased tadpoles	Lifestage 3	Lifestage 4
Diseased	25	7
Healthy	77	5
Total	102	12
Percentage (%) diseased	25%	58%
Tadpole size (mm)	Lifestage 3	Lifestage 4
Diseased	43.88 (n=25; range=32-55mm)	65.57 (n=7; range=50-78mm)
Healthy	36.01 (n=77; range=25-55mm)	62.2 (n=5; range 50-82mm)

3.3 Spatial differences in the incidence of chytridiomycosis in tadpoles.

To compare the incidence of chytridiomycosis in different streams, the columns of the contingency table containing counts of diseased and non-diseased tadpoles were classed into seven sub-catchments in order to obtain as many cell values of the contingency table to be greater than 5 so that an adequate sample size could be obtained. However, even with this procedure, several cells still had counts below 5. The chi-squared value obtained was not significant (chi-squared = 19.2; d.f.=6; p=0.15). Therefore, there is no strong evidence for between-stream differences in the incidence of the disease. When examining the two long term study sites, 42% of the tadpoles sampled had disease at site 1, whilst only 22 % of the tadpoles had disease at site 2 (Table 3.5). However the corresponding Fishers' Exact Test revealed no significant (p=0.1149) difference in disease incidence between these two sites.

The short term study sites revealed a few cases of disease, but as these sites were only sampled once our sample of diseased individuals is relatively small (Table 3.5).

3.4 Night sampling

A total of 15 night surveys were performed during my study period (April 2013-February 2014). Within this period a total of 9 adult frogs were encountered during the summer months (November 2013 - January 2014) of the study period and examined for chytridiomycosis disease. However, no clear cuticular chytridiomycosis (i.e. epidermal sloughing and ulceration), or abnormal posture (Daszak *et al.* 1999) could be identified in any of these individuals.

Table 3.5. Abundance of *Hadromophryne natalensis* tadpoles and the altitude of each study site as site 1 and site 2 (in bold) represent the permanent study sites. Sites are arranged according to altitude from highest to lowest.

Site	Altitude	Number of tadpoles caught	Number of photographed individuals per site	Number of diseased individuals (determined from photographed individuals)	Percentage (%) of diseased individuals
Site 9	1528-1585	3	2	1	50
Site 8	1513-1598	1	1	0	0
Site 13	1508-1534	3	1	0	0
Site 12	1499-1569	10	10	4	40
Site 10	1491-1677	12	12	0	0
Site 1	**1352**	**186**	**43**	**18**	**42**
Site 2	**1292**	**187**	**23**	**5**	**22**
Site 14	999-1011	24	19	1	5
Site 11	891-1005	3	3	3	100
Site 7	890-884	1	1	0	0

4 DISCUSSION

A recent study to examine the prevalence of *Bd* in South Africa's threatened frog species discovered that the *Bd* incidence was highest in Kwa-Zulu Natal (79 of 348 individuals [=22%] testing positive for *Bd*) and the Western Cape (200 of 616 individuals [=32%] testing positive for *Bd*), while Limpopo revealed a very low prevalence of *Bd* (12 of 219 individuals [=5%] testing positive for *Bd*) (Tarrant *et al.* 2013). With 32 of 114 photographed tadpoles [=28%] at Mariepskop showing visible signs of the disease, our study indicates that the area with a high incidence (encountered further south in the Western Cape and KwaZulu-Natal lowlands, by Tarrant *et al.* 2013) extends much further northwards than reported to date.

The local spatial distribution of chytridiomycosis

A Chi-squared test ($p>0.05$) revealed no significant between-stream differences in the incidence of chytridiomycosis in cascade frog (*H. natalensis*) tadpoles. This was also reflected in the comparison which revealed no significant ($p>0.05$) difference in disease prevalence between our two long term study sites. We deduce that chytridiomycosis is widespread among all streams and most sample locations at Mariepskop, with no effect of altitude and no difference in prevalence across different streams. This probably reflects the general environmental requirements of the *Bd* fungus, with frogs only being an incidental part of the much wider ecosystem-wide distribution of the fungus (Powell 1993).

