Seasonal variation and the influence of environmental gradients on *Batrachochytrium dendrobatidis* infections in frogs from the Drakensberg Mountains

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"His lightning's enlightened the world: the earth saw, and trembled.
The hills melted like wax at the presence of the Lord, at the presence of the Lord of the whole earth. The heavens declare his righteousness, and all people see his glory."

Psalm 97: 4-6

Thank you to the Lord who gave me the opportunity to do this study in the amazing Drakensberg Mountains that he created. It is there where I realized how big and mighty the Lord is and how small we really are.

I would like to dedicate this dissertation to my loving parents Hennie and Hannelie Meyer, who supported me in ways nobody else could. They are the two most amazing people in my life and I am truly grateful. They mean the world to me and I sincerely respect them and love them very much. You are my world.

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Abstract

The *Batrachochytrium dendrobatidis* fungus has been implicated in the decline of many frog species as well as the extinction of some throughout the world. Apart from this, declines in some amphibian populations are also caused by variations in temperature. It has been proposed that the cause of the decline or apparent extinctions of at least 14 high elevation species of the Australian tropics were due to *B. dendrobatidis.* The main aim of this study was to determine the effect of seasonal variations on *B. dendrobatidis* infections and the influence these have on frog populations in the Drakensberg Mountains in South Africa.

In one part of this study, frog populations from different altitudes in the Royal Natal National Park and Mont-aux Sources in the Drakensberg region were monitored; *Hadromophryne natalensis* from low altitude sites and *Amietia vertebralis* from high altitude sites. *Batrachochytrium dendrobatidis* was detected in the field by using a 10x hand lens and in the laboratory with a compound microscope. No mortality has yet been observed in *H. natalensis*, but *A. vertebralis* is disease-susceptible and die-offs do occur. Most of the mortalities have therefore occurred at high altitudes where temperature levels vary from cold to moderate. This pattern of susceptibility with regard to altitudinal gradient is reflected in case studies from the Australian and American tropics. Although *B. dendrobatidis* is prevalent throughout the year at both high and low altitudes, prevalence levels peak in winter and spring. It is important for conservation strategies of montane amphibian communities to determine whether the observed mortalities constitute evidence of actual declines or whether these can be regarded as part of natural fluctuations in population size. Although no declines have been observed as yet, the chance exists that declines could occur because *A. vertebralis* is susceptible to the pathogen.

Another part of this study was conducted with emphasis on the breeding behaviour of *A. vertebralis* which is a semi-aquatic, high-elevation frog endemic to the Drakensberg Mountains and the Lesotho highlands. This species breeds in slow-flowing streams and associated pools with sandy bottoms. Published data indicates that breeding occurs after the first spring rains in September and continues until March. The objective of this part of the study was to gain insight into the breeding biology of *A. vertebralis* by studying empirical data gained from its tadpoles. Tadpoles were collected on a bimonthly basis over a two-year period for staging and measurement. Casual observations on adults and egg clutches were also documented.

Contrary to what has been documented, amplecting *A. vertebralis* pairs were observed as early as July; however, this could be an indication that they are opportunistic breeders. Tadpoles of different lengths and stages were collected throughout the year, supporting the notion that these frogs have an extended breeding season or that the breeding season is correctly described in the literature, but the development of the tadpoles takes place over an extended period of time.

A preliminary study was conducted on the distribution of *B. dendrobatidis* along an altitudinal transect. Frogs were collected and DNA swabs were taken of each specimen and analysed with qPCR sequencing. Infection was found at every site across the transect except for one. Altitude did not play an influential role in infection levels of this pathogen. Rainfall had a negative correlation with prevalence at some stages when floods occurred, otherwise prevalence increased gradually according to rainfall. Temperature did influence prevalence infections, but a consistent pattern according to correlation with prevalence infections was not observed.

In conclusion, chytrid has a widespread distribution across southern Africa and has no preference to infect only certain species. Most of the species that have been sampled were found to have been infected.

Opsomming

Die *Batrachochytrium dendrobatidis*-swam word beskou as bydraend tot die afname in baie paddaspesies en ook tot die uitsterwing van sommige spesies regoor die wêreld. Afnames ten opsigte van amfibiese populasies word verder ook veroorsaak deur variasies in temperatuur. Daar is al aan die hand gedoen dat *B. dendrobatidis* die oorsaak was van die afname of duidelike uitsterwings van sowat 14 paddaspesies wat by hoë hoogtes voorkom in die Australiese trope. Die doel van hierdie studie was om die effek van seisoenale variasies op *B. dendrobatidis* infeksies te bepaal, tesame met die invloed wat dit het op paddapopulasies in die Drakensberge.

In een deel van die studie is paddapopulasies wat by verskeie hoogtes voorkom in die Royal Natal Nasionale Park en Mount-aux Sources in die Drakensberge gemoniteer. *Hadromophryne natalensis* het by lae hoogtes voorgekom en *Amietia vertebralis* het by hoër hoogtes voorgekom. *Batrachochytrium dendrobatidis* is waargeneem in paddavisse deur gebruik te maak van 'n 10x handlens in die veld en 'n standaard-elektronmikroskoop in die laboratorium. Geen mortaliteite is tot dusver in *H. natalensis* waargeneem nie, maar *A. vertebralis* is baie vatbaar vir die siekte en mortaliteite het wel voorgekom in die spesie. Die meerderheid van hierdie mortaliteite het voorgekom by hoë hoogtes waar temperatuurvlakke gefluktueer het tussen koud en middelmatig. Die patroon ten opsigte van vatbaarheid vir die swam met inagneming van die hoogtegradiënt word in gevallestudies van die Australiese en Amerikaanse trope gereflekteer. Alhoewel *B. dendrobatidis* teenwoordig is regdeur die jaar by beide hoë en lae hoogtes, bereik prevalensie 'n piek tydens die winter en lente. Dit is belangrik vir bewaringstrategieë wat gemoeid is met bergagtige paddapopulasies dat daar bepaal word of die waargenome mortaliteite wel aanduidend is van 'n afname in populasies en of dit bloot deel uitgemaak het van natuurlike fluktuasies in populasiedigthede. Alhoewel geen afnames in populasies al waargeneem is nie, bestaan daar wel 'n kans dat 'n afname kan plaasvind aangesien *A. vertebralis* baie vatbaar is vir die siekte.

Die volgende deel van die studie was begaan met die broeigedrag van *A. vertebralis* wat 'n semi-akwatiese padda is wat by hoë hoogtes voorkom en wat endemies is tot die Drakensberge en die Lesotho-Hoëveld. Dié paddaspesie broei in stadig-vloeiende

strome en poele met sandagtige substrate. Gepubliseerde data dui daarop dat die spesie broei vanaf September tot Maartmaand. Die doel van hierdie deel van die studie was om insig te verkry oor die broeigedrag van dié spesie deur empiriese data van sy paddavisse te bestudeer. Paddavisse is versamel op 'n tweemaandlikse basis oor 'n periode van twee jaar. Daar is klem gelê op paddavisstadiums en die lengtes van die paddavisse. Observasies van volwasse paddas en van eierpakkies is ook aangeteken.

Ten spyte van wat tipiese gedokumenteerde data aandui ten opsigte van die broeigedrag van die spesie, is paddas wat in ampleksus was waargeneem so vroeg as Juliemaand. Hierdie waarnemings kan moontlik aanduidend daarvan kan wees dat hulle opportunistiese broeiers is. Paddavisse van verskeie groottes en stadiums is versamel regdeur die jaar; dit ondersteun die idee dat die spesie heeljaar broei of is aanduidend daarvan dat hulle'n verlengde broeiseisoen het. 'n Ander gedagte wat ook vorendag kom is dat die spesie wel die broeitydperk beset soos bekend vanaf die literatuur, maar dat die paddavisse'n langer ontwikkelingstydperk het.

'n Voorstudie is gedoen ten opsigte van die verspreiding van *B. dendrobatidis* oor 'n hoogtegradiënttransek. Paddas is versamel by verskeie persele op die transek en DNA watte pluis is geneem van elke padda. Die watte pluis is geanaliseer met qPCR-analisering. Infeksie is by elke perseel op die transek gevind, behalwe by een perseel. Hoogte bo seespieël het nie 'n beduidende rol gespeel by die infeksievlakke van die patogeen nie. Reënval het 'n negatiewe korrelasie getoon ten opsigte van prevalensie veral met sekere tye wanneer vloede plaasgevind het. Andersins het prevalensie stelselmatig toegeneem in samehang met reënval. Temperatuur het nie 'n groot invloed op prevalensie-infeksies gehad nie, maar 'n konstante patroon is nie waargeneem in korrelasie met prevalensie nie.

Ten slotte: chytrid het wydverspreid voorgekom in suider-Afrika en het geen voorkeure getoon ten opsigte van watter spesies dit geïnfekteer het nie.

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Chapter 2

This chapter presents the general material and methods for each of the different field surveying techniques and their different objectives. It includes different ways of collecting frogs and field sampling techniques. Furthermore, it describes the study areas, site allocations and provides an overview of the two main focal areas studied in the Drakensberg Mountains.

Chapter 3

In this chapter, data that were collected and analysed regarding the influence of temperature and the effect it has on *B. dendrobatidis* is discussed. The data on the effect of *B. dendrobatidis* on frog populations in the Drakensberg Mountains and across an altitudinal transect from KwaZulu-Natal to the North-West Province is presented in the chapter. Data for the breeding behaviour of *A. vertebralis* are also included.

Chapter 4

In this chapter, a general discussion is offered on all the results and findings of this case study. Included in this chapter are recommendations for future research.

Chapter 5

In this chapter specific possibilities for further research that can be conducted in the mountains on the two frog species and their populations are presented. Suggestions regarding possible improvements in terms of research in this study are also offered.

1 Introduction and Literature Study

1.1 Importance of amphibians

Amphibians were the first group of vertebrates to colonise land approximately 360 million years ago. This invasion of land required remarkable morphological and physiological changes to the amphibian body and constituted perhaps the most dramatic event in animal evolution. Amphibians have since radiated and today they are found on all hospitable continents and on most of the larger islands. They display a far greater diversity and modes of life history than any other vertebrate group. Extant amphibians are divided into three main groups; the order Gymnophiona or Apoda (wormlike, legless amphibians), the Caudata or Urodela (salamanders) and Anura or Salientia (frogs and toads).

Amphibians are integral components of many ecosystems (Burton & Likens, 1975), they serve as prey and as predators in ecosystems (Porter 1972) and the larvae can be important herbivores (Dickman, 1968; Seale, 1980; Morin *et al.,* 1990) as well as prey (Duelman & Trueb, 1986) in aquatic environments. Amphibians are also good environmental indicators because they are in contact with both water and land. The absence of amphibians at aquatic sites is a clear indication of poor environmental quality.

1.2 Amphibian declines

A worldwide decline in amphibians could have an important impact on other animals and the ecosystem in general. Amphibian species have been declining over the years, and a sudden decline in species occurred from the 1970s in the western United States, Peurto Rico and north-eastern Australia (Drost & Fellers, 1996; Burrows *et al.,* 2004; Czechura & Ingram, 1990). There are currently over 6 500 described amphibian species, with 5 787 being Anura, 582 Caudate and 176 Gymnophiona (AmphibiaWeb, 2009). Over 32% of amphibians are listed as globally

endangered and 43% are declining in population size (Frost, 2007); furthermore, almost 200 species are likely to have become extinct since the 1980s (Stuart *et al.,* 2004).

There has been a great deal of debate about the reasons for these declines. Some of these reasons are discussed below:

Global warming, which refers to the increase in the average measured temperature of the earth's near-surface air and oceans since the mid-twentieth century, and its projected continuation. The Intergovernmental Panel on Climate Change (IPCC) concludes that, "most of the observed increase in globally averaged temperatures since the mid-twentieth century are very likely due to the observed increase in anthropogenic (man-made) greenhouse gas concentrations" via an enhanced greenhouse effect. Natural phenomena such as solar variation combined with volcanoes probably had a small warming effect from pre-industrial times until 1950 and a small cooling effect from 1950 onward (Kiesecker *et al.,* 2001; Pounds *et al.,* 2006; Hegerl *et al.,* 2007; Ammann *et al.,* 2007).

The loss of habitat due to deforestation and human interference, which destroyed or fragmented suitable habitats for amphibian species (Alford & Richards, 1999; Blaustein *et al.,* 1994; Hayes & Jennings, 1986; Phillips, 1990; Tyler, 1997; Wyman, 1990).

Chemical pollution has been shown to cause deformities in frog development such as extra limbs or malformed eyes. Some chemicals are also known to have an effect on the central nervous system of frogs. Other pollutants such as atrazine are known to cause a disruption in the production and secretion of hormones (Bridges & Semlitsch, 2000; Phillips, 1990; Sparling *et al.,* 2000; Wyman, 1990).

Acid precipitation has harmful effects on the environment, habitats of animals and on structures. Acid rain is mostly caused by emissions due to human production of sulphur and nitrogen compounds which react in the atmosphere to produce acids (Phillips, 1990; Wyman, 1990).

Increased ultra violet radiation: recent studies by Blaustein *et al.* (2003) indicated that the depletion of stratospheric ozone and an increase in UV-B radiation reduce the hatching success of embryos and tadpole survival in some amphibian species.

The introduction of exotic species into the wild: non-native predators and competitors have also been found to affect the viability of frogs in their habitats. Introducing non-native fish into habitats for recreational purposes such as fishing can have a detrimental effect on frog populations because the tadpoles and developing frogs fall prey to these fish (Hayes & Jennings, 1986; Phillips, 1990; Tyler, 1997; Wyman, 1990).

Natural population fluctuations, harvesting by humans and diseases also cause declines in amphibian populations (Phillips, 1990; Wyman, 1990). In particular, diseases such as "red leg" disease (*Aeromonas hydrophila*), ranavirus, anuraperkinsus and chytridiomycosis have been related to die-offs of species.

1.3 The amphibian chytrid *Batrachochytrium dendrobatidis*

Batrachochytrium dendrobatidis was detected in dead and dying anurans in 1998 (Longcore *et al.,* 1999) and since then research has shown that the fungus is widespread, occurring over five continents: North and South America, Australia, Europe and Africa (Longcore *et al.,* 1999; Berger *et al.*, 1999b; Lips, 1999; Mutschmann *et al.,* 2000; Bosch *et al.,* 2001; Fellers *et al.,* 2001; Speare *et al.,* 2000; Bradley *et al.,* 2002; Weldon *et al.,* 2004.). The earliest global record was found in *Xenopus laevis* from South Africa that was collected in 1938 (Weldon *et al.*, 2004). This formed part of a historical survey that was conducted on 697 archived specimens of 3 species of *Xenopus* collected from 1879 to 1999 in southern Africa. *Batrachochytrium dendrobatidis* is a fungal pathogen that has started to cause observable declines since the 1970s and has been implicated in the decline and extinction of many frog species in the world; it was described in both wild (Berger *et al.,* 1998; Bosch *et al.,* 2001) and captive amphibians (Pessier *et al.,* 1999; Mutschmann *et al.,* 2000) as a cutaneous disease, chytridiomycosis (Pessier *et al.,* 1999). Chytrid fungi (Chytridiomycota) constitute a large and diverse group and have been found in many different types of environments, including rainforests, deserts and arctic tundra (Powell, 1993). They are mostly found in water where they degrade chitin from dead insects, cellulose from vegetable matter, and pollen and keratin from hair and skin. They play an important role in the ecosystem as biodegraders (Barr, 1990).

1.3.1 Life cycle of *Batrachochytrium dendrobatidis*

Batrachochytrium dendrobatidis has a life cycle that progresses from zoospore to a growing thallus, which produces a single zoosporangium (sporangium) that in turn produces zoospores. The zoospores exit the sporangium through one or more discharge papillae. Although sexual

reproduction has not been observed, it is known that colony development results from more than one sporangium that develops from one zoospore (asexual amplification) (Berger *et al.,* 2005). According to Longcore *et al.* (1999), the formation of more than one sporangium from one zoospore is the only known variation of the cycle.

Zoospores are discharged through an inoperculate opening in both colonial or monocentric thalli (Longcore *et al.,* 1999). Sporangia infect the cells in the stratum granulosum and the stratum corneum that are situated in the superficial epidermis in amphibians. Immature sporangia usually occur inside the deeper cells that are more viable, while the mature and empty zoosporangia occur in the outermost layers that are keratinised. Zoospores are released into the environment by discharge tubes that are projected towards the skin surface to facilitate the release of zoospores. When the sporangia infect amphibians they form clusters where the zoospores become encysted. Clustering may either be caused by zoospores that are attracted to foci of infection, or the zoospores infect the surrounding cells in the skin because they have a limited time of mobility that facilitates dispersal before they encyst (Piotrowski *et al.,* 2004). According to Weldon and Du Preez (2006), sporangia aggregate in clusters in the cell as a result of the colonisation strategy of the organism. It is said that the colonies have a tendency to expand concentrically from the point that was originally infected and this, in turn, results in a core of hyperkeratotic tissue devoid of sporangia. When *B. dendrobatidis* is grown on agar, a similar pattern can be observed. Zoospores encyst themselves on the edges of already existing colonies and, in turn, the colony expands as a result of this behaviour (Weldon & Du Preez, 2006). Some zoospores can be released into intercellular spaces, preventing them from escaping the infected area and forming new clusters (Berger *et al.,* 2005). Sporangia in infected tadpoles are distributed among the keratinised areas and as the tadpoles develop, the distribution disperses among the keratinised parts (Marantelli *et al.,* 2004). The distribution of sporangia in adult frogs is an indication that a multilayered, keratinised epidermis must be present for *B. dendrobatidis* to occur as a parasite (Berger *et al.,* 1998; Marantelli *et al.,* 2004). Infection can spread from tadpoles to metamorphs and adult frogs (Rachowicz & Vredenburg, 2004).

Two hypotheses have been proposed with a view to explaining how it could be possible for a fungus that is restricted to the superficial epidermal layer to kill its amphibian host. The first hypothesis proposes that *B. dendrobatidis* releases proteolytic enzymes or other compounds that break down proteins and these are taken up by the permeable skin of frogs. The second hypothesis is that the skin is damaged and its functions related to respiration and water or electrolyte balance are disturbed, which ultimately results in death (Berger *et al.,* 1998; Pessier *et al.,* 1999).

Because this fungus is situated in the epidermal layer, a loss of infection can occur when frogs shed their skin at high temperatures (Berger *et al.,* 2004; McDonald *et al.,* 2005; Weldon & Du Preez, 2006). High temperatures (i.e. >25°C) increase the rate of epidermal turnover and reduce the growth of the chytrid (Piotrowski *et al.,* 2004). According to Berger *et al.* (2005), this could be because the fungus does not have sufficient time to complete its life cycle before the frog sheds its skin.

1.3.2 Morphology

Batrachochytrium dendrobatidis can be identified by the presence of spheroid, walled, and sometimes septate sporangia that typically occur in clusters (figure 1). The fungus was originally isolated from the blue poison dart frog (*Dendrobates auratus)* and described by Longcore *et al.* (1999). Chytridiomycota are characterised by the presence of chitin in the cell wall and the production of motile zoospores (3-5 µm diameters) with a single posterior directed flagellum which develops into stationary sporangia (Longcore *et al.,* 1999). These sporangia form discharge papillae through which the zoospores are released (Berger *et al.*, 1999a). The zoospores of *B. dendrobatidis* are waterborne and they can live for up to 24 hours; they are infective to both amphibian larvae and adults. The zoospores do not have a cell wall and they require water for dispersal; furthermore, they can only swim short distances - less than 2 cm (Piotrowski *et al.,* 2004). This suggests that the zoospores are unable to swim long distances in search of hosts. Piotrowski *et al.* (2004) also suggest that this could be the explanation for the clustering of chytrid sporangia on the skin and mouthparts of amphibians.

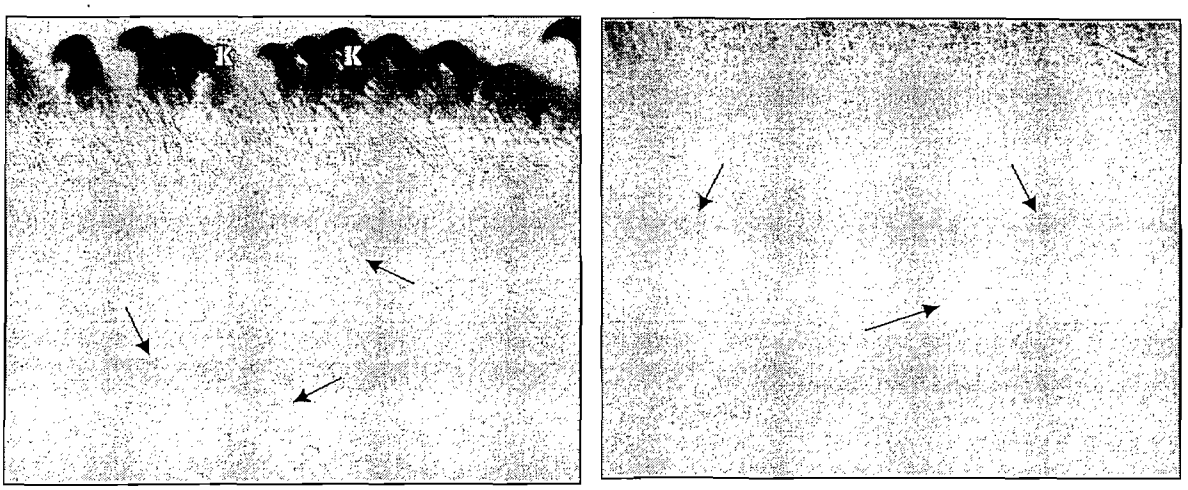

Figure 1: *Batrachochytrium dendrobatidis* infection between the keratodonts (K) of an *Amietia vertebralis* tadpole. The arrows indicate clusters of spherical sporangia of *Batrachochytrium dendrobatidis.*

1.4 Chytridiomycosis and amphibian declines

Chytridiomycosis has been implicated as the cause of amphibian deaths and some population declines (Berger *et al.,* 1998; Lips, 1999; Bosch *et al.,* 2000; Bradley *et al.,* 2002). There is epidemiological, pathological, and experimental evidence that some amphibian populations suddenly declined due to mass mortalities caused by chytridiomycosis (Berger *et al.,* 1999a). The disease has been connected with amphibian declines across the world: North America, Central America, Australia, Europe and Africa (Berger *et al.,* 1999b; Carey *et al.,* 1999; Bosch *et al.,* 2001; Lips *et al.,* 2003, Weldon & Du Preez, 2004). It has been proposed that in Queensland, Australia, the cause of the decline or apparent extinctions of at least 14 high elevation species of rainforest frogs was *B. dendrobatidis* (Retallick *et al.,* 2004). Although *B. dendrobatidis* has a broad amphibian host range and is currently widespread among many species, not all of the species that are susceptible have declined. The selectivity of the declines may be due to a combination of environmental factors and host biology that provide the necessary conditions for expression of the disease, as well as making species less able to recover after populations have declined dramatically. Declining species from high altitude rainforests have restricted geographical ranges and smaller clutch sizes, and their larvae are associated with streams - the adults inhabit streams (Williams & Hero, 1998; McDonald & Alford, 1999). This can be an indication that the disease responsible for these declines is waterborne (Kriger & Hero, 2008). A seasonal peak of infection was found during the cooler months by Retallick *et al.* (2004). This fungus infects two of the three amphibian orders (Anura and Caudata). Chytridiomycosis is one of the few causes, together with other factors, that caused extinction of about 200 species in 14 families since the 1980s (Stuart *et al.,* 2004).

1.4.1 Pathogenesis

This amphibian chytrid fungus is known to cause widespread infection of the skin. Some of the lesions that chytridiomycosis cause in tadpoles include structural damage to the keratinised mouthparts and depigmentation of the keratodonts and rostrodonts. The depigmentation is most common in the upper keratodonts of the tadpole's mouth. According to Berger *et al.* (1999b), *B. dendrobatidis* infects the keratinised cutaneous epithelium of adult amphibians and the keratinised mouthparts of tadpoles. Smith *et al.* (2006) found that infected tadpoles may suffer reduced growth and developmental rates as a result of oral chytridiomycosis and that these possibly affect adult fitness. Infected tadpoles can also serve as reservoirs for *B. dendrobatidis* capable of transmitting the disease to post-metamorphic individuals with consequent disease outbreaks (Rachowicz &

Vredenburg, 2004). The damage to mouthparts in infected tadpoles can result in decreased feeding efficiency (Parris & Cornelius, 2004). Thus, impaired growth due to oral chytridiomycosis may result from reduced feeding. Severely diseased frogs have slowed reactions and show strange behavioural changes such as abnormal sitting posture with back legs pulled into the sides of the body (Speare, 1994), lethargy and slow response to tactile stimuli (Berger *et al.*, 1999a).

1.4.2 Epidemiology

There are two hypotheses that can be proposed in order to explain the outbreaks of *B. dendrobatidis* infections. The first is that *B. dendrobatidis* may be endemic to the regions that are infected and, because of changes in the environment, have become more chytrid virulent. The second hypothesis is that *B. dendrobatidis* could have been introduced to these infected regions recently and is infecting novel hosts (Berger *et al.,* 1999a). Individual frogs contract the disease when their skin comes into contact with water or substrates that contain zoospores from infected animals or from direct contact with infected frogs. International dissemination is most likely due to human-assisted translocation of infected amphibians through the pet trade (Aplin & Kirkpatrick, 1999), scientific trade (Parker *et al.,* 2002), and food trade (Mazzoni *et al.,* 2003) or through contaminated water (Rowley & Alford, 2007).

Other declines in amphibian populations have been correlated with climate events. Spear *et al.* (2004) found a seasonal peak of infection from 1993 to 2000 in the cooler months with no interannual variation with 53% of cases in a number of wild frog species in Queensland and New South Wales. They also found that the rate of infection was higher during winter and spring than during summer and autumn. Annual variation in rainfall can have an effect on the number of eggs that are laid in a given year and the amount and timing of precipitation can affect the yearly reproductive output of an amphibian population (Carey & Alexander, 2003).

Pounds *et al.* (2006) proposed that global warming is the cause of temperatures rising at many highland localities and that these temperatures are shifting towards the growth optimum of *B. dendrobatidis* and thus encourage outbreaks of chytridiomycosis. Longcore *et al.* (1999) found the optimal growth temperature for *B. dendrobatidis* to be 23°C, but the fungus also grows well at lower temperatures. The minimum and maximum temperature ranges are 4°C and 29°C. Berger *et al.* (2004) conducted a transmission experiment at different temperatures and confirmed that temperature is an important factor in the pathogenesis of chytridiomycosis. Their results showed that more frogs died at 17°C and 23°C as compared with 27°C. Furthermore, sporangia and

zoospores do not survive desiccation (Berger *et al.,* 2004). At 37°C, 100% mortality of the pathogen occurs within four hours (Johnson *et al.,* 2003).

It has become clear that infectious disease is one of several causes of amphibian declines (Carey & Alexander, 2003). There are many possibilities for the indirect effects of climate change. Analyses of temperature and moisture anomalies for four different areas in which amphibian mass mortalities have been attributed to *Batrachochytrium* (north-eastern Queensland, Puerto Rico, the central Colorado Rockies, Costa Rica and Panama) showed that no extreme climate events occurred prior to the onset of mass mortality (Alexander & Eischeid, 2001). Also, no similarities in weather patterns were evident among the four sites at the onset of the mortalities. The study thus showed that climate was not an indirect cause of the outbreak of the disease. More research must therefore be conducted on climate change and amphibian declines and more information is also needed on the role that climate change plays on the reproductive rate of amphibians and on the incidence of infectious diseases.

According to Young *et al.* (2001), most amphibian declines in Central America occurred above 500 m in elevation. Pounds *et al.* (2006) hypothesised that shifts in temperature at a micro-scale – (both increases and decreases in temperature) that result from a large-scale warming and cloud cover that changes frequently - can favour the development of chytridiomycosis. Scherer *et al.* (2005) found that xeric conditions could cause reduced survival rates in toads, because toads are more prone to dehydration or inhibited gas exchange than treefrogs. Pounds *et al.* (1999) suggest that reduced survival rates of toads during xeric conditions could be caused by the aggregation of infected toads in pools with little water in them and this, in turn, increases the transmission of the chytrid fungus. Xeric conditions could also have a reduced effect on the transmission of the chytrid fungus because of the inhibition of the chytrid zoospores (Daszak *et al.,* 1999). According to Collins *et al.* (2003), thermal and hydric environments that cycle together may impact the growth of chytridiomycosis. When *Litoria chloris* were experimentally exposed to continuous mist, the disease developed faster than in animals that were exposed to rain or dry air with access to water (Collins *et al.,* 2003). Chytrid fungus virulence could be affected by air temperatures during the growing season (Berger *et al.,* 2004) and higher air temperatures may inhibit the chytrid fungus growth (Longcore *et al.,* 1999) and the impact of the fungus on amphibian hosts could be reduced in this manner.

1.5 Frogs of the Drakensberg Mountains

The Drakensberg is the highest mountain range in southern Africa, rising to 3 482 m in height. The range is located in the eastern part of South Africa, running for some 1 000 km from south-west to north-east. The mountains are drained on the western slopes by the Orange and Vaal Rivers, and on the east and south by a number of smaller rivers, the Tugela being the largest of these. The range separates KwaZulu-Natal Province from the Free State Province, lurking over the nearby coast of Natal. The fungal pathogen of amphibians (*Batrachochytrium dendrobatidis*) is widespread throughout southern Africa; however, *B. dendrobatidis* is not known to have caused population declines and is only rarely associated with amphibian mortality. Because *B. dendrobatidis* is associated with mortality events that are rare in southern Africa, my study aims to identify the influences that environmental fluctuations have on *B. dendrobatidis* and the effect it has on amphibians. The following amphibian species occur in the KwaZulu-Natal Drakensberg Mountian Region: *Xenopus laevis*, Semnodactylus wealii, Kassina senegalensis*, Amietophrynus rangeri, Amietophrynus gutturalis, Strongylopus fasciatus*, Amietia angolensis*, Amietia fuscigula*, Amietia dracomontana*, Amietia umbraculata*, Strongylopus grayii*, Cacosternum boettgeri*, Phrynobatrachus natalensis, Hadromophryne natalensis* and Amietia vertebralis** (Lambiris, 1988). Most of these species occurring in this region were found to be infected with the amphibian chytrid (*). Mortalities were found in one species occurring at a high altitude (*A. vertebralis*).

1.6 Study objectives

Objective 1: To determine the influence of seasonal and environmental fluctuations on infection rate of *Batrachochytrium dendrobatidis* and mortality rate among frogs of the Drakensberg Mountains.

This case study will focus on two species that occur in Royal Natal National Park, namely *Hadromophryne natalensis* (Natal Cascade Frog) from low altitude sites and *Amietia vertebralis* (Phofung River Frog) from high altitude sites. The distribution of the two species did not overlap and none of the species were present at the same sites. By monitoring disease levels over a full season, we will be able to determine the effect of environmental fluctuations (temperature and altitude) on disease prevalence. This is the main focus of this study.

Objective 2: To study the correlation between tadpole size and infection.

In this objective, the correlation between tadpole size and the infection of the tadpoles will be determined. The tadpole size will be determined by making use of the Gosner stages (1960).

Objective 3: To study the breeding behaviour of *Amietia vertebralis*.

The breeding behaviour and breeding pattern of *Amietia vertebralis* will be studied to determine the exact months in which breeding takes place and the duration of the breeding season.

Objective 4: To compare the *Batrachochytrium dendrobatidis* infection between two species; one high altitude and one low altitude species.

Two frog species, *Amietia vertebralis* (high elevation) and *Hadromophryne natalensis* (low elevation), will be monitored on a bimonthly period. The difference in prevalence between the two species will be evaluated and compared to determine the effect of prevalence on two different species at different altitudes.

Objective 5: To determine the environmental niche of *Batrachochytrium dendrobatidis* along an altitudinal transect.

This objective sets out to determine the occurrence of *B. dendrobatidis* across an environmental gradient that includes altitude, rainfall and temperature. All frog species will be screened at selected sites along a 700 km transect stretching from Vernon Crookes Nature Reserve on the east coast to Swartruggens in the North-West Province of South Africa. This is only a preliminary study and will be monitored every year to determine a pattern of chytrid distribution.

2 General Material and Methods

2.1 Study area

2.1.1 Lesotho and the Drakensberg Mountains

The study was conducted in the foothills and on top of the Drakensberg Mountains of Lesotho and KwaZulu-Natal (Figure 2). The country of Lesotho is very small and is situated inside South Africa. Lesotho is situated entirely above 1000 m in elevation and is the only country in the world to be located above this elevation. Lesotho covers an area of 30 355 km^2 and comprises of a lot of different terrain such as high plateaus, low hills and mountains such as the Drakensberg Mountains. The highest mountains in Lesotho is the Thabana Ntlenyana with a height of 3 482 m in elevation. The lowest point of Lesotho is 1 400 m in elevation and is situated in the junction of the Orange and the Makhaleng Rivers. Lesotho is situated between latitudes 28°34' and 30°31'S and longitudes 27°00' and 29°28'E.

The Drakensberg Mountains is an amazing mountain range that runs From the Free State into KwaZulu-Natal. The mountain range stretches across KwaZulu-Natal for about 1 000km and is orientated in a south-west to north-east direction. The border of Lesotho is also part of the Drakensberg Mountains that is situated at the north-eastern bend of the mountain range. Some of the big rivers such as the Orange and Vaal Rivers flow on the mountains in a western direction. There are also some smaller rivers that flow in an eastern direction that conduit the mountain. The Tugela River is also situated on the mountain and the Tugela waterfall is the highest waterfall in South Africa with a drop of 946m across the Amphitheatre.

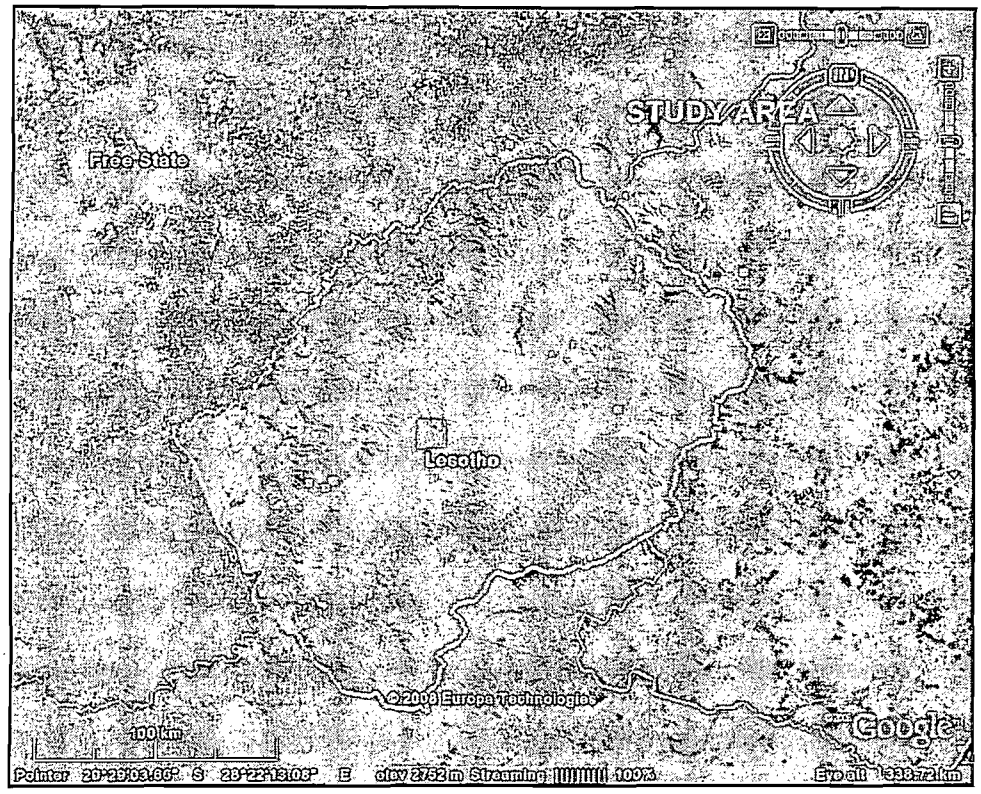

Figure 2: Map of Lesotho and the surrounding Drakensberg Mountains showing the location of the study area (Modified from Google maps).

2.1.2 Climate

The Drakensberg Mountain range is characterised by cold, dry winters and hot, humid summers with a unique combination of weather patterns. The weather can fluctuate very rapidly throughout the day (Suchet, 2006). Snow falls regularly in the winter months, when the rivers may be frozen and streams hardly flowing (Figure 3). Rain and mist can occur through the entire year with frequent thunderstorms at high altitudes. Temperatures can rise to 30°C in the valleys of the mountains during the summer months and as low as -11°C during the cold winter months; the average annual temperature for the area is 5.8°C (Nel and Sumner, 2008). The distribution of rainfall across South Africa reduces westwards from the escarpment all along the interior of South Africa. Due to irregularities in the topography between the escarpment and the ocean, there is an increase of density of rainfall in the eastern and southern coast. When looked at satellite imaging, the Drakensberg Mountains runs almost parallel to the coast for most parts of the eastern side of

the country and this causes rainfall to increase in the eastern part of South Africa. Southern Africa receives about 300 mm of rain per annum in 35% of the country and this is caused by the presence of subtropical high pressure cells which holds back rainfall production mostly because of subsiding air (Nel and Sumner, 2008).

The mean annual precipitation for Lesotho is 725 mm and this rainfall occurs mainly during the spring and summer months (October to April). Hail storms and ghastly thunderstorms with strong gale force winds are common in the mountains. Lesotho experiences some of the highest occurrences of thunder and lightning in the world, with approximately 5-12 strikes per km^2 per year. Because of the high rate of lightning, a great deal of lightning-induced fires occurs, and these have a bearing on the habitats of amphibians and habitat destruction. During the winter months precipitation occurs in the form of snow, but it has been known to snow during the summer as well. Mean precipitation during the winter has been recorded at 10-15 mm (Nel and Sumner, 2008; Nel and Sumner, 2007).

Figure 3: The Tugela river on top of the Drakensberg Mountains freezes in winter.

Weather stations are situated at Royal Natal National Park for the Royal Natal data and at Sentinel peak for the Mont-aux-Sources data. The rainfall data for the duration of the sampling period was acquired from the Weather Bureau of South Africa.

2.1.3 Site allocations for the Drakensberg Mountains

Twenty-eight sites were selected and monitored on a bi-monthly basis. Eighteen sites were located at a mid altitude of 1 800-2 000 m and ten sites were located at a high altitude of 3 000-3 400 m (Figure 4). Different species assemblages occur at these two different altitudes; *Hadromophryne natalensis* was selected for monitoring at the mid altitude and *Amietia vertebralis* at the high altitude.

The following high altitude sites were selected:

Tugela	28.74994 S	28.88302 E
Bilanjil	28.76051 S	28.89949 E
Ribbon Falls Lower	28.76268 S	28.91794 E
Ribbon Falls Middle	28.76398 S	28.91790 E
Ribbon Falls Upper	28.76614 S	28.91761 E
Thukelahed	28.75856 S	28.87812 E
Khubnam	28.76435 S	28.86215 E
Nampolice	28.75558 S	28.86734 E
Vemvame Upper	28.74913 S	28.87186 E
Vemvame Lower	28.74851 S	28.87581 E

The following low altitude sites were selected:

The Gully	28.74684 S	28.91279 E
Devilstooth	28.74613 S	28.91364 E
Sentinal Gully	28.74218 S	28.91424 E
Tugela 1	28.74185 S	28.91460 E
Butterfly	28.73437 S	28.91403 E
Junction	28.71347 S	28.93356 E
Mahai Upper	28.69039 S	28.91098 E
Mahai Lower	28.68644 S	28.92853 E
Gudu Upper	28.67674 S	28.92808 E
Gudu Lower	28.68286 S	28.92960 E
Golide Upper	28.66247 S	28.93648 E

✦ Golide Lower	28.69069 S	28.94523 E
✦ Zigubudu	28.65609 S	28.95610 E
✦ Gezana	28.66874 S	28.95230 E
✦ Sunday Falls	28.40151 S	28.57108 E
✦ Vemvaan Upper	28.72230 S	28.91598 E
✦ Vemvaan Lower	28.71986 S	28.92375 E
✦ Devilshoek	28.71116 S	28.92181 E

The high elevation sites are situated along the escarpment of the Amphitheatre and into Lesotho, while the low elevation sites are situated east of the Amphitheatre. These sites are spread out over a distance of ± 115 km and the sampling was conducted over five days time by hiking to each site. Sampling was difficult at times due to the harsh weather conditions. During winter the surface of the iced-up rivers had to be broken to collect tadpoles of *A. vertebralis*, and this was obviously very time-consuming. Rain also made sampling difficult, especially detection of *H. natalensis* tadpoles, because of rivers flowing very strongly and running very deeply during the rainy season.

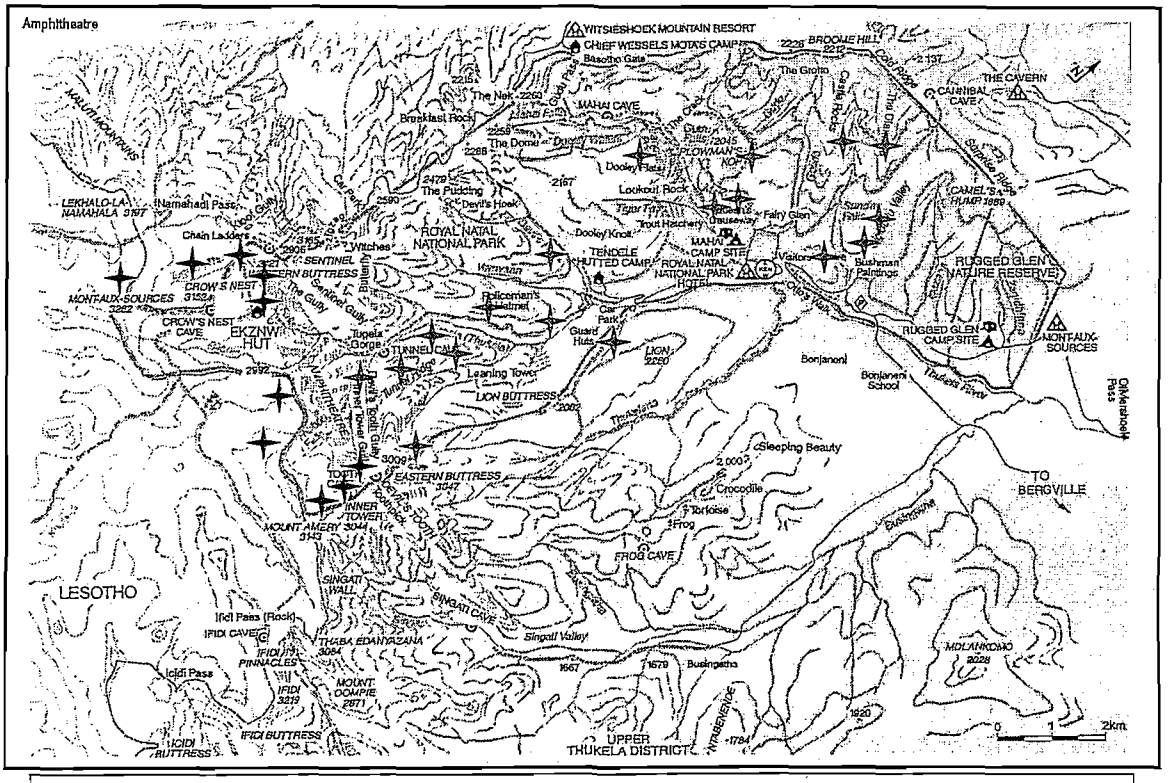

Figure 4: Map of the Royal Natal National Park. Blue stars indicate study sites above 3 000 m and the red stars indicate study sites below 2 000 m (Map acquired and modified from Bristow, 2007).