The effect of tadpole size and lifestage on the incidence of chytridiomycosis

There was a strong positive correlation between cascade frog tadpole size and the incidence of chytridiomycosis, however, this relationship was not reflected in the analysis of lifestages,

with no significant correlations between lifestage and the prevalence of disease (Table 3.3). No significant relationship was found between altitude and disease incidence (Table 3.3).

Smith *et al.* (2007) found that infected tadpoles of several different species of African amphibians were larger than non-infected individuals. They suggested that this could be explained on three grounds: 1) the disease only manifests at a later stage of development; 2) infections with the chytrid fungus may only be visible after the individual has been exposed numerous times and is only apparent when 'infection has reached a critical magnitude'; 3) infected tadpoles may compensate their reduced ability to feed effectively by a more rapid rate of metamorphosis. An additional explanation why larger tadpoles may be more infected with chytridiomycosis, is that of the skin area of the larger tadpoles. Larger individuals should have a larger oral surface area and therefore a higher likelihood of coming into contact with fungal zoospores, leading to a higher possibility of contracting chytridiomycosis. For the frog *Strongylopus hymenopus*, when tadpoles from comparable Gosner stages were compared, Smith *et al.* (2007) found no size difference, suggesting no effects of *Bd* on tadpole growth rate. However, Smith *et al.* (2007) did not demonstrate the same effect for *H. natalensis*, which they also studied.

Within each lifestage we found no significant difference in the size of diseased *H. natalensis* tadpoles compared to healthy individuals (Table 3.4). This suggests that *Bd* is not affecting the growth and development of infected individuals at Mariepskop. However, despite the non-significant result, our data are consistent with the explanation that larger tadpoles are more susceptible to the disease (Smith *et al.* 2007). Thus, more developed tadpoles are expected to have a greater prevalence of chytridiomycosis than younger tadpoles (Table 3.4).

The importance of long term monitoring of populations

It appears that there are no immediate signs that the *H. natalensis* population at Mariepskop is under threat but this does not mean that the population is not declining, as we have no available data prior to the chytrid infection of the population. Superficially, however, the tadpole population of *H. natalensis* at Mariepskop, appears to be healthy. Firstly, breeding is occurring as numerous pre-metamorphic individuals were found and post-metamorphic individuals were encountered during the summer months. Secondly, *Bd* does not seem to affect the growth and development of infected tadpole individuals. Currently, the pre-metamorphic and post-metamorphic population appears to be coping with the disease at Mariepskop, as there were no visible signs of disease in the *H. natalensis* adult lifestage and they appear to be breeding successfully, however, we cannot make unequivocal assumptions about the state of the post-metamorphic population at Mariepskop due to a low sample size.

Additionally, our results indicate that we caught far more individuals of lifestage three when compared to lifestage four (Table 3.4). This ratio could possibly reflect natural mortality of tadpoles at later lifestages due to predation, or signify that the larger tadpoles are harder to catch whilst sampling, as they may be able to swim faster than smaller tadpoles. Overall, our observations are encouraging in the sense that strong population level consequences of the disease (Ouellet *et al*. 2005) were not observed. However, only systematic monitoring can reveal the longer term effects of the fungus on cascade frogs and the true status of the *H. natalensis* populations at Mariepskop can only be ascertained after longer term monitoring of population size and breeding success, especially across all lifestages. This would require monitoring protocols that are repeatable and rapid. An appropriate methodology might be to use permanent transects along streams to obtain a) an indication of population size (e.g. no. of tadpoles / count), b) monitor disease prevalence and correlate this with adult tadpole and frog abundance. This could be done by sampling a 100 m