The sites in the Mont-aux Sources area were situated in open grasslands in full sunlight, with no trees or shrubs providing shade to cover the streams (Figure 5).

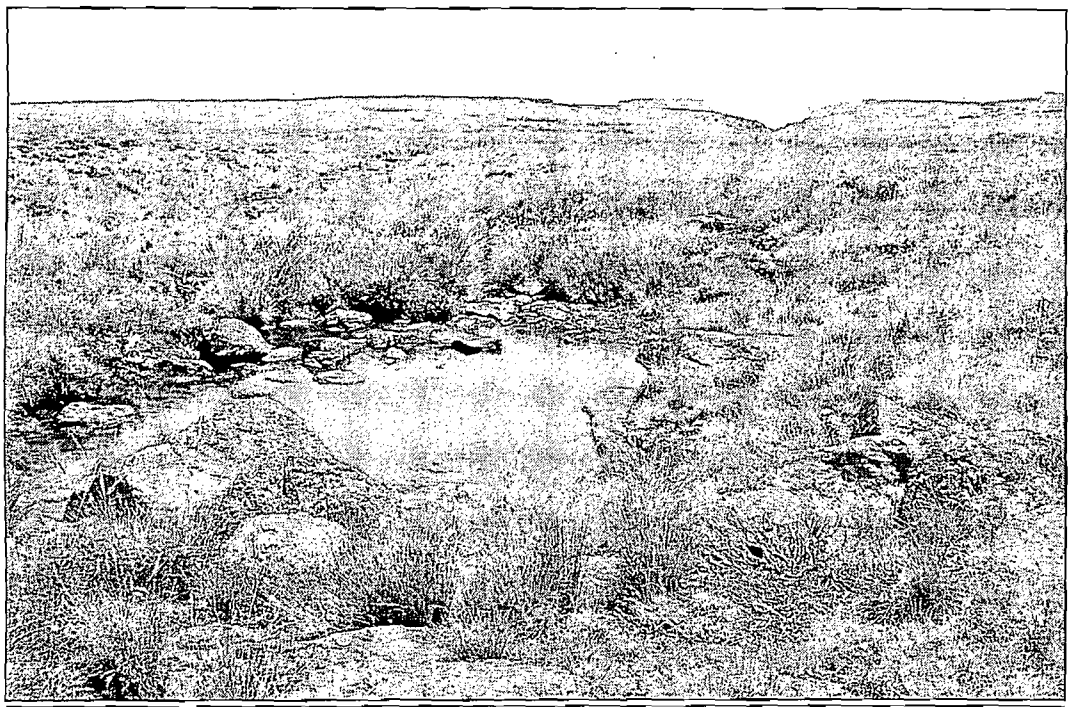

Figure 5: Sites on top of the Drakensberg Mountains at Mont-aux Sources were situated in open grasslands with the sun shining directly on the rivers and ponds. *Amietia vertebralis* were sampled in these streams and ponds.

The sites in the Royal Natal National Park area were completely different from the sites in the Mont-aux Sources area. These sites were situated in patches of forest with large trees and shrubs covering the streams. These sites were situated in the shade with little to no sunlight shining directly on these sites (Figure 6)

Figure 6: Sites in Royal Natal were situated in the shade with little direct sunlight on the river streams and ponds. *Hadromophryne natalensis* were sampled in these streams and ponds.

2.2 Site allocations for the transect

The transect that was sampled stretches over a distance of ±900 km from the KwaZulu-Natal coast, through the Free State Province and into the North-West Province. Ten sites were sampled over different seasons to determine the altitude and niche that *B. dendrobatidis* prefers. The ten sites that were sampled can be seen in Figure 7. These were sampled during October 2007, January 2008, May 2008 and June 2008. The sites were distributed over different provinces with a view to obtain a gradient of different altitudes and climate zones. This also enabled the collection of a larger diversity of frogs. The Vernon Crookes Nature Reserve forest and Vernon Crookes Nature Reserve Loopdam data were pooled together, because these two sites are situated very close together.

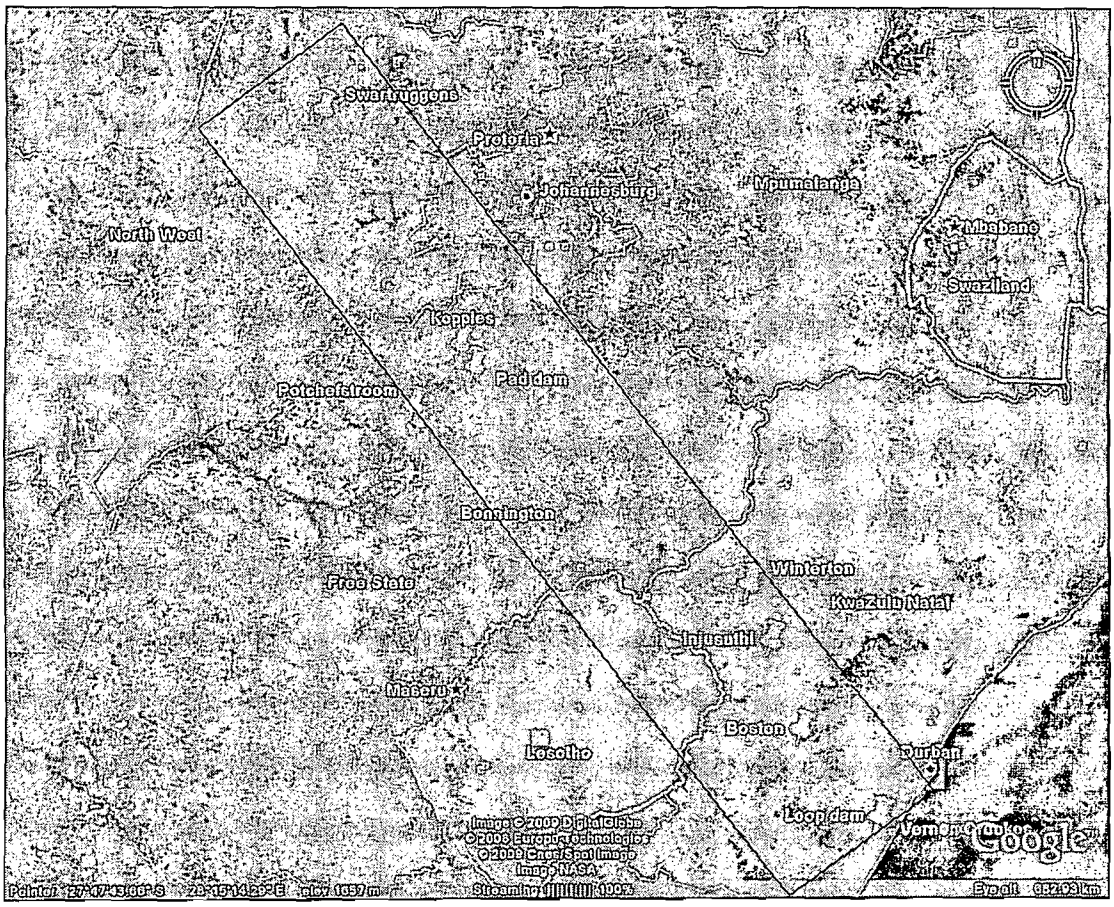

Figure 7: The location of all sites from which frogs were sampled on the transect (modified from Google maps).

The grid references below are for the sites in Figure 7:

Camp site	30.26072 S	30.61093 E
Loopdam	30.16252 S	30.37319 E
Boston	29.70132 S	30.01444 E
Winterton	28.75219 S	29.61961 E
Bonnington	28.29888 S	28.31833 E
Koppies	27.20085 S	27.51591 E
Pad dam	27.34629 S	27.62487 E
Potchefstroom	27.58413 S	27.17053 E
Swartruggens	25.64582 S	26.54173 E

2.3 Species description

2.3.1 Description and distribution of *Amietia vertebralis,* Phofung river frog

Amietia vertebralis (Figure 8) belongs to the anuran family Pyxicephalidae and it occurs in the high altitude streams and rivers of Lesotho and the adjacent Drakensberg Mountains of KwaZulu-Natal and the Free State Province. This species occurs at altitudes between 1 800 – 3 200 m (Bates & Haacke, 2003). This frog is commonly found in streams and rivers that flow eastward into South Africa.

Adults of *A. vertebralis* are medium-sized frogs, reaching sizes of 45-55 mm SVL (Poynton, 1964; Tarrant, 2008). With its compact body shape, this frog is fully aquatic and has a relatively broad head with a rounded snout that gives it a squat appearance. The colour of the dorsal part varies from light to dark brown with dark markings scattered all across the body in a 'V' or 'X' shape (Poynton, 1964; Tarrant, 2008). A number of warts are scattered on the back. An umbraculum is present above the eye that protects it from harsh sunlight and UV light. The tympanum is partially obscured by warts. The underside of this frog is smooth and white with mottling over the gular region. Fairly extensive webbing is present, but two to three toes are without any webbing (Lambiris, 1987).

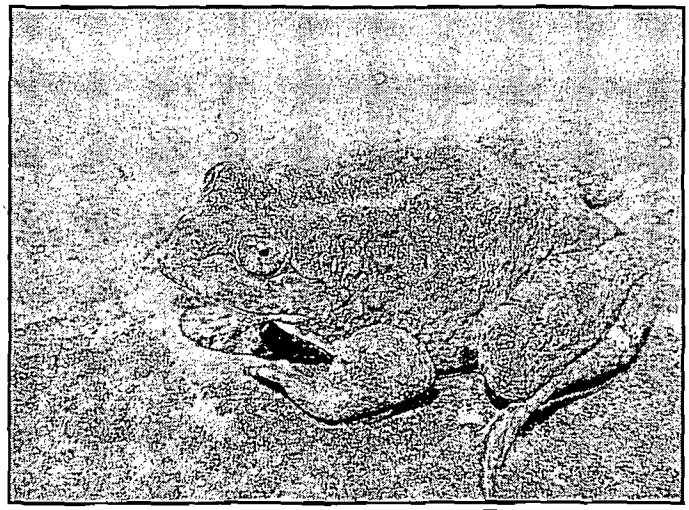

Figure 8: An adult *Amietia vertebralis* (Phofung river frog).

The tadpole of this species reaches a total average length of ±45 mm. They have rounded bodies and the anterior fifth of the fin is somewhat low with the tip of the tail rounded, as can be seen in Figure 9. The body and the tail have a brownish-grey colour with dark blotches and the fins are striped. The underside of the tadpole is white. An umbraculum is also present in the eye of the tadpole. The mouth of the tadpole has a double row of papillae below and three to four rows at the oral angle with a single row at the upper part of the mouth. The jaw sheaths are pigmented to the base and the labial tooth row formula is 3(2-3)/3 or 3(2-3)/3(1-2) (Du Preez & Carruthers, in press).

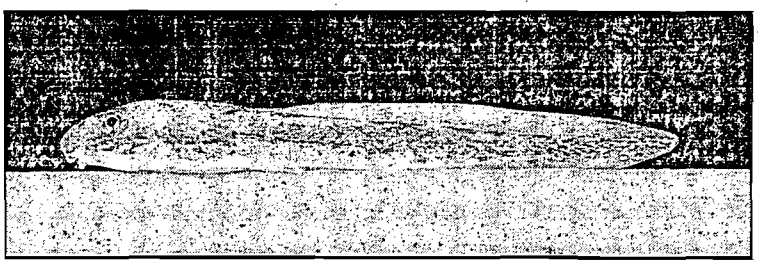

Figure 9: Tadpole of *Amietia vertebralis* with the tip of tail rounded.

2.3.2 Description and distribution of *Hadromophryne natalensis*, Natal Cascade frog

Hadromophryne natalensis (Figure 10) belongs to the anuran family Heleophrynidae and occurs throughout the Drakensberg and Maluti Mountains and also along the escarpment of South Africa, Swaziland and Lesotho. This species occurs at altitudes between 580 – 2700 m above sea level. This frog inhabits clear, swift-flowing streams that run through forests and mountainous areas (Minter *et al.*, 2004).

Adults of *H. natalensis* are medium-sized animals, but larger than *A. vertebralis,* reaching sizes of up to 50 – 60 mm SVL (Hewitt, 1913; van Dijk, 2008). This frog has a dark brown colour with greenish yellow spots on its back. The horizontal line in the eye is less conspicuous than in other frogs of this family - but it has large bulging eyes with a vertical cat-like pupil. The belly is granular and white with off-white patches, sometimes with markings, on its throat. Like ghost frogs, *H. natalensis* has a flattened body and it has small triangular–shaped pads on the tips of its front and back toes (Hewitt, 1913; van Dijk, 2008).

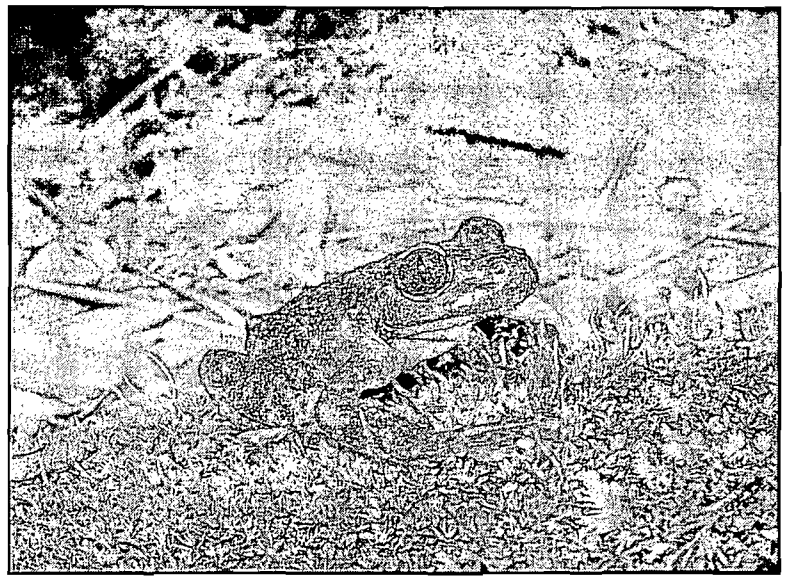

Figure 10: An adult *Hadromophryne natalensis* (Natal Cascade frog). The horizontal stripe in the eye is barely visible.

Tadpoles of this species reach a total average length of ± 80 mm (from nose to the tip of the tail) and have a streamlined body. The tadpoles are very distinct and their colour is sandy brown, sometimes a little mottled, to blend in with the substrate. The tail is muscular and the fin starts in the middle of the tadpole; there is no tailfin in the first third of the tail and it is similarly pigmented - as can be seen in Figure 11. The trunk of this tadpole is flattened and appears trapezoidal in the dorsal view. The eyes are small and the fin arises from behind the trunk with a rounded tip; the tip is sometimes black. The tadpole is semitransparent below and a dark intestine is visible. A suprarostrodont is absent in this species. It has an enormous oral disc with which it attaches to rocks in the fast-flowing mountain streams and the mouth is completely circled by two rows of papillae above and four rows below. The jaw sheaths are pigmented to the base and the labial tooth row formula is 4/14 to 4/17 (Hewitt, 1926).

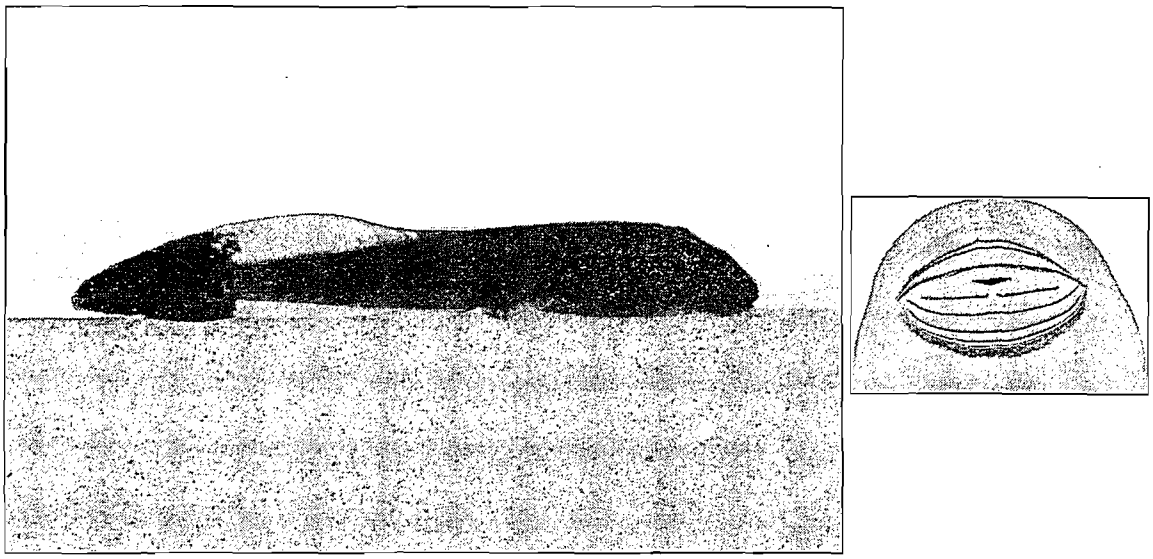

Figure 11: Tadpole of *Hadromophryne natalensis* and the mouthparts with pigmentation markings. The tip of the tail is pigmented.

2.4 Frog sampling techniques

2.4.1 Sampling of *Hadromophryne natalensis* and *Amietia vertebralis*

The collection of *H. natalensis* tadpoles took place at the Royal Natal National Park at 18 different sites by means of dip nets of 10 cm x 10 cm. To catch this species, rocks had to be turned over in the pools and in rapid parts of the streams in order to detect them (Figure 12). A net was held downstream of a rock and the rock turned while sweeping the net underneath the rock. Up to ten tadpoles, where possible, were collected at each site. *H. natalensis* tadpoles were screened in the field and released again. *H. natalensis* tadpoles have large oral discs. The hyperpigmentation that occurs in the oral discs of *H. natalensis* is caused by *Batrachochytrium dendrobatidis*, which makes it possible to screen them in the field (insert in Figure 11).

The collecting of *A. vertebralis* took place at the top of the Drakensberg Mountain at Mont-aux-Sources. There were ten sites at Mont-aux Sources that were monitored on a bi-monthly basis. Up to ten specimens were collected at each site, and the tadpoles were euthanased by immersion in MS222 (Tricaine Methane Sulphonate) and preserved in 70 % ethanol for laboratory examination of *B. dendrobatidis*. To catch this species, dip nets were used to scoop them out of the ponds and rocks were turned over and scooped underneath with the net. Water temperature was taken at each site before the start of sampling.

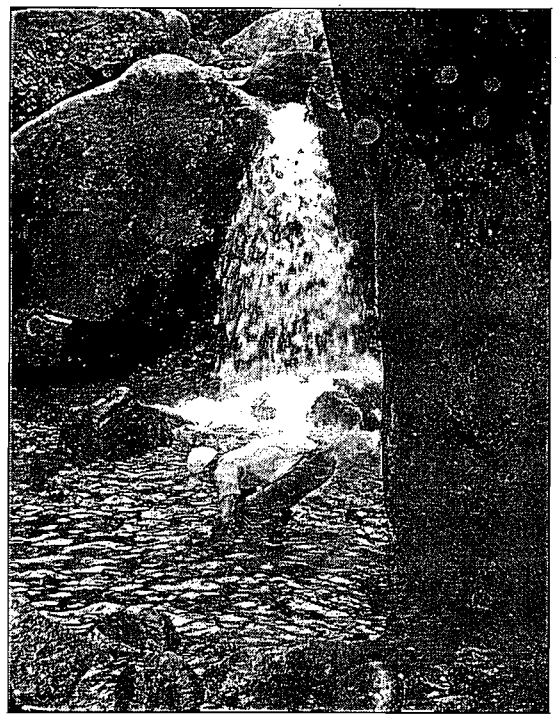

Figure 12: Sampling for *Hadromophryne natalensis* done in Royal Natal by means of using a 10 cm x 10 cm sampling net.

2.4.2 Visual encounter sampling

When making use of visual encounter sampling, field surveying must be conducted at night. This sampling technique was used to detect river frogs, tree frogs, reed frogs and other ground-dwelling frogs that included toads around streams and ponds. The collecting of these frogs was done by hand. Frogs were spotted by means of a flashlight during the evenings at ponds and the area around the ponds in the vegetation where frogs seek shelter and safety from predators. One would listen to the calls of male frogs and walk in the direction of the calls while looking for the males that called, and females that proceeded towards the calls. When frogs were spotted, they were caught and placed into plastic containers. Each species was placed in a separate container. A total of 10 to 20 specimens per species were collected at each site. Not all species were found to be abundant at all sites. DNA swabs were taken of each specimen in the field and the specimens were released at the sites where they were collected. The DNA swabs were sent to the National Zoological Gardens, Pretoria, for qPCR testing.

2.4.3 Dip net sampling

A dip net was used to sample tadpoles in a dam after the site was surveyed for frogs. A dip net with a size 300 x 250 mm opening and a mesh of 2 mm was used to sweep through the aquatic vegetation near the banks of the water body. Dip net sampling must be used after visual encounter sampling, because dip net sampling is an invasive method of sampling and disturbs the habitat and can therefore chase away frogs that are in close vicinity. The captured tadpoles were euthanased in MS222 and preserved in 70 % ethanol and taken back to the lab to be screened for *B. dendrobatidis*.

2.4.4 Aquatic traps

Aquatic bucket traps were used to collect *Xenopus laevis*. This species is fully aquatic and can only be collected by setting traps for them in the water. Traps were made from 20 L buckets fitted with a funnel on the side of the bucket. Holes were drilled in the bottom of the bucket to allow ventilation (Figure 13). The bucket was turned upside down with air holes at the top so that fresh air could enter the bucket. Bait that has a strong odour, such as chicken liver and chicken hearts, was used to attract the frogs to the traps because *X. laevis* relies on its olfactory sense to detect food. The traps were set along the periphery of water bodies with a few centimeters sticking out above the water. A stone or heavy object was placed on top of the trap to weight it down. Traps were left overnight. The traps were removed the next morning and the captured *X. laevis* transferred to a bucket and swabbed on site. All the frogs were released back into the same dam where they were caught. The DNA swab data collected were preserved in a 2 ml Eppindorf filled with 70 % ethanol. The data was sent to the National Zoological Gardens for qPCR testing.

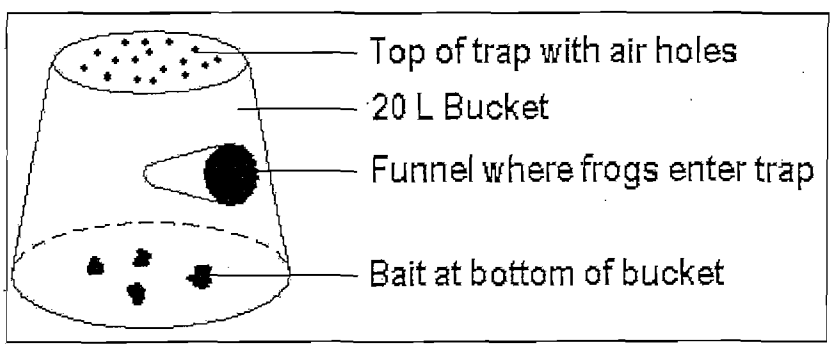

Figure 13: Illustration of a funnel trap with bait at the bottom of the trap to lure *Xenopus laevis*.

2.4.5 Identification of frogs

Frogs that were collected in the field were identified by their morphological characteristics and by the calls of the males. Lambiris (1987) was used to identify tadpoles that were collected and Carruthers (2001) was used to identify frog specimens that were collected. Most frogs are limited to a certain locality, and by identifying the locality of the specimens that were collected, they could be identified. Distribution patterns were helpful to support the identification.

2.5 Screening for *Batrachochytrium dendrobatidis*

Tadpoles that were brought back from Mont-aux-Sources were screened in the lab by means of a dissecting microscope with 0.8 up to 5 x 10 lenses. Mouthparts were also examined under a 10x dissecting microscope for malformations or loss of keratinised mouthparts. The lower oral disc of the mouthparts containing the lower keratodonts and rostrodont were surgically removed and temporary slides were made of the lower part of the mouth of *A. vertebralis* by mounting the oral disc in a drop of water on a microscope slide and by covering it with a cover slip. Slides were screened under 100x and 400x magnification with the aid of a standard compound microscope. Infected tadpoles were identified by the presence of spheroid, walled, septate sporangia that occur in clusters and sometimes contain zoospores.

In the field, a 20x hand lens was used to screen the mouthparts of *H. natalensis* tadpoles. Positive infections were determined by checking the keratodonts for hyperpigmented spots that appear as brown spots (Smith *et al.,* 2007).

2.5.1 Swabbing technique

Frogs that were collected at each site were kept in unused 20 x 25 cm plastic zip lock bags. Each frog was screened for *B. dendrobatidis* by firmly running a cotton swab ten times over the ventral surface of the frog, the sides of the frog including the thighs, and the feet and webbing of the frogs (Kriger *et al.,* 2006a). The swabbing technique can be seen in Figure 14. Swabs were placed in Eppindorf tubes filled with 70 % ethanol and then placed in a cooler bag. After handling each frog, hands were rinsed to prevent disease transmission between individuals. Swabs were sent to the National Zoological Gardens, Pretoria (NZG) for *B. dendrobatidis* testing using quantitative (real-time) polymerase chain reaction (qPCR) described by Boyle *et al.* (2004) and Kriger *et al.* (2006b).

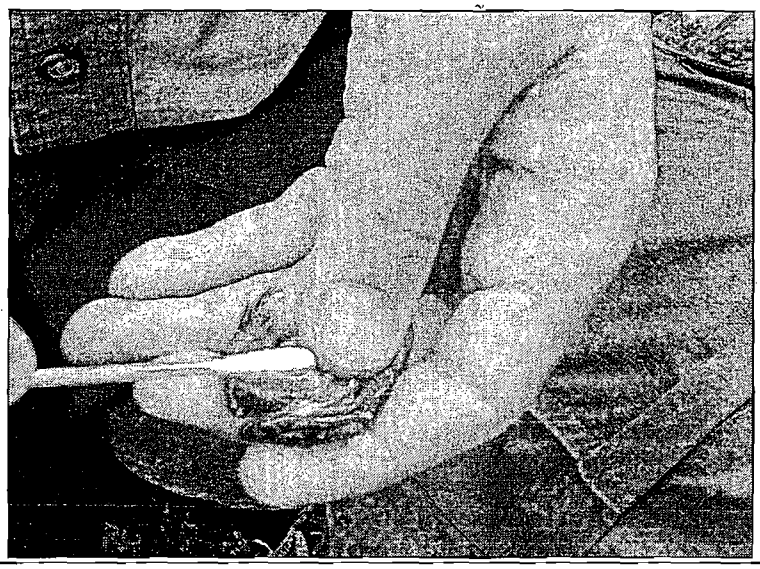

Figure 14: The technique for swabbing an adult frog to detect whether it is infected with amphibian chytrid (*Batrachochytrium dendrobatidis*).

2.6 Tadpole development

As tadpoles proceed through metamorphosis, they undergo major morphological changes and this development can help to determine the larval stage of the tadpole. Tadpoles hatch from eggs and then transform into free-swimming larvae - and these larvae undergo many changes in order to transform into a frog. K.A. Gosner compiled a table in 1960 in order to determine the different stages that anuran embryos and larvae undergo. In this table all the different stages and transformations of a tadpole metamorphosing into a frog are indicated. There are 46 stages from tadpole to complete metamorphosis into a frog. These 46 stages can, in turn, be grouped into eight different stages (Berrill M., pers comm*). These eight stages can be used on any frog species, in the same way as the Gosner stages.

* Berrill, M., Trent University, Canada

In table 1, the 46 Gosner stages are grouped into the eight different field stages:

Table 1: Explanation of how Gosner stages are grouped into the different field stages.

Description	No limb buds	limb buds	ankles and small feet	large feet	arm buds	arms and tail	tail resorbing	tail resorbed
Gosner Stage	25 & lower	26-30	31-35	36-40	41	42	43-45	46
Field Stage	1	2	3	4	5	6	7	8

Field stage 1 consists of Gosner stage 25 and lower (Figure 15), where the mouth starts to form and mouthparts begin to develop and become obvious. Field stage 2 consists of Gosner stages 26-30 (Figure 16), where the hind limb bud starts to develop. Field stage 3 consists of Gosner stages 31-35 (Figure 17), where the foot paddle starts to appear and indentations in the foot paddle begin to form. Field stage 4 consists of Gosner stages 36-40 (Figure 18), where large feet are formed and the toes are separated, and foot tubercles are formed. Field stage 5 consists of Gosner stage 41 (Figure 19); during this stage the forelimbs become visible and the mouthparts deteriorate; the vent tube also disappears. Field stage 6 consists of Gosner stage 42 (Figure 20); during this stage the forelimbs emerge and the mouthparts shift towards an anterior position in relation to the nostrils. Field stage 7 consists of Gosner stages 43-45 (Figure 20). During stage 43 (Figure 20), the mouth has shifted to between the nostril and the eye and the tail starts to deteriorate. In stage 44 (Figure 20), the mouth has moved to beneath the eye and the tail is greatly reduced; and then during stage 45 (Figure 20), the mouth stretches posterior to the eye and the tail is almost resorbed; only a stub remains. Field stage 8 consists of Gosner stage 46 (Figure 20), and this is the last stage that the larvae undergoes. During this stage the tail has been resorbed completely and metamorphosis is complete.

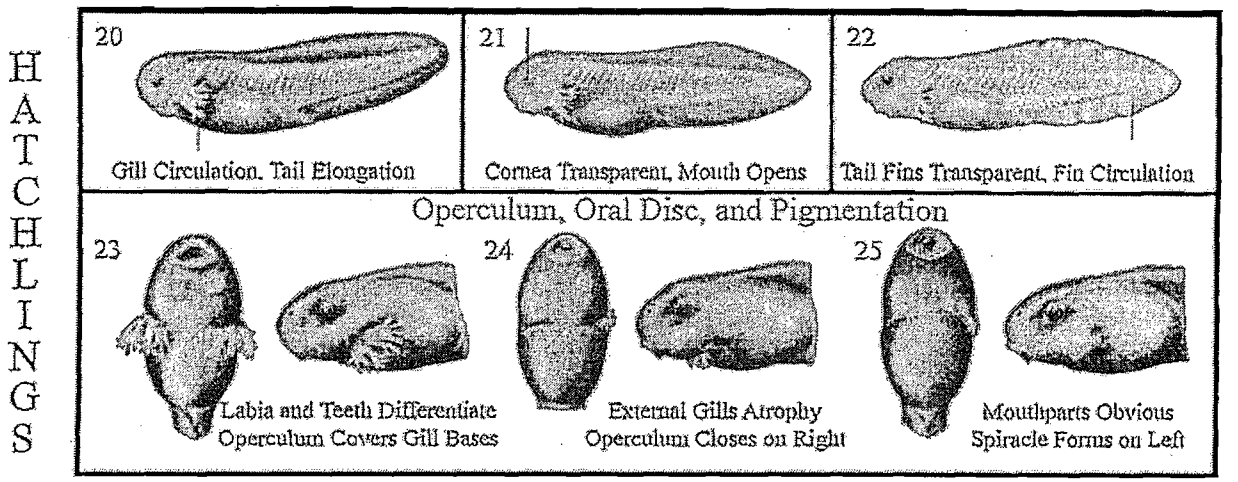

Figure 15: The different stages of operculum and oral disc formation constitute field stage 1 (Gosner stages 20-25) (Duelman and Trueb, 1986).

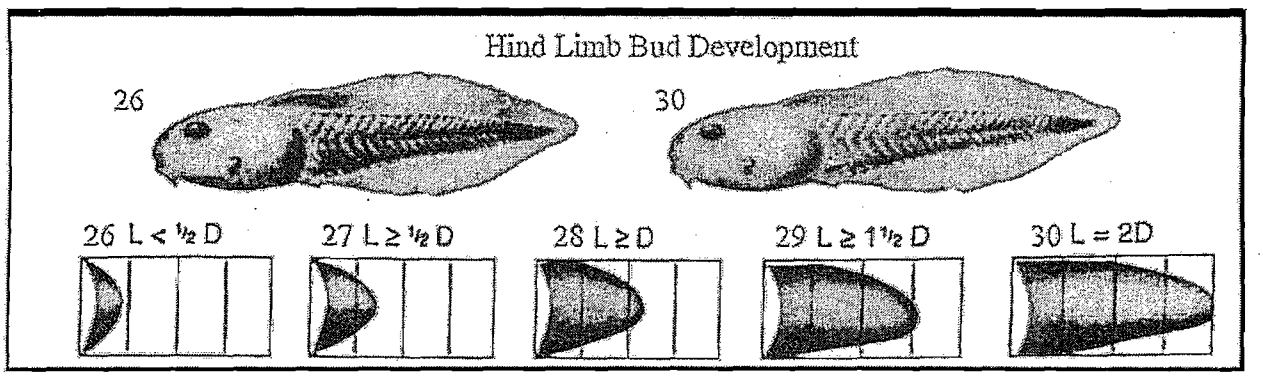

Figure 16: The different stages where the hind limb buds start to develop, constitute field stage 2 (Gosner stages 26-30) (Duelman and Trueb, 1986).

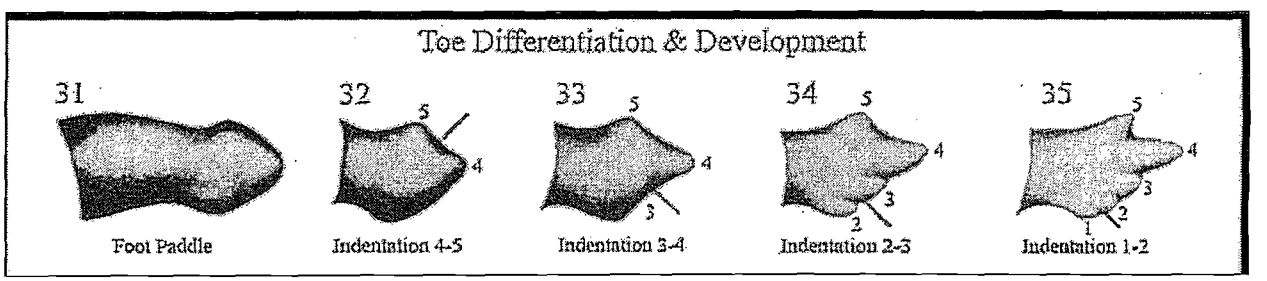

Figure 17: The different stages where toe differentiation and development take place constitute field stage 3 (Gosner stages 31-35) (Duelman and Trueb, 1986).

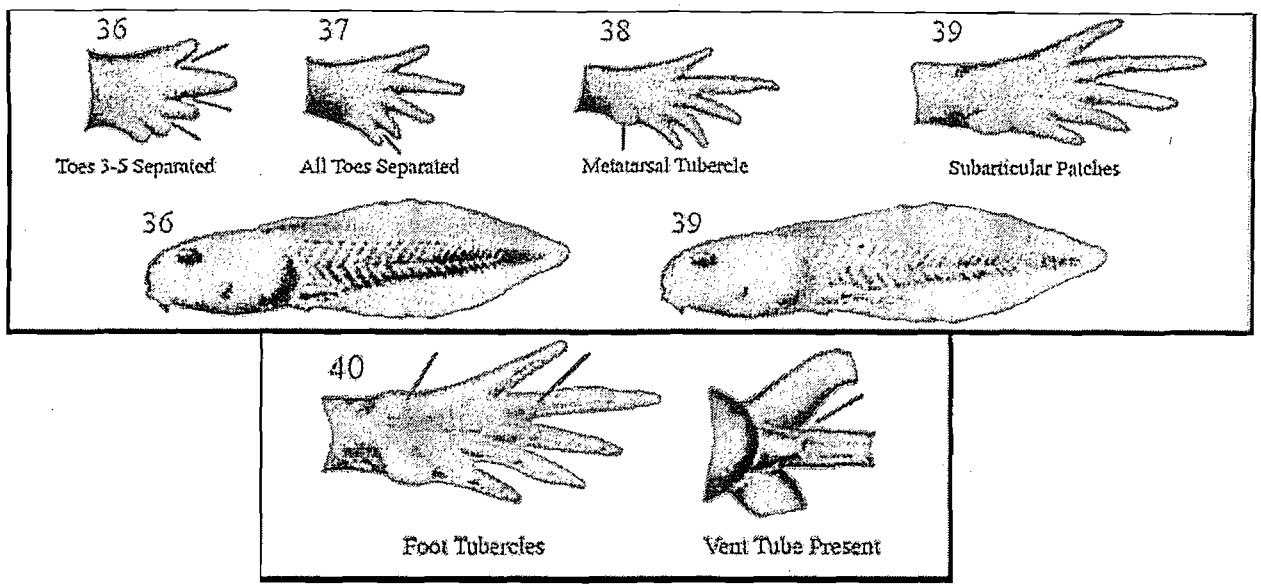

Figure 18: The different stages where the toes are separated completely and foot tubercles are formed constitute field stage 4 (Gosner stages 36-40) (Duelman and Trueb, 1986).

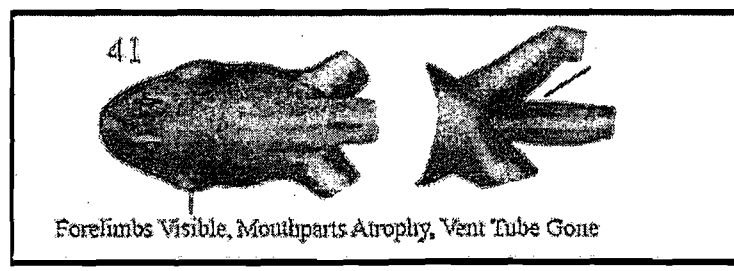

Figure 19: Gosner stage 41 is where the forelimb is visible and the vent tube has disappeared. The mouthparts are also starting to disappear in this stage, which constitutes field stage 5 (Duelman and Trueb, 1986).

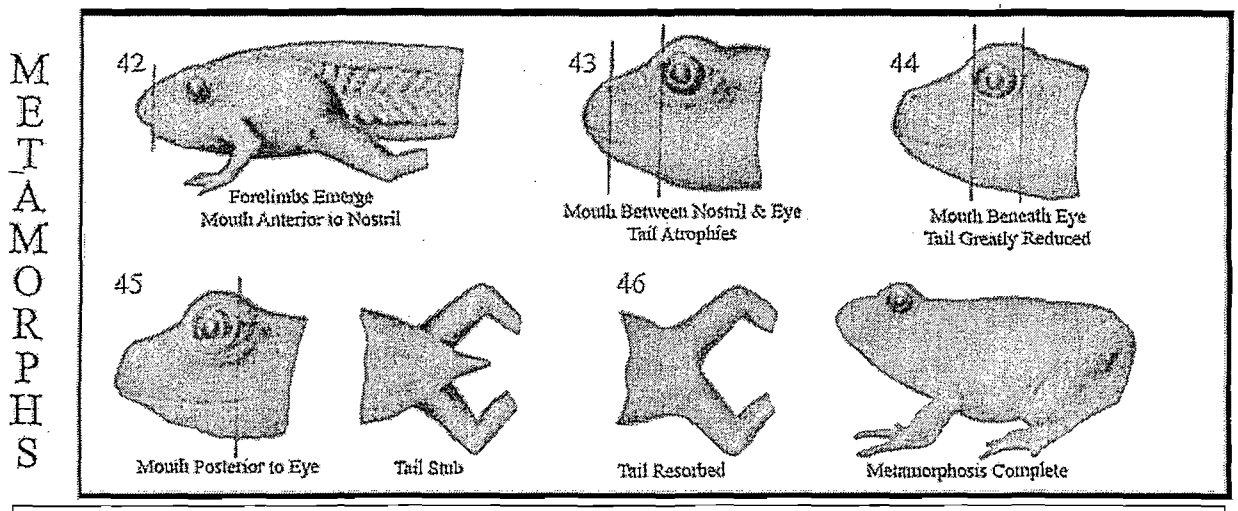

Figure 20: Gosner stages 42-46 are shown in the figure above. Stages 42-45 indicate the shifting of the mouth posterior to the eye. Gosner stage 42 constitutes field stage 6 and stages 43-45 constitute field stage 7. Gosner stage 46 indicates the resorption of the tail until metamorphosis is complete and constututes field stage 8 (Duelman and Trueb, 1986).

2.7 Statistical analyses

Statistical analyses were conducted by means of the statistical program STATISTICA 8.0 (Statsoft, 2007). As the size of data sets increases, the statistical significance tests that are conducted have a tendency to produce small p-values, which indicate the significance of the data. Ellis and Steyn (2003) suggested a model that can determine the effect size of a data set. The effect size does not depend on the sample size and it is a measure of practical significance. If the effect is large enough, it can be observed in the field. This means that the effect is large enough that it can be called important in practice. This effect is also described for differences in means such as the correlation between two-way frequency tables and multiple regression fits.

Statistical significance was tested by means of T-tests for the comparative analysis of particular groups (e.g. infection rates at different Gosner stages). For the data to be statistically significant, the p value has to be smaller than 0.05 ($p<0.05$). Because large data sets tend to yield small p values, it does not nessesarily follow that the same result is relevant in practice (Ellis & Steyn, 2003). Effect sizes were thus determined for the differences in the means of the data to see whether these were statistically significant in practice, since effect size is not dependant on sample size (Ellis & Steyn, 2003). For differences between means, data that has an effect size of $d=0.2$ has a small effect. Data that has an effect size of $d=0.5$ has a medium effect and data with an effect

size of $d \geq 0.8$ has a large effect. Data with an effect size of $d \geq 0.8$ can be considered to be practically significant. To determine effect size (d), the maximum standard deviation (S_{max}) of each sample was used in the following formula: The difference between two means divided by the estimate for standard deviation.

$$d = \frac{|\bar{x}_1 - \bar{x}_2|}{s_{\max}}$$

There are also some instances where relationships between two variables, i.e. Gosner stage and prevalence, are practically significant. Chi-square tests are used in these samples to determine the statistical significance of these relationships. The aim of this Chi-square test is to determine whether the relationships are significant enough to be important in a practical sense. The effect size given in a different formula, in this instance $w = \sqrt{\frac{X^2}{n}}$, and $X^{2,}$ is the Chi-squared statistic that is used for the contingency table and n is the sample size (Steyn, 1999; 2002). The effect size in this case is also not dependant on sample size. Cohen (1988) offered guidelines for the interpretation of these:

When $w=0.1$ it has a small effect. When $w=0.3$ it has a medium effect, and when $w \geq 0.5$ it has a large effect, and when $w \geq 0.5$ it can be considered as practically significant.