transect of stream using the dip-netting technique, followed by measuring and counting of individual tadpoles as well as photographing the mouthparts of all tadpoles for the presence of disease. A more time consuming activity is night observations (e.g. numerous night samples per trip) to determine what effect chytridiomycosis has on post-metamorphic individuals. We suggest that these methods be used regularly over several years at permanent sites that are easily accessible by road. An additional method to aid in long term monitoring may be to use laboratory studies to examine the survivorship of individuals (infected with chytridiomycosis and not infected with chytridiomycosis) at different lifestages in order to determine how proficient each lifestage of *H. natalensis* is with coping with disease as well as how susceptible *H. natalensis* is to disease. These results may help us obtain a more detailed understanding of how this species is coping with disease.

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CHAPTER 4

CONCLUSION

One of the major findings of this study is the apparent winter breeding period of *Hadromophryne natalensis* at Mariepskop, which opposes all previous studies done on this species (Boycott 2004, Rivers-Moore & Karssing 2014). Furthermore, breeding in this species did not appear to be triggered by rainfall events, but rather occurs when water levels are low.

As *H. natalensis* is a forest specialist with a significant affinity for vegetation around streams (i.e. forest habitat), it is clear that this habitat is of particular significance to this species and should be conserved. Additionally, structural habitat characteristics which offered a degree of cover (loose rock and sticks), had a significant positive effect on the abundance of *H. natalensis* and could be an important habitat determinant in this species. Tadpoles are abundant in all streams at Mariepskop that have sufficient cover, a fast water flow and have an absence of predators.

The *H. natalensis* population at Mariepskop, are able to survive at low and high altitudes (Wager 1965), as well as at warmer temperatures (Rivers-Moore & Karssing 2014) within forested environments. Climate change is unlikely to have a direct effect on this species with regards to changes in water temperature. Although there appears to be no direct effects of increased temperature through climate change on *H. natalensis*, indirect effects of changing temperatures and precipitation could result in forest distribution shifts which could potentially result in range extensions of this species. Conversely, if climate change causes the forest range to shrink, *H. natalensis* distribution in South Africa is likely to become more concentrated and threatened.

Overall, *H. natalensis* at Mariepskop appears to be breeding successfully and is under no immediate threat, even though chytridiomycosis disease was detected in the population. Superficially, the population of cascade frogs at Mariepskop appears to be coping well with the chytridiomycosis infection, as it is apparent that although the pre-metamorphic population was infected with disease, the post-metamorphic population showed no visible signs of disease. However, we cannot make unequivocal assumptions about the state of the post-metamorphic population at Mariepskop due to a low sample size. It is also imperative that we remember that as we have no available data prior to chytridiomycosis being among the population, we cannot make assumptions about whether the population is declining, or what the long terms affects of disease on the population are. This however, highlights the urgency for long term monitoring so that the true population status for *H. natalensis*, across all lifestages, at Mariepskop can be ascertained. It is clear from this study that areas with high chytridiomycosis incidence extends further north that previously thought.

In Australia, chytridiomycosis has caused population declines in 'regionally endemic rain forest specialists with low fecundity that reproduce in streams and live at high altitudes' (Daszak *et al.* 1999). Thus, as our study species fulfils this criteria and chytridiomycosis has been detected in the population it is imperative that a long term monitoring programme be considered to get an idea of the full extent of chytridiomycosis in this area as well as the repercussions of this disease on the population of Natal cascade frogs at Mariepskop.

In conclusion, this study provided valuable information regarding the geographic range of chytridiomycosis (area with high incidence where chytridiomycosis is common and endemic), which we discovered extends much further northwards in South Africa than reported to date. This also highlights the need for long term monitoring to find out what effects chytridiomycosis may be having on this population. Additionally, although the *H. natalensis* appears to be stable at Mariepskop, it is important that we conserve the forested

habitats of this species, as changes in forested habitats brought on by climate change may have extremely detrimental effects on this species.

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