3 Results

3.1 Seasonal amphibian chytrid monitoring in the Drakensberg Mountains

3.1.1a Climate data - (Temperature)

It has been well documented that amphibian activity is to a large extent regulated by climatical conditions, in particular rainfall and temperature. Pounds *et al.* (2006) concur that the role of climate on amphibian chytrid infections needs to be re-evaluated. The current study in the Drakensberg Mountains aimed to determine the influence of climate, elevation and average water temperature on chytrid infection levels.

During sampling at the Mont-aux-Sources sites, the average water temperature was taken on the day the sampling was done. Collecting of tadpoles was quite difficult during winter at Mont-aux-Sources sites, due to the rivers being frozen (Figure 21). The average water temperature varied significantly between day and night at the Mont-aux-Sources sites and to a lesser extent at the Royal Natal National Park sites. These sites did not freeze during winter months.

The Mont-aux Sources sites started to freeze as early as April and the ice only began to melt during August. For all the years when sampling was conducted, snow was still present in August, especially on the eastern slopes of the Lesotho Drakensberg. During May and July, the rivers were frozen completely. The thickest ice layers on rivers occurred during July, and this made sampling very difficult (Figure 21). Tadpoles still survived in these cold and harsh conditions. The frozen layers on the rivers had to be broken so that tadpoles could be caught for screening of *B. dendrobatidis.* The ice started melting when temperatures increased, but some rivers still had thin layers of ice covering them in September 2007. Snow was also still present as late as September 2007, especially on the eastern slopes of the Lesotho Drakensberg.

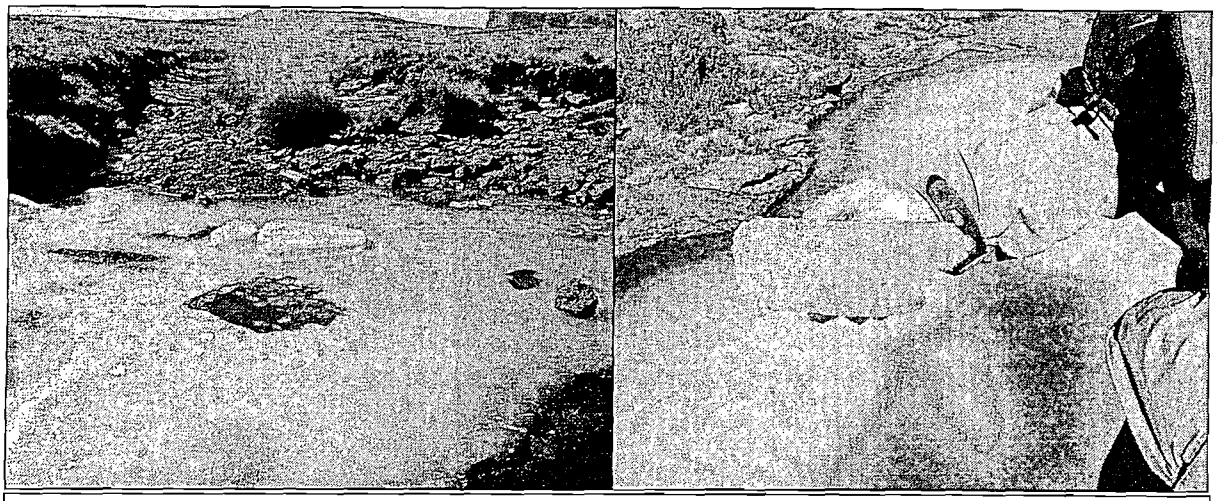

Figure 21: The Tugela river froze during the winter so that the frozen layers had to be broken in order to reach the tadpoles underneath the ice (left). A fragment of ice that indicates the thickness of the frozen layers on rivers during July 2007 (right).

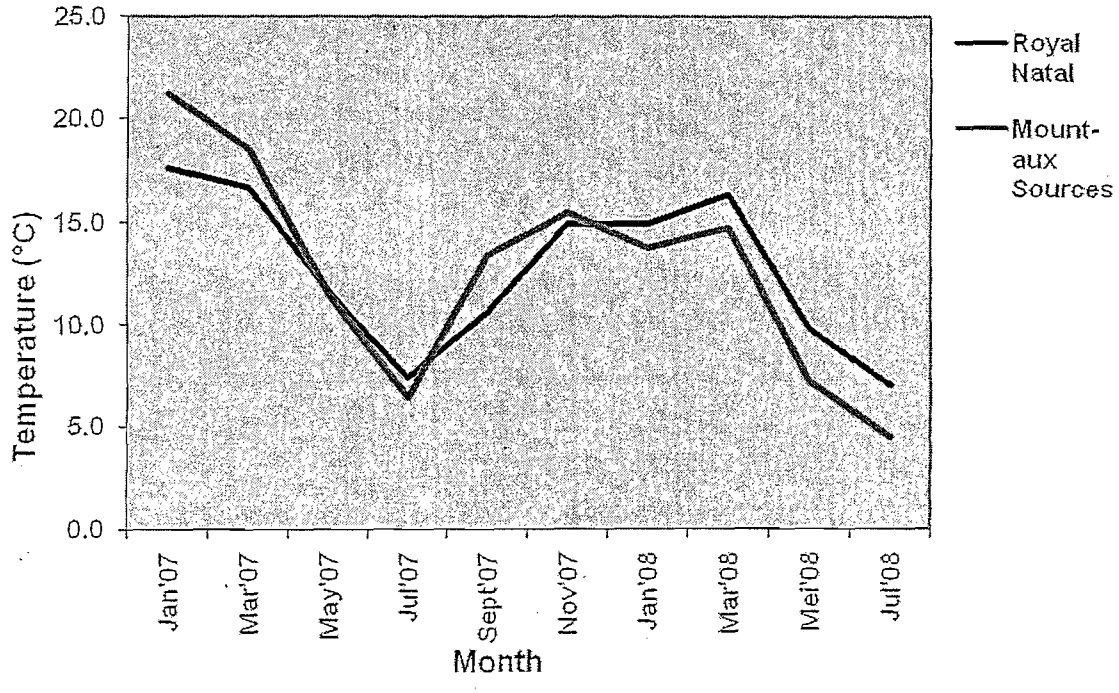

Figure 22: The combined monthly average water temperatures of the Royal Natal National Park sites and the Mont-aux Sources sites over a period of a year and a half.

The average water temperature followed a pattern that one would expect. A clear distinction could be made between the colder and the warmer months. From January through to July, the average water temperature decreased and started to increase from September through to March; then a decrease in average water temperature occurred again when autumn approached (Figure 22). Tables 2 and 3 indicate the relevant temperatures per sampling month per species.

Table 2: The amount of tadpoles sampled per season and the relevant seasonal temperatures at the sites of *Amietia vertebralis*.

Season	Date	n Tadpoles	Average water temperature (°C)
Summer	Jan'07	118	21.2
Summer	Mrt'07	104	18.53
Autumn	May'07	104	11.54
Winter	Jul'07	104	6.42
Spring	Sept'07	112	11.9
Summer	Nov'07	107	13.8
Summer	Jan'08	76	13.8
Summer	Mrt'08	100	14.7
Autumn	May'08	107	7.2
Winter	Jul'08	98	4.4

The average water temperatures for the Mont-aux Sources sites and the Royal Natal National Park sites were very similar. In Figure 22, the lines that indicate average water temperature for each month that sampling was conducted follow much the same pattern. Air temperature differed a great deal between the Mont-aux Sources and Royal Natal National Park sites, but the average water temperature did not differ as much. The average water temperature at Mont-aux Sources was colder during winter and warmer during summer when compared to the average water temperature of Royal Natal National Park. When comparing the Mont-aux Sources sites against the Royal Natal National Park sites, Mont-aux Sources was warmer during January 2007 and September to November 2007. During July 2007 and July 2008, the Mont-aux Sources sites were

colder than Royal Natal National Park (Figure 22). These were also the months when snow occurred at Mont-aux Sources and the rivers were frozen (Figure 21).

During winter nights, the air temperature dropped to below 0°C, which caused the rivers to freeze. During the day a minimum average daytime water temperature of 4.4°C was measured in July 2008 for the Mont-aux Sources sites, and 6.8°C for the Royal Natal National Park sites. During the summer months the average daytime water temperature reached measurements of 13.8°C up to 21.2°C respectively for the Mont-aux Sources sites and an average of 14.3°C up to 16.5°C for the Royal Natal National Park sites (Figure 22, Tables 2 and 3). Tables 2 and 3 present a summary of data that was collected. These tables show the number of tadpoles and average water temperature measured for the two sampling areas for every month sampling was conducted.

Table 3: The amount of tadpoles sampled per season and the relevant seasonal temperatures at the sites of *Hadromophryne natalensis*.

Season	Date	n Tadpoles	Average water temperature (°C)
Summer	Jan'07	121	16.3
Summer	Mrt'07	131	16.5
Autumn	May'07	125	11.7
Winter	Jul'07	148	7.4
Spring	Sept'07	146	10.7
Summer	Nov'07	51	15.1
Summer	Jan'08	64	14.3
Summer	Mrt'08	87	16.2
Autumn	May'08	116	9.8
Winter	Jul'08	98	6.8

As the rivers froze during the winter, they also started to dry up and shallow pools formed. Tadpoles clustered together in these shallow pools and some tadpoles even died because the pools also dried up (Figure 23).

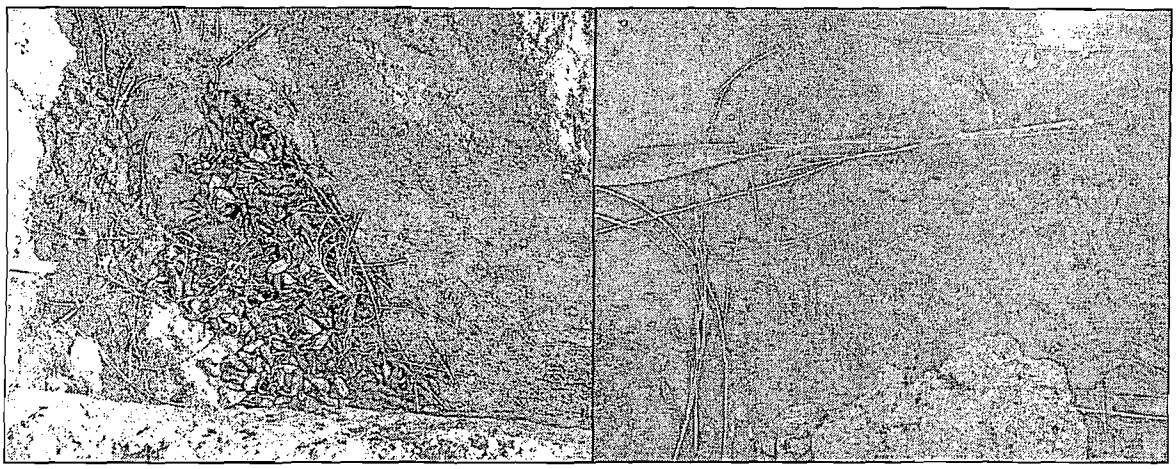

Figure 23: Tadpoles die when the rivers dry up (left). The clustering of tadpoles in a shrinking river pool can increase the transmission of chytrid between individuals (right).

3.1.1b Climate data - (Precipitation)

Precipitation at the Mont-aux Sources and Royal Natal National Park sites followed the same pattern to an extent, but higher rainfall was recorded at Royal Natal National Park than at Mont-aux Sources. During the summer months, there was a great deal of precipitation that made the rivers flow stronger and sampling was difficult during some sampling sessions. High rainfall figures were reported for Mont-aux Sources for the months September 2006 and November 2006, but for these two months no rainfall data is available for Royal Natal National Park (where a fair amount of rain occurred). Mont-aux Sources received some good showers, especially during November 2006. Precipitation occurred in September and November at Royal Natal National Park during previous years and one could infer that rainfall did occur during those months for which data was not available. The cold winter months of May and July received little or no rain. There was precipitation in May 2007 at Royal Natal National Park but it was a small quantity of 0.8 mm. In May 2008 there was quite a bit more precipitation than in May 2007. Precipitation was high during the summer months, peaking during January and March 2008 at Royal Natal National Park. Precipitation at Mont-aux Sources peaked during November 2006 and January 2008. The highest rainfall for Mont-aux Sources during the sampling year of 2007 was in January. Precipitation was higher during the 2008 sampling than during the 2007 sampling, but a pattern was observed to an extent (Figure 24).

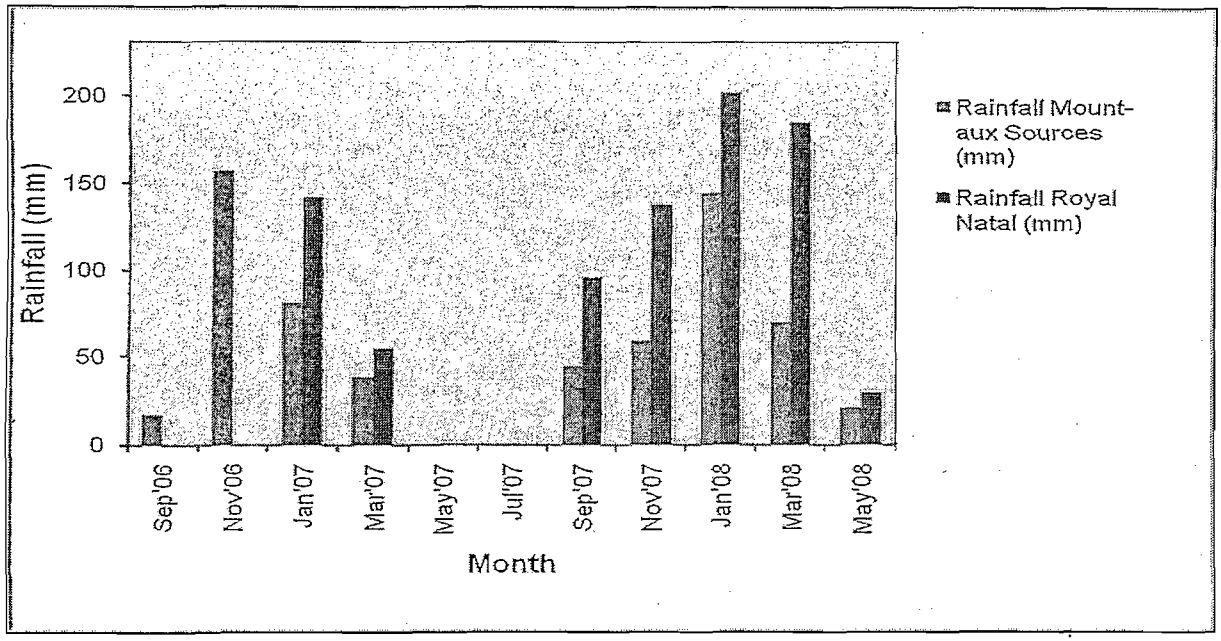

Figure 24: Combined monthly precipitation Figures for the Royal Natal National Park sites and the Mont-aux Sources sites over a period of 18 months followed a pattern according to season.

3.1.2 Monitoring of *Batrachochytrium dendrobatidis* in the Drakensberg Mountains

3.1.2a Distribution of *Batrachochytrium dendrobatidis* at Mont-aux Sources and Royal Natal National Park

All the data for the upper ten sites situated at Mont-aux Sources was distributed along the escarpment of the Drakensberg Mountains and all tadpoles collected for data were found to be infected *A. vertebralis* tadpoles were found at each of these sites. At each site ten tadpoles were collected on a bimonthly basis, and in total for the entire sampling season a total of 1 208 *A. vertebralis* tadpoles (Table 4) were collected and screened for *B. dendrobatidis*. The total average prevalence for each site is shown in Table 4. The number of tadpoles collected per site is also indicated in Table 4. The highest infection levels were recorded at both Site Ribbon Falls Lower and Site Ribbon Falls Middle, with a prevalence of 55% and 51% respectively. The sites with the lowest prevalence were Nampolice and Tugela Head with a prevalence of 20% and 34% respectively. The other sites were ranged closely together in terms of prevalence.

Table 4: Table of the number of *Amietia vertebralis* tadpoles and their *B. dendrobatidis* infection status for each of the collecting sites during the entire sampling period.

Site	n Tadpoles Positive	Prevalence %	n tadpoles
Bilanjil	63	41	153
Khubnam	49	41	120
Nampolice	22	20	109
Ribbon Falls Lower	61	55	110
Ribbon Falls Middle	58	51	114
Ribbon Falls Upper	45	41	109
Tugela Head	39	34	115
Tugela	64	50	128
Vemvame Lower	54	43	125
Vemvame Upper	50	40	125
Totals	505	42	1208

Prevalence differed so that statistically significant differences between these sites existed ($p<0.00059$), although the data was not practically significant ($w=0.16$). The data for the different sites was therefore pooled to construct a graph of infection compared to temperature over time for the species (Figure 25).

Table 5: Table of the number of *Hadromophryne natalensis* tadpoles and their *B. dendrobatidis* infection status for each of the collecting sites during the entire sampling period.

Site	n Tadpoles Positive	Prevalence %	n tadpoles
Gully	69	61	114
Devils tooth	65	56	116
Sentinel Gully	38	34	113
Tugela 1	79	67	118
Butterfly	36	31	116
Junction	16	64	25
Mahai Lower	8	21	38
Gudu Lower	6	35	17
Gudu Upper	59	47	125
Mahai Upper	35	38	93
Golide Lower	21	57	37
Golide Upper	43	37	116
Zigubudu	22	42	53
Gezana	32	62	52
Sunday Falls	11	48	23
Vemvaan	45	47	96
Vemvaan Lower	39	42	92
Devils Hoek	45	48	93
Totals	669	47	1437

The 18 sites allocated in Royal Natal National Park were distributed across the base of the mountain and infected *H. natalensis* tadpoles were found at each of these sites. At each site a total of ten tadpoles were collected, where possible, on a bi-monthly basis. The total number of tadpoles that were collected during the entire sampling season at these sites was 1 437 (Table 5). These collected tadpoles were screened in the field for *B. dendrobatidis* and released into the same river. The total average prevalence for each site situated in Royal Natal National Park as well as the total number of tadpoles that were infected at each site during the sampling season is shown in Table 5. There is not a significant difference between these sites according to prevalence. The highest

prevalence was found at Tugela 1 with a prevalence of 67%. Another site with a high prevalence was Junction with a prevalence of 64%. Some of the sites with a high prevalence were those where a small number of tadpoles were collected. The only site with a reasonable number of tadpoles that also showed a high prevalence was Tugela 1. The site with the lowest prevalence was Mahai Lower with a prevalence of 21%.

Of the species that were collected, 42.4% of 1 208 tadpoles of *A. vertebralis* were found to be infected and 47.0% of 1 437 tadpoles of *H. natalensis* tadpoles (Table 6) were infected (including September and November 2006 data).

Table 6: Annual breakup of the sampling data, including the number of frogs and *Batrachochytrium dendrobatidis* prevalence for *Hadromophryne natalensis* and *Amietia vertebralis*.

Royal Natal National Park	**Species**	**n**	**Prevalence (%)**
Year 1	*Hadromophryne natalensis*	338	69.4
Year 2	*Hadromophryne natalensis*	735	44.5
Year 3	*Hadromophryne natalensis*	364	31.3
		1437	47
Mont-aux Sources	**Species**	**n**	**Prevalence (%)**
Year 1	*Amietia vertebralis*	170	36.5
Year 2	*Amietia vertebralis*	657	59.1
Year 3	*Amietia vertebralis*	381	16.3
		1208	42.4

3.1.2b Correlation between prevalence and temperature

The average water temperature data in Figure 22 follows a distinct pattern and one may infer that chytrid prevalence in tadpoles would also follow a distinct pattern according to temperature. This was, however, not the case during some months; in Figures 25 and 26 an irregular pattern is indicated regarding the prevalence data for the two infected frog species. Prevalence did, however, decrease during the colder months. Data for September and November 2006 were collected by

Kevin Smith and Ché Weldon and is included in Figures 22, 23, 24, and 25 as well as in Table 6. This data was included to enlarge the data set.

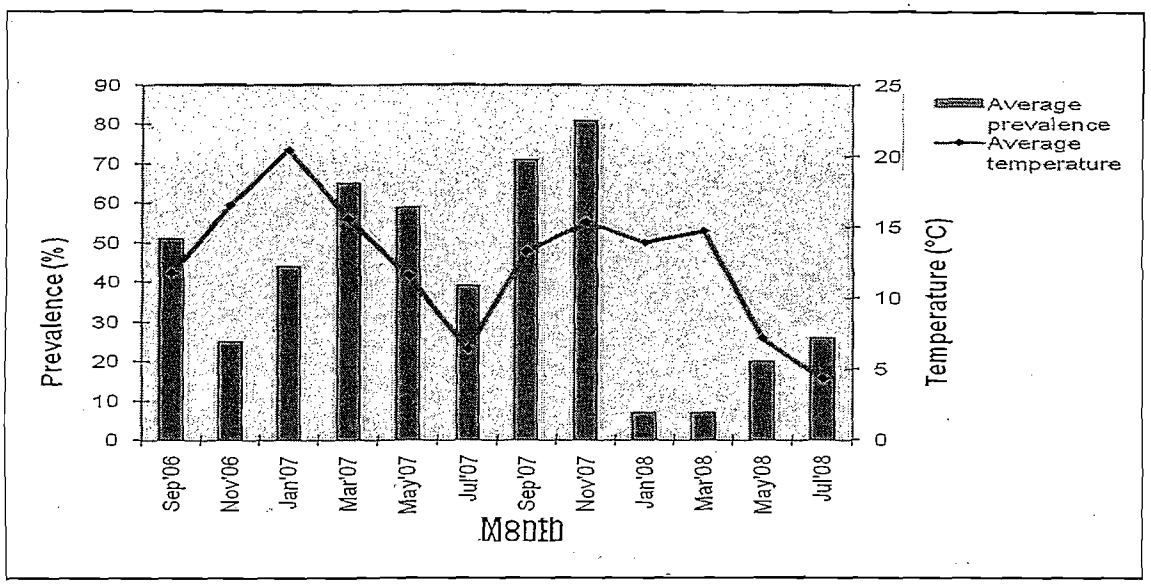

Figure 25: The average prevalence of all the sites at Mont-aux Sources, where *Amietia vertebralis* occurs, is plotted with the average water temperature at the time of collection for all the sites over a period of 18 months.

Prevalence data for Mont-aux Sources followed a pattern for temperature for the first 14 months, but was lagging behind slightly the last six months (Figure 25). Average water temperature peaked in January 2007 while the chytrid prevalence peaked in March 2007, followed by a decline and reaching a low point for both temperature and prevalence during July 2007. Thereafter we observed an increase in prevalence as the weather warmed up again. On the other hand, prevalence data for Royal Natal National Park, to some extent, followed the temperature profile (Figure 26). Prevalence peaked in November while average temperature increased and peaked during March. In the winter months of May and July, prevalence decreased but these were not the months with the lowest prevalence (Figure 26). The overall prevalence at Royal Natal National Park was higher than at Mont-aux Sources. As for Mont-aux Sources, the prevalence peaked during the summer months (Figure 27).

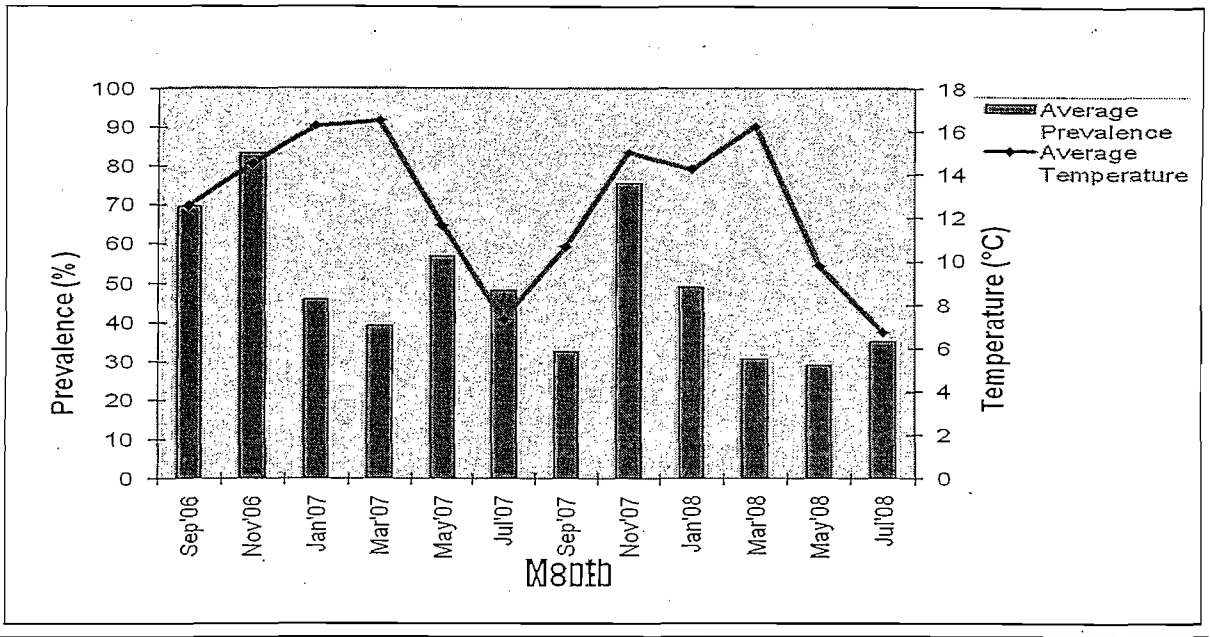

Figure 26: The average prevalence of all the sites at Royal Natal National Park where *Hadromophryne natalensis occurs,* are plotted with the average water temperature at the time of collection for all the sites over a period of 18 months.

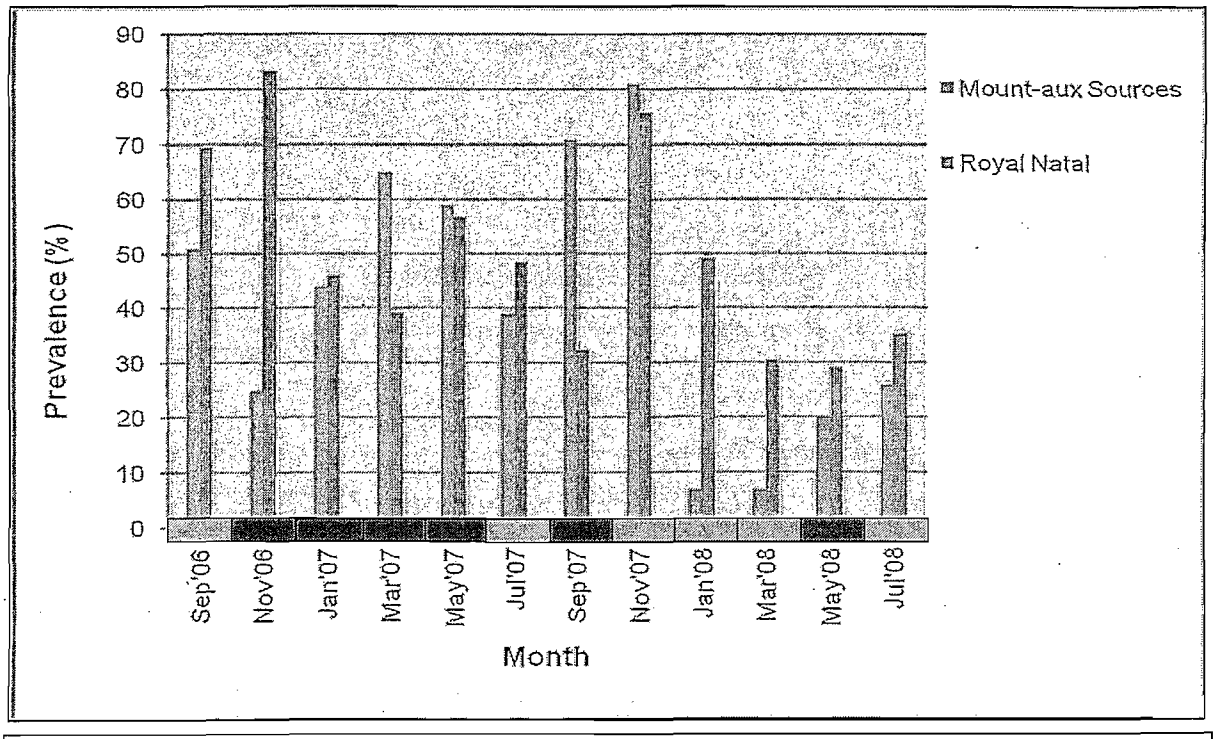

Figure 27: Comparison of prevalence data for the Royal Natal National Park and Mont-aux Sources sites. The green bars indicate where both localities followed the same tendency and red where they followed opposing tendencies.

Amietia vertebralis and *Hadromophryne natalensis* tadpoles were collected throughout the year and during different weather conditions and temperatures. A standard student T-test was performed on the data for all the tadpoles that were collected during the sampling year. Sampling months were classified as either warm or cold months with September, November and January being the warm months and March, May and July being the cold months. The temperatures shown in Figure 22 for the respective sampling areas (the Mont-aux Sources and Royal Natal National Park sites) were used to determine these warm and cold month groups.

The test was performed to establish whether there was any statistical significance between prevalence and temperature. It was found that prevalence for *A. vertebralis* was the highest during the warmer months with a mean prevalence of 47%, and the colder months had a low prevalence of 36% (Figure 28). The *p* value for this test for *A. vertebralis* species was 0.00001, showing statistical significance. The effect size for the test was d=0.60 which meant it had a medium effect in practice and could partially be observed in the field. A total of 1 208 *A. vertebralis* tadpoles were collected during the sampling period and of these, 558 tadpoles were collected during the warm months and 650 were collected during the cold months.

The prevalence for the Royal Natal National Park sites, where *H. natalensis* tadpoles were collected, showed the same results as the Mont-aux Sources sites. Prevalence was highest during the warmer months (59%) and low during the cold months (40 %) (Figure 28). This data was statistically significant (p = 0.00001) and had an effect size of d=0.90, implying that a high prevalence during the warmer months could be observed from the field data. A total of 1 313 *H. natalensis* tadpoles were collected during the sampling period and, of these, 633 tadpoles were collected during the warm months and 680 during the cold months.

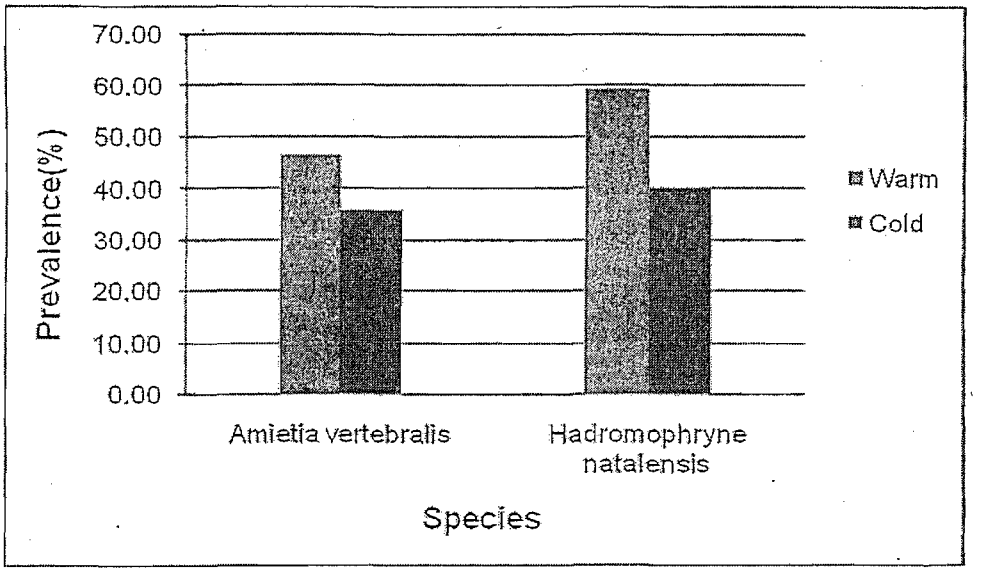

Figure 28: Comparing warm and cold month prevalence between Mont-aux Sources (*Amietia vertebralis*) and Royal Natal National Park (*Hadromophryne natalensis*).

3.1.2c Correlation between prevalence and precipitation

A pattern was observed when precipitation data was correlated with prevalence data for the Mont-aux Sources sites. Prevalence was low during the cold months but increased after the winter when it started raining. During September it usually starts to rain. A peak in precipitation was observed during November 2006 and during January 2008. During these two months, prevalence decreased to a very low point (Figure 27). When precipitation was low or constant, prevalence seemed to increase slightly or remained constant. This was seen during the months of January 2007 up to November 2007. Data in figures 26 and 27 includes Kevin Smith and Ché Weldon's data.

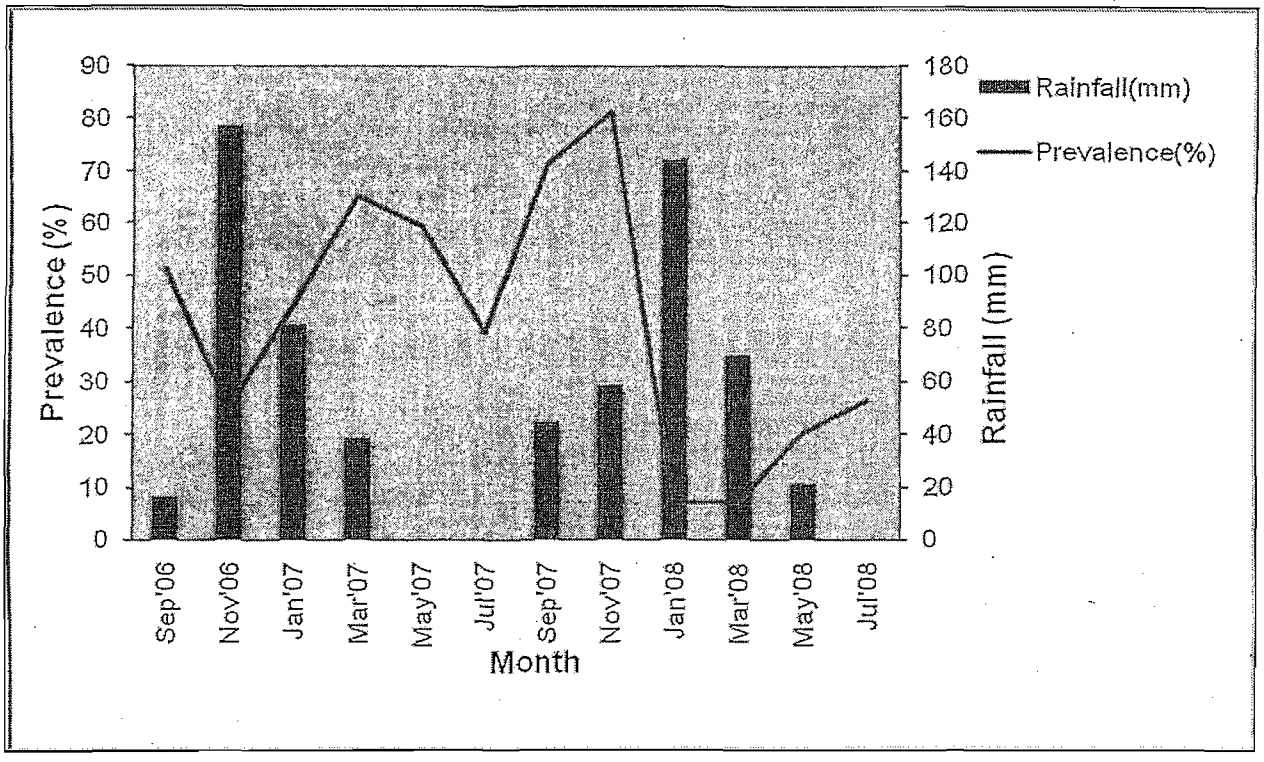

Figure 29: The average prevalence of all the sites at Mont-aux Sources is plotted with the average precipitation for each month when sampling was done for all the sites over a period of 18 months.

Regarding the Royal Natal National Park precipitation data, a similar pattern was observed. Prevalence was low during the cold months but increased after the first rains occurred in September. No data was available for September and November 2006 due to problems with the weather stations. It could only be assumed that precipitation would have occurred during these months where data was not available because it usually begins to rain in September. Prevalence increased or remained constant between January 2007 and November 2007. Precipitation peaked in January and March 2008. During these two months, prevalence decreased rapidly and then stabilised as precipitation decreased (Figure 29).

Prevalence for the Mont-aux Sources sites, where *A. vertebralis* was found during sampling periods, ranged from as high as 81.3% (November 2007) to as low as 6.9% (January 2008), as indicated by Figure 29.

Regarding the *H. natalensis* data collected during November 2006 and November 2007 (Figure 30), prevalence was the highest during these two months (83.4% and 75.9 % respectively), but for

A. vertebralis (Figure 29) during the same months, the results were different. During November 2006 the prevalence was very low at 25% and in November 2007 the prevalence was very high with a percentage of 81%. During November 2006, the precipitation was very high where the prevalence was low and during November 2007 the precipitation was low where prevalence was very high.

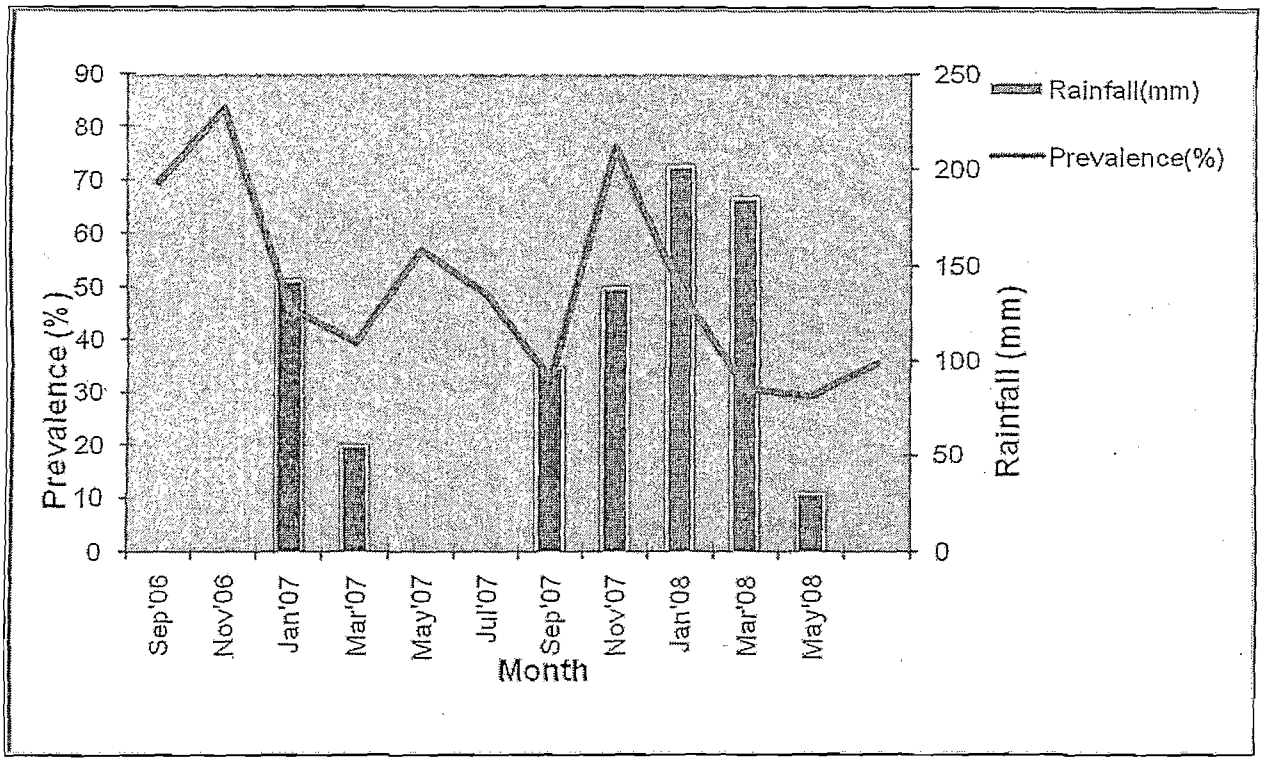

Figure 30: The average prevalence of all the sites at Royal Natal National Park is plotted with the average precipitation for each month when sampling was done for all the sites over a period of 18 months.

3.1.2d Correlation between tadpole size and infection

Amietia vertebralis tadpoles were found and collected at ten sites across the escarpment of the Amphitheatre in the Mont-aux Sources region. Screening of tadpole mouthparts indicated that individuals of *Amietia vertebralis* were frequently infected with *B. dendrobatidis.* Across all the surveys, the prevalence of oral chytridiomycosis was 38.9% (range: 0%-100%) for *A.vertebralis.* Mouthpart depigmentation was observed in 221 out of the 1 208 (18.3%) tadpoles that were collected, out of which 202 (91.4 %) were diagnosed with oral chytridiomycosis. In Figure 31, it is indicated that most of the tadpoles had a positive infection, with the highest prevalence in stages 34 up to 38. Tadpoles that were positively infected were at a later developmental stage than the non-infected tadpoles (Figure 31).

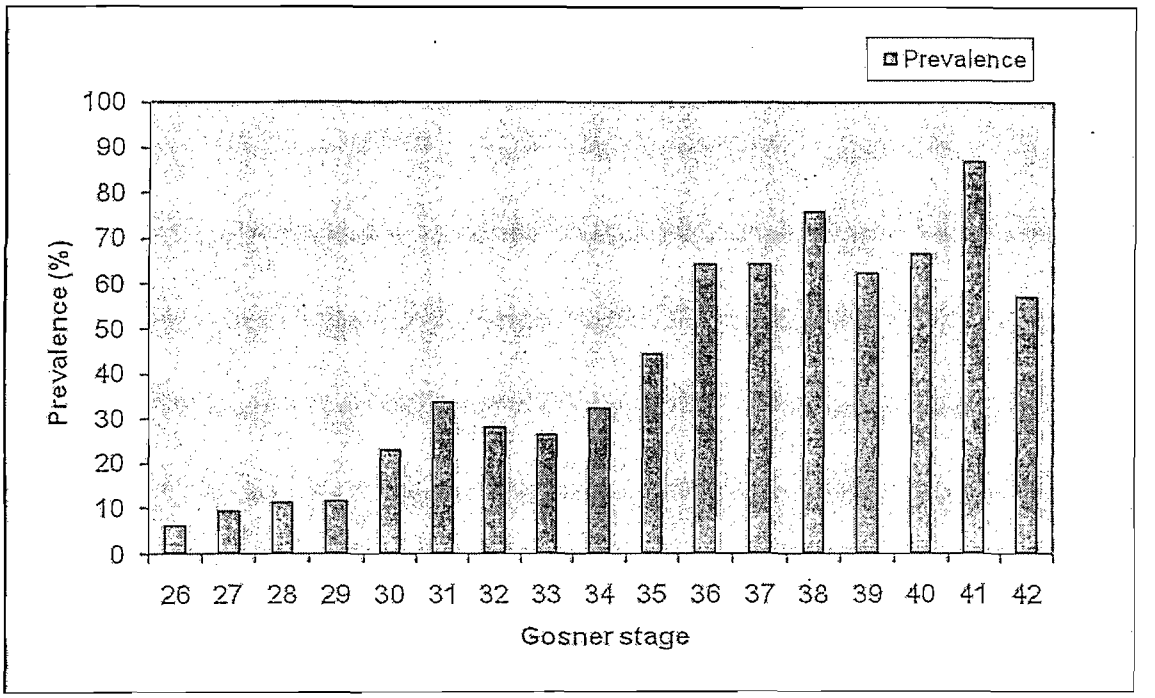

Figure 31: Average prevalence for each of the larval stages of *Amietia vertebralis* that were collected.

Table 7 shows the results of a standard T-test done to determine if tadpoles that were more developed were also more likely to be infected. The infected larvae of *A. vertebralis* were significantly more developed than non-infected larvae (Student's T=-14.00, $p<0.001$). The mean Gosner stage for infected larvae was 36.058 (SD = 3.486) while the mean Gosner stage for non-infected larvae was 32.628 (SD = 4.134). The *p* value for the T-test in Table 10 is 0.00016 and is

statistically significant, and the effect size was d=0.83. This means that this result was practically significant with a large effect.

Table 7: A standard T-test to determine at which developmental stages infection occurred most often.

	Mean: Non-infected stage	Mean: Infected stage	t-value	p	Valid n: Non-infected	Valid n: Infected	Std.Dev.: Non-infected	Std.Dev.: Infected
Stage:	32.62848	36.05869	-14.0023	0.001	611	426	4.134355	3.485612

As tadpoles developed, they were increasingly infected with chytridiomycosis (Figure 31). Prevalence at the earlier larval stages, 26-29, was below 10%, and from stages .30-35, the prevalence was above 10%, but below 50%. In the later stages of larval development the prevalence was above 50% - up to 90% at some stages (stages 36-42). Thus, the larger and more developed the tadpoles, the more infected they tended to be (Figure 31).

Tadpoles that were more developed and more infected had oral deformities and depigmentation in their labial tooth rows. The tooth rows consists of keratin and *B. dendrobatidis* completes its lifecycle in these dead keratinised cells which cause the depigmentation and deformities (Figure 32).

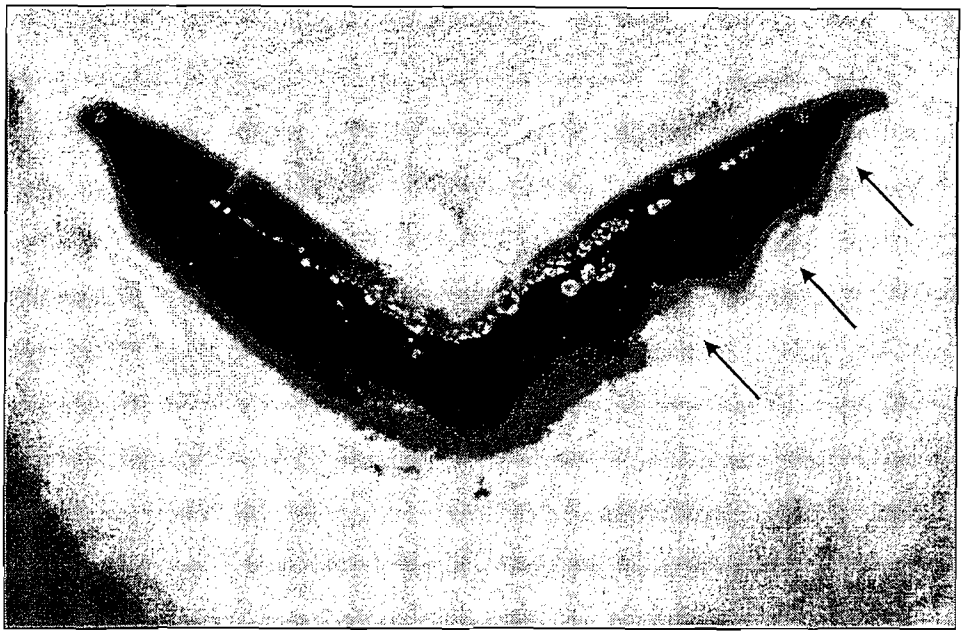

Figure 32: Chytridiomycosis can be the cause of oral depigmentation in tadpoles. The arrows indicate depigmentation of the lower rostrodont.

3.1.2e Correlation between prevalence and site allocation in a river

There were two rivers at the Mont-aux Sources sampling area for which an upper and lower collecting site were selected for sampling. Ribbon Falls Upper and Ribbon Falls Lower were two sites in the Ribbon Falls River and were roughly 200 m apart with approximately 100 m difference in elevation between these. The second river, Vemvame, had two sampling areas, namely Vemvame Upper and Vemvame Lower. These two sites were also roughly 200 m apart with a small difference in elevation of about 20 m between them. The upper sites were situated close to the head waters while the lower sites were close to the escarpment in both of the rivers.

Table 8: A two-way table analysis that indicates the difference in prevalence between the upper and lower sites of Ribbon Falls River.

Site	Chytrid Status: n Positive	Prevalence (%)	n Tadpoles
Ribbon Falls Lower	54	52	103
Ribbon Falls Upper	41	40	102

Chytrid status was determined for the two different sites allocated in the Ribbon Falls River, the Ribbon Falls Upper site and the Ribbon Falls Lower site (Table 8). The total number of tadpoles that were sampled for these sites was 205, of which 103 were collected at the lower site and 102 collected at the upper site. Ribbon Falls Lower (52%) had a higher prevalence than Ribbon Falls Upper (40%). The data in Table 8 was not statistically significant with p=0.79 and the effect size was w=0.12, indicating no difference.

Table 9: Two-way table analysis that indicates the difference in prevalence between the upper and lower sites of Vemvame River.

Site	Chytrid Status: n Positive	Prevalence (%)	n Tadpoles
Vemvame Lower	43	42	102
Vemvame Upper	43	41	105

The Vemvame River is situated on the western side of the mountain. The chytrid status was determined for the two different sites allocated in the Vemvame River. The total number of tadpoles that were sampled for these sites were 207, of which 102 were collected at the lower site and 105 at the upper site. Vemvame Lower (42%) showed almost the same prevalence as Vemvame Upper (41%) (Table 9). The data in Table 9 was not statistically significant with p=0.86 and was also not effective in practice with w=0.01.

Table 10: Levene's Test to determine what mean stages were present at the Upper and Lower sites of Ribbon Falls River and Vemvame River.

Site	Stage: Mean	Stage: Std.Err.	Stage: -95.00%	Stage: +95.00%	n tadpoles
Ribbon Falls Lower	35.50485	0.412834	34.69331	36.31640	103
Ribbon Falls Upper	32.84314	0.414852	32.02762	33.65865	102
Vemvame Lower	35.04902	0.414852	34.23350	35.86453	102
Vemvame Upper	34.48571	0.408883	33.68193	35.28949	105

In Table 9, the upper and lower sites for the two rivers were compared according to the different life stages at upper and lower sites. For the upper and lower sites of Ribbon Falls, the lower site

contained larger and morphologically more advanced tadpoles (mean Gosner stage: 35) than the tadpoles found at the upper site (mean stage: 32). At the Vemvame Lower site, there were larger and more developed tadpoles (mean stage: 35), and at the Vemvame Upper site there were smaller and less developed tadpoles (mean stage: 34). The result was not the same as at the Ribbon Falls River, but a pattern could be observed. The effect size for the comparison between the upper and lower sites at Ribbon Falls for practical significance was d=0.63, which means that there was a medium effect in practice which could, to some extent, be observed in the field. The effect size for the comparison between the upper and lower sites at Vemvame River for practical significance was d=0.13, which means that it has a small effect in practice that could not be observed in the field.

3.1.2f Mortalities of *Amietia vertebralis*

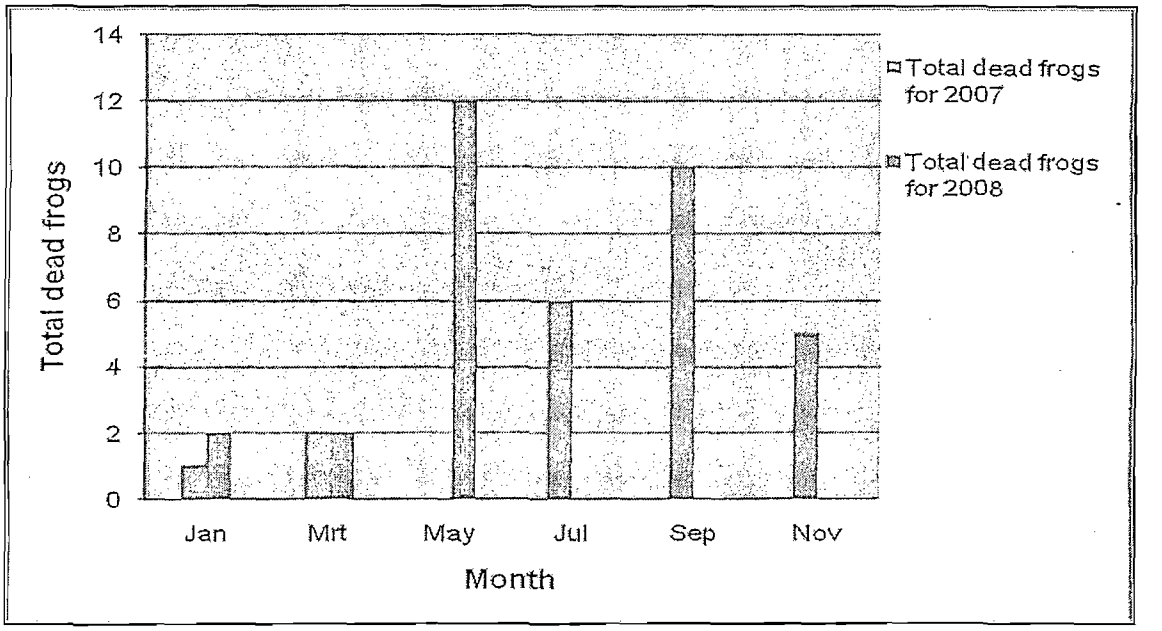

Figure 33: The total number of dead *Amietia vertebralis* metamorphs collected through the sampling period at Mont-aux Sources during 2007 and 2008.

During the sampling period when *A. vertebralis* tadpoles were collected at the Mont-aux Sources area, mortalities of metamorphs and adults of this species were found and the carcasses were screened for amphibian chytrid infection. All the dead specimens collected were infected with *B. dendrobatidis*. Mortalities were found during 8 of the 10 sampling months. The months where mortalities were not found were May 2007 and July 2008. During July 2007, September 2007, November 2007 and May 2008 the highest mortalities were observed. Over the entire sampling

period, a total of 40 frog mortalities were observed. The month with the highest mortality rate was May 2008, with 12 observed mortalities. No correlation between season and dead metamorphs could be gleaned from Figure 33. Die-offs did not occur during the same months. Kevin Smith and Ché Weldon's data was not included in this figure. Tadpoles were found feeding on the dead frogs that were present in the streams and pools (Figure 34). Dead frogs could have served as reservoirs for the pathogen.

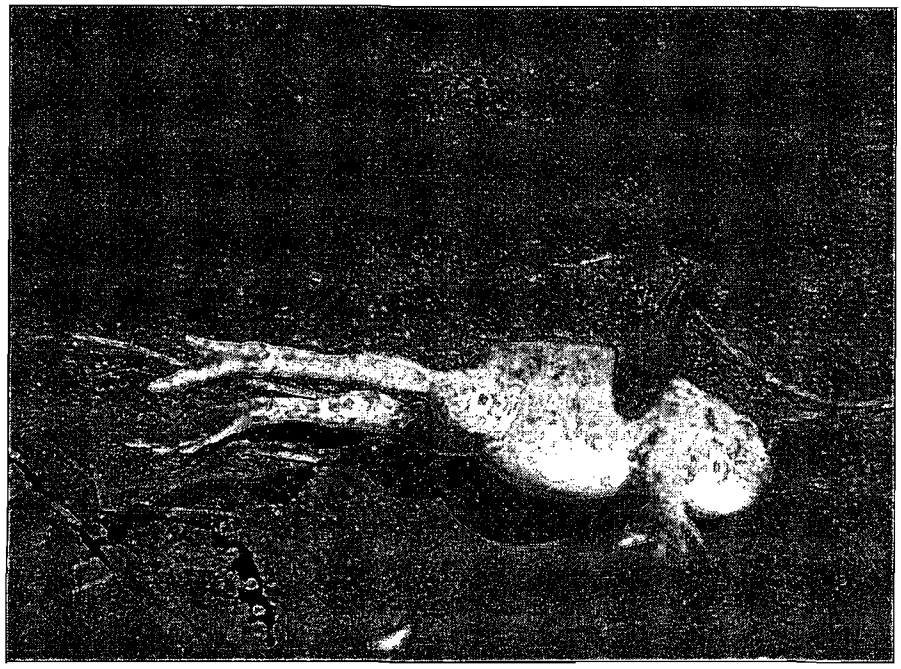

Figure 34: A dead *Amietia vertebralis* found at a sampling site with a tadpole of the same species feeding on the carcasses.

3.1.3 Breeding behaviour of *Amietia vertebralis*

The fact that fairly little is known about this species and its breeding biology is not due to the fact that this frog is so rare, but rather because it does not occur in easily accessible habitats. Specimens were encountered throughout the year. Tadpoles of various stages were collected every month that sampling was done. Furthermore, every Gosner stage from stage 26 up to stage 40 was present throughout the year, indicating that this species is an opportunistic breeder with a prolonged breeding season (Table 11).

Table 11: Range of developmental stages of *Amietia vertebralis* tadpoles that were collected for the six sampling events during 2007

Month	Gosner stages collected
January	26-42
March	26-44 and 46
May	26-41 and 46
July	26-40 and 46
September	26-42 and 46
November	26-42 and 46

Some stages were not present in all the data for each month because these stages were not found during these sampling sessions. The stages that were not included were stages 41-45 (Table 11). At each sampling site metamorphs were collected (stage 46). This data was based on a total of 639 tadpoles that were collected during the sampling period.

All the different stages of tadpole development were found throughout the field season, but in various proportions. Certain stages were consistently more predominant than others. The stages that were more predominant were stages 31-41. Tadpoles between stages 42-46 were particularly scant during most of the months, but this number peaked in January (Figure 35).

Figure 36 shows the degree to which tadpole body length increases in relation to tadpole development. Four of the eight different field stages can be witnessed in Figure 35. Body length increased gradually during field stage 2 (Gosner stage 26-30). This was followed by a somewhat slower increase in body length during field stage 3 (Gosner stage 31-35). The fastest increase in body weight takes place during field stage 4 (Gosner stages 36-40). During field stages 5, 6, 7, and 8, development is slow and maximum body length has been reached. These stages are closely related with minor developmental changes that differentiate them. These stages were between Gosner stages 41-46 (Figure 36).

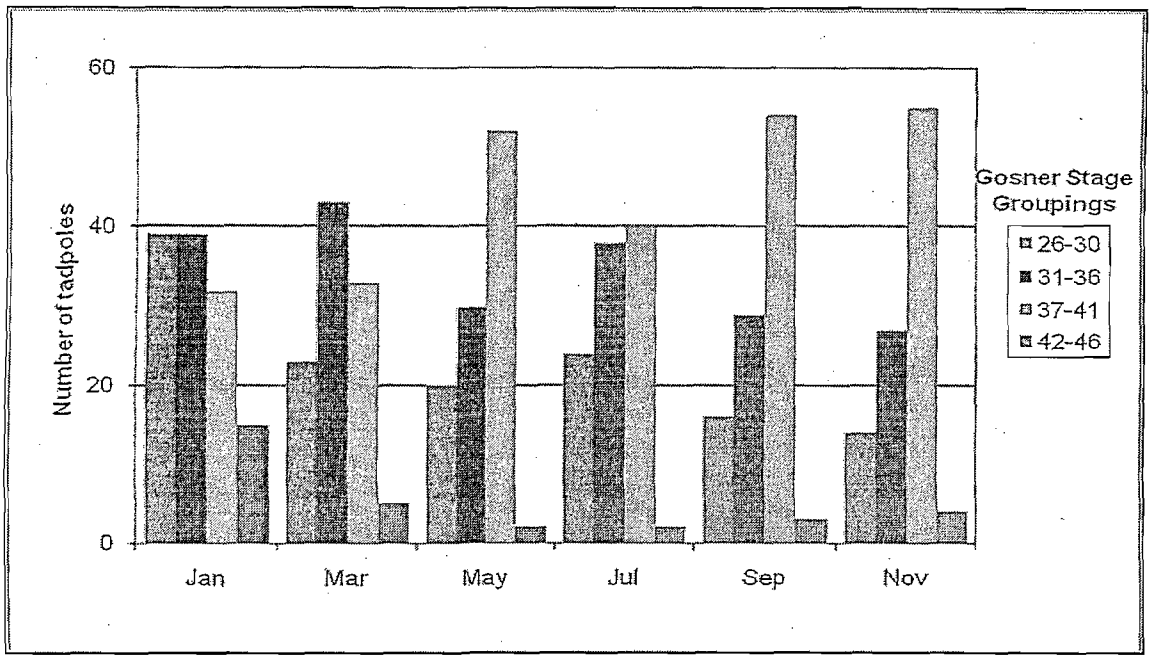

Figure 35: The number of tadpoles caught for each of the different physiological larval stages of *Amietia vertebralis* during one field season.

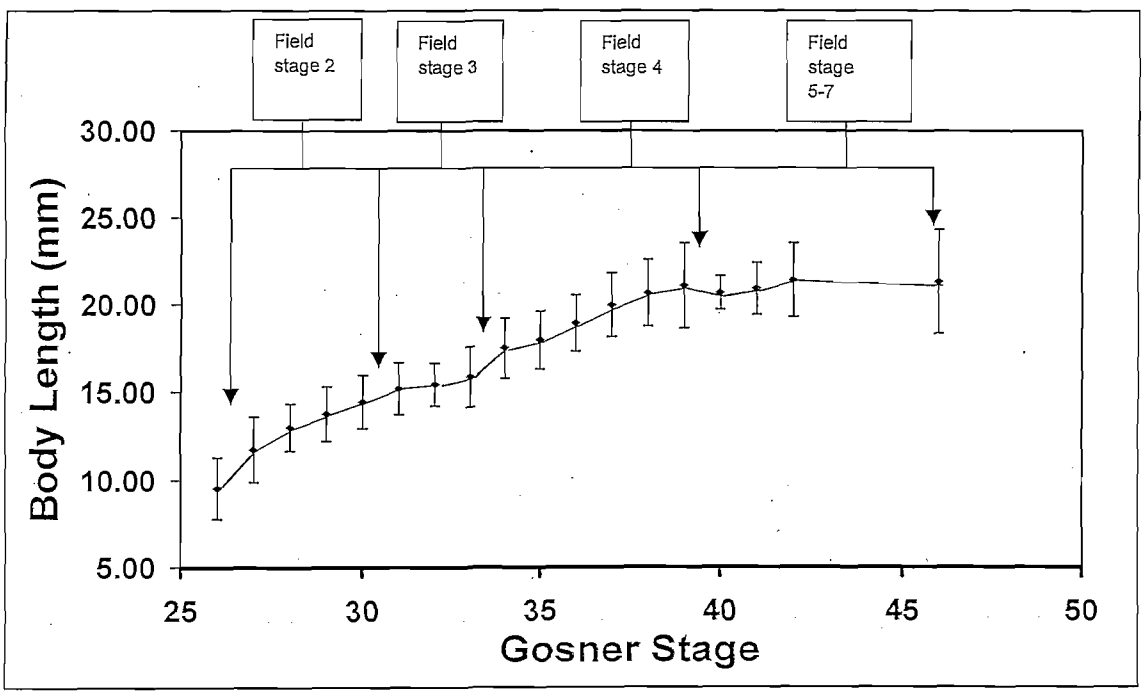

Figure 36: Average body length of *Amietia vertebralis* larvae through metamorphic climax.

Adult frogs of *A. vertebralis* and their tadpoles have a colour pattern that blends in with their environment. I assume that this colour pattern protects amplecting pairs during the breeding season from predators because the colour pattern of the frogs and tadpoles mimics the colour of the substrate. This colour pattern can then protect the tadpoles as well because the streams that they occur in are open and there are few rocks to hide under (Figure 37). This statement remains to be proven

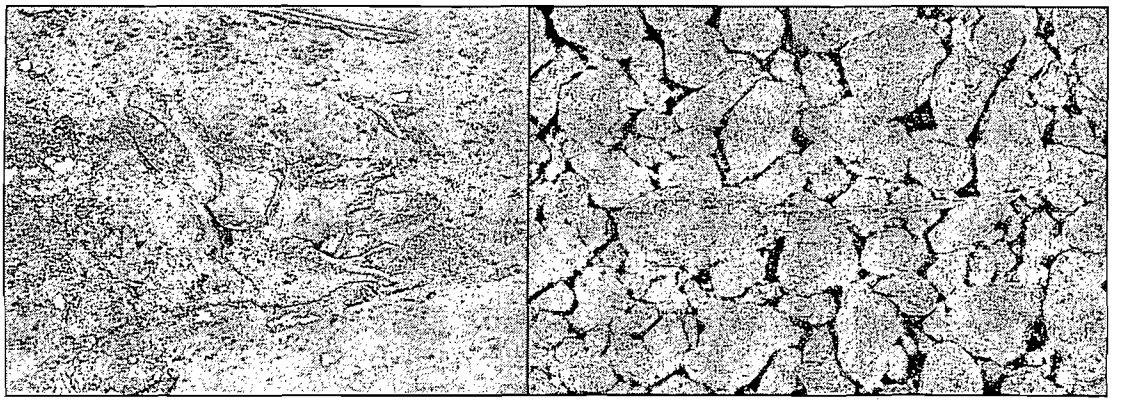

Figure 37: *Amietia vertebralis* adult frogs and tadpoles has a distinct colour pattern that mimics the colour pattern of the substrate and the assumption is made that it is so for protection from predators. This enabled the adult frogs to breed during day time in the streams and pools. This remains to be proven.

3.2 Anuran infection monitoring across an altitudinal transect

KwaZulu-Natal has a climate that varies and is very green thanks to diverse, complex topography. Generally, the coast of KwaZulu-Natal is subtropical with the inland regions becoming progressively colder. Vernon Crookes Nature Reserve on the south coast has an annual rainfall of roughly 1 009 mm, with daytime maximum temperatures peaking from January to March at 28°C with a minimum of 21°C, dropping to daytime highs from June to August of 23°C with a minimum of 11°C. Temperatures dropped towards the interior, with Boston being similar in the summer, but much cooler in the winter. Winterton in the Tugela River Valley reached 30°C in the summer, but dropped below freezing point in the winter evenings. The Drakensberg Mountains experienced heavy winter snow, with light snow occasionally experienced on the highest peaks in summer. The south coast was very humid. The Free State experienced a continental climate, characterised by warm to hot summers and cool to cold winters. Bethlehem is situated in the east of the Free State Province and experienced frequent snowfalls in the winter especially on the higher ranges. The summer months were the months when most precipitation occurred. The annual precipitation for Bethlehem was known to be roughly 680 mm, with daytime maximum temperatures peaking at

27°C from January to March with a minimum of 13°C. The daytime maximum temperatures dropped to an average maximum of 16°C to a minimum of -2°C in July. Koppies is also situated in the Free State Province and had a similar climate as Bethlehem. Potchefstroom is situated in the North-West Province and temperatures ranged from 17° to 31°C in the summer and between 3° to 21°C during winter. The annual rainfall for this region was roughly 360 mm, with almost all of it falling during the summer months, from September to April. Swartruggens also falls in the North-West Province with roughly the same temperatures and rainfall as Potchefstroom (South African Weather Bureau).

Frogs that were collected on the transect that stretched from Vernon Crookes Nature Reserve in KwaZulu-Natal, through the Free-State and into the North-West Province at Swartruggens, are listed in Table 12 below. The transect was sampled three times: twice during the warm, rainy season and once during the cold, dry season. A total of 10 to 20 frogs of each species were collected at each site, where possible. A total of to 20 frogs were not always found, but the number of frogs that were collected per species was still sampled. A total of 366 frogs were collected which represented a total of 18 species. The sites where sampling was done were spaced along an altitudinal gradient (Figure 38). Bethlehem is situated at the highest altitude and Vernon Crookes Nature Reserve at the lowest altitude. Also, Vernon Crookes Nature Reserve is close to the coast whereas Bethlehem is situated well into the interior. The highest diversity of frogs was caught at Vernon Crookes Nature Reserve, with a total of 15 species.

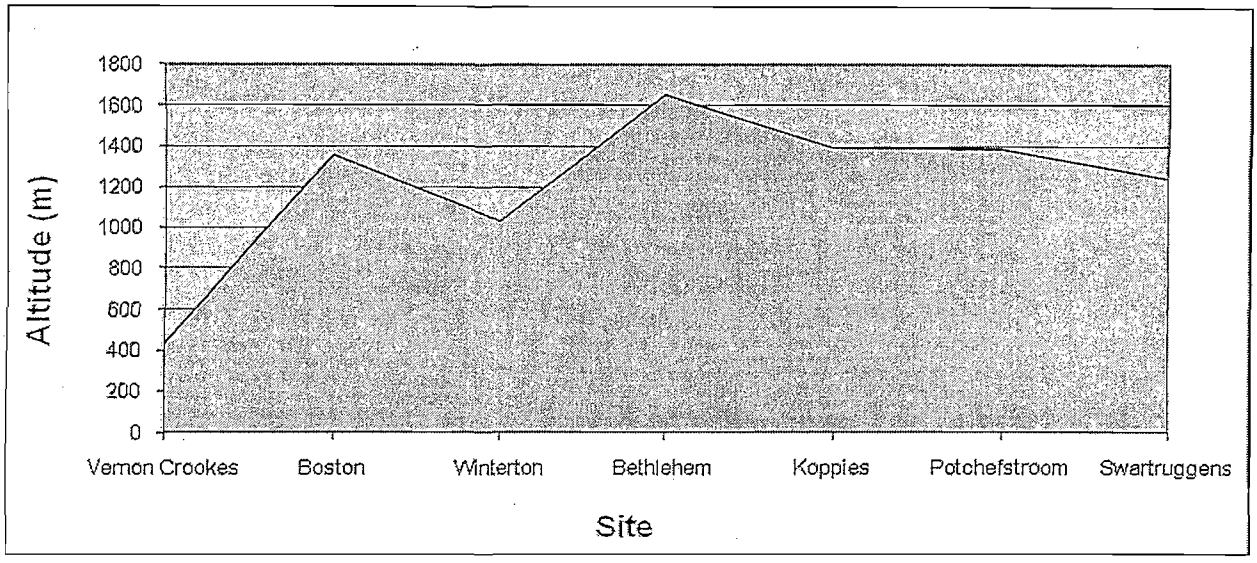

Figure 38: The altitude of each site along the transect.

Table 12: Frog species that were collected on a transect from Vernon Crookes Nature reserve in KwaZulu-Natal to Swartruggens in the North-West Province.

Date	Locality	Altitude (m)	Frog species	n adults
Oct'07	Vernon Crookes Nature Reserve	431	*Afrixalus delicatus*	8
			Amietophrynus gutturalis	4
			Amietophrynus rangeri	2
			Arthroleptis wahlbergi	2
			Hyperolius marmoratus	16
			Hyperolius pusillus	4
			Kassina senegalensis	14
			Leptopelis natalensis	6
			Natalobatrachus bonebergi	1
			Phrynobatrachus natalensis	7
			Ptychadena oxyrhynchus	4
			Schismaderma carens	4

			Strongylopus fasciatus	1
			Strongylopus grayii	2
Oct'07	Boston	1360	*Amietia angolensis*	8
			Amietophrynus gutturalis	10
			Amietophrynus rangeri	1
			Strongylopus fasciatus	5
			Xenopus laevis	10
Oct'07	Bethlehem	1654	*Amietia angolensis*	3
			Amietia fuscigula	2
			Amietophrynus rangeri	10
			Tomopterna cryptotus	10
			Xenopus laevis	1
Oct'07	Swartruggens	1244	*Cacosternum boettgeri*	2
			Kassina senegalensis	1
			Schismaderma carens	2
Oct'07	Potchefstroom	1388	*Cacosternum boettgeri*	2
			Kassina senegalensis	4
			Tomopterna cryptotus	4
Nov'07	Koppies	1395	*Amietophrynus rangeri*	2
			Cacosternum boettgeri	7
Jan'08	Vernon Crookes	431	*Afrixalus delicatus*	13
			Amietophrynus rangeri	10
			Hyperolius marmoratus	6
			Hyperolius pusillus	17
			Leptopelis natalensis	4
			Phrynobatrachus natalensis	2
			Ptychadena oxyrynchus	3
			Xenopus laevis	1
Jan'08	Boston	1360	*Amietia angolensis*	4
			Amietophrynus gutturalis	2
			Hyperolius marmoratus	12
			Xenopus laevis	9
Jan'08	Winterton	11031	*Amietia angolensis*	5

			Kassina senegalensis	5
			Phrynobatrachus natalensis	3
			Schismaderma carens	2
Jan'08	Bethlehem	1654	*Amietia angolensis*	3
			Amietophrynus gutturalis	1
Feb'08	Koppies	1395	*Schismaderma carens*	6
			Xenopus laevis	23
May'08	Vernon Crookes	431	*Arthroleptis wahlbergi*	11
			Cacosternum boettgeri	1
			Phrynobatrachus natalensis	1
			Strongylopus fasciatus	11
			Xenopus laevis	10
May'08	Boston	1360	*Amietia angolensis*	10
			Strongylopus fasciatus	11
			Xenopus laevis	10
Jun'08	Winterton	1031	*Xenopus laevis*	10
Jun'08	Bethlehem	1654	*Xenopus laevis*	6

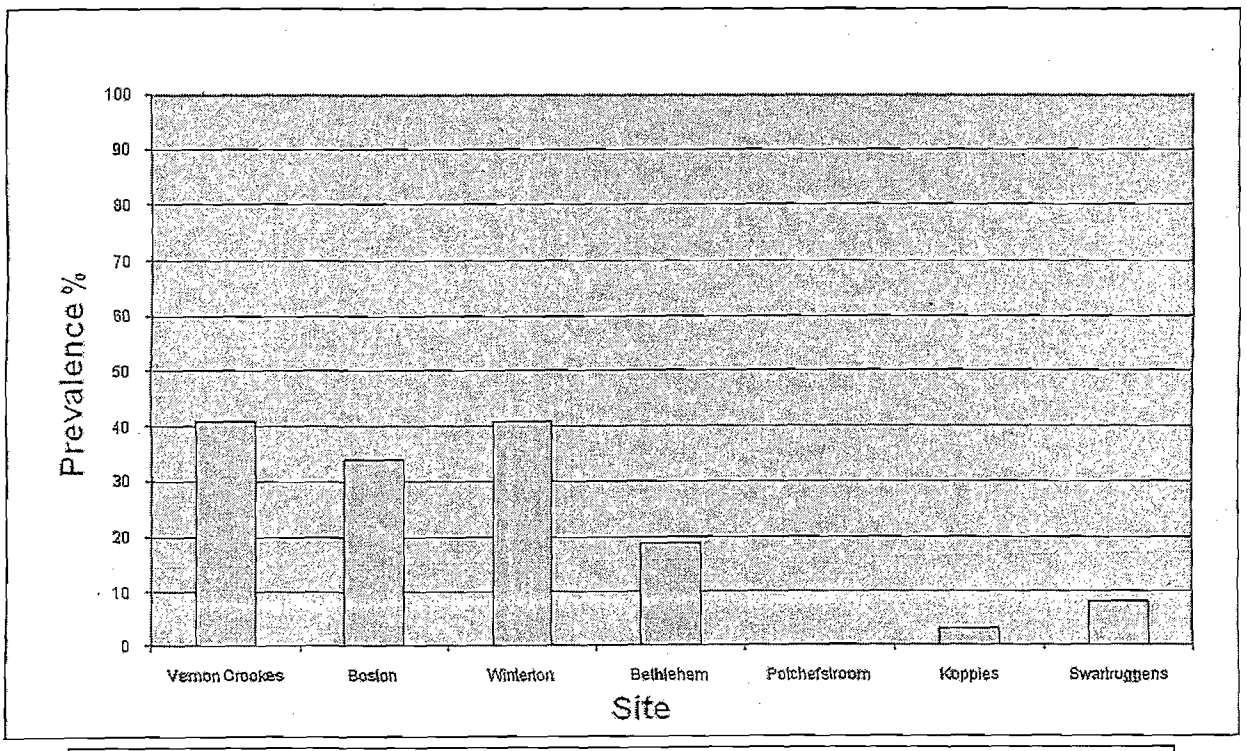

Figure 39: The prevalence of *Batrachochytrium dendrobatidis* for the different sites on the transect.

Prevalence data for the frogs listed in Table 14 is shown in Figure 39. The prevalence was determined by swabbing frogs with sterile medical swabs, and these were analysed with qPCR for *B. dendrobatidis*. The different sites had different prevalence levels, but *B. dendrobatidis* was present at all the sites except at Potchefstroom. The prevalence was highest at the Vernon Crookes Nature Reserve site and decreased as the sampling proceeded inland.

Sampling was conducted during different months to include different seasons. October was regarded as the start of the warm season, January as the warmest month and July as the coldest month (July is in the middle of winter). During the October sampling, infection was found at four sites except at Swartruggens, Potchefstroom and Winterton. Sampling at the Winterton site only began in January 2008. During the January sampling, infection was found at four sites except at Bethlehem, Potchefstroom and Koppies. The prevalence at the infected sites remained fairly constant at about 10%. During the July sampling, which was the coldest sampling month, infection was found at four sites. The prevalence was very high at the infected sites with the prevalence at each site reaching infection levels of between 70% and 90%. No infection was found at the Potchefstroom site and little infection was found at the Swartruggens and Koppies sites (Figure 40). The sampling month with the highest prevalence was May 2008, and January 2008 was the month with the lowest prevalence.

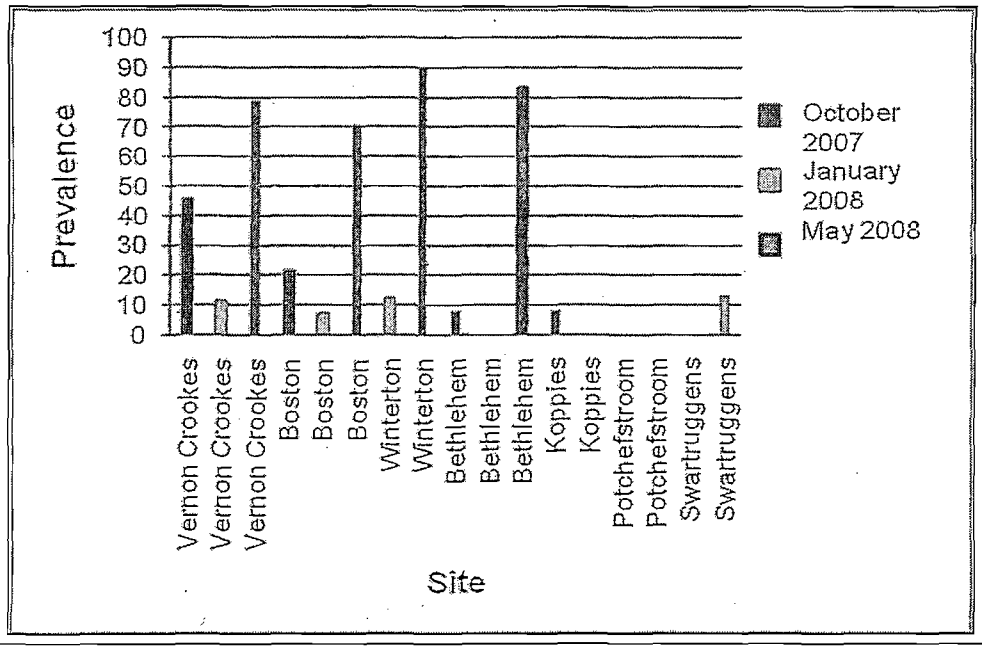

Figure 40: Comparison of the chytrid prevalence for each site.

4 Discussion

4.1 Seasonal variation in *Batrachochytrium dendrobatidis* infections

Tadpoles of *Amietia vertebralis* that were monitored were found to be infected with *B. dendrobatidis* to varied extents. The average prevalence for each site at Mont-aux Sources throughout the 18 months was fairly constant, with the exception of some sites where low average prevalences were found. If prevalence followed a seasonal pattern, it would be expected that the average water temperature would either be low or high during prevalence peaking months. However, this was not the case. Prevalence was fairly low during July 2007 and 2008 when temperatures were low, but generally, prevalence fluctuated too randomly to indicate a clear correlation with temperature. Water temperatures at which *A. vertebralis* tadpoles were collected did not seem to have a significant influence on prevalence for this species. Prevalence has fluctuated frequently among the sampling months and a pattern has been observed for the first 14 months but the latter months were lagging behind in terms of an observable pattern. In the two successive sampling seasons (2006 and 2007) at Mont-aux Sources, prevalence reached peaks during different months, which also indicated that no observable pattern was formed. During July 2007 and July 2008, the prevalence was low at Mont-aux Sources. These were the coldest sampling months of the entire sampling period. During these months the rivers were frozen and the ice had to be broken in order to reach the tadpoles. Low prevalence in this instance could have been attributed to the extremely low temperature since cold influences the growth and infection rate of *B. dendrobatidis.* However, *Batrachochytrium dendrobatidis* was still present even though the rivers were frozen. Only the surface of the rivers freeze and the water still flowed under the frozen surface. Water temperatures did not decrease below 4°C. The water at the tranquil pools seemed to have been colder or completely frozen.

Seimon *et al.* (2008) found three anuran species in deglaciated ponds and two of these species were found to be infected with *B. dendrobatidis*. Zoospores in the surface layer of the river that

freezes die, but the zoospores in the flowing water under the frozen surface remain alive and active. Although zoospores were lethargic they were still infective during cold and harsh conditions. Rivers and ponds in Royal Natal National Park did not freeze during winter and the tadpoles did not occur in such harsh conditions as the tadpoles at the Mont-aux Sources sites.

Tadpoles found at the Royal Natal National Park sites were also infected with *B. dendrobatidis*. *Hadromophryne natalensis* infections seemed to follow a pattern determined by temperature. As average water temperatures increased from September 2006, the prevalence also increased. Prevalence followed the temperature gradient: when it started to get colder, prevalence decreased. This indicated that the lower altitude sites at Royal Natal National Park followed a pattern that correlated with temperature while high altitude sites at Mont-aux Sources showed a less distinct pattern. The reason for *B. dendrobatidis* infection levels not following a pattern consistent with temperature changes can be attributed to different temperature regimes at the two areas. The Mont-aux Sources area, as mentioned, is situated at high altitudes and is associated with very cold temperatures. This area is also situated in open grasslands on top of the plateau. These sites were exposed to full sunlight, explaining the increased influence on the water temperature at each site, but the high altitude had a counter-effect and lowered the temperature. Temperatures observed at the Mont-aux Sources sites were within the viable temperatures for chytridiomycosis. The Royal Natal National Park sites are situated in small patches of forest and are shaded. The lack of sunlight on these waterbodies explains the low recorded temperatures. These sites are also located at lower altitudes and the air temperature at lower altitudes tends to be higher than air temperatures at high altitudes. The prevalence at Royal Natal National Park was higher than at Mont-aux Sources for both the cold and the warm seasons. The average prevalence for each site at Royal Natal National Park throughout the 18 months of sampling was fairly constant, with the exception of some sites that had low average prevalences, but the average prevalence for Royal Natal National Park was higher than at Mont-aux Sources. Prevalence was higher at Royal Natal National Park because of the low altitude of this area and the concomitant warmer temperatures of these sites. The observed chytridiomycosis prevalences closely matched the seasonal temperature pattern at the Royal Natal National Park sites.

When compared according to chytrid prevalence, the Mont-aux Sources and the Royal Natal National Park sites, followed different patterns. Prevalence at Royal Natal National Park and Mont-aux Sources differed significantly throughout the 18 months that sampling period. The months when Royal Natal National Park and Mont-aux Sources followed the same tendencies were September 2006, July 2007, and November 2007 to March 2008 and in July 2008. Temperature on

the other hand, for both the sampling areas followed the same general patterns. After the high prevalence during November, a sharp decrease in prevalence occurred during both sampling years (2006 and 2007) at Royal Natal National Park and Mont-aux Sources areas. This could not be ascribed to a fluctuation in temperature, but rather to another factor that influenced infection prevalence. Because no constant pattern was observed between prevalence and temperature at the different areas, even though temperature at both areas followed the same pattern, other factors together with temperature had to play a role regarding the fluctuation of prevalence.

The amphibian chytrid fungus is known to grow best between 17°C and 25°C, with an optimum growth at 23°C (Daszak *et al.,* 2003; Berger *et al.,* 2004; Piotrowski *et al.,* 2004). Because of the fog and frequent cloud cover on top of the mountains, the amphibian chytrid fungus is probably favoured by the climatic conditions. Since this fungus stops growing at 28°C and dies at 30°C, the cloudiness and fog made the area humid and maintained the moist conditions that aided the survival, growth and reproduction of the pathogen. The temperatures at the Royal Natal National Park sites did not exceed 30°C and fell within the optimum temperature range for amphibian chytrid growth. This could be ascribed to that fact that the sites at Royal Natal National Park are situated in the shade and the temperatures were thus lower. The Mont-aux Sources sites are situated in full sun with the temperatures also reaching the optimum temperature range for amphibian chytrid growth. Temperatures at the Mont-aux Sources sites that were heated by the sun during the day helped with the growth and transmission of amphibian chytrid. It was suggested by Retallic *et al.* (2004) that warmer sites could have a higher prevalence, but the climate in South Africa is not the same as in a rainforest. The temperatures did not exceed 30°C during either the winter or summer months.

It has been shown that the fungus tends to be more lethal under moist conditions (La Marca *et al.,* 2005; Piotrowski *et al.,* 2004). In the Drakensberg Mountains, the conditions are typically very wet and humid, especially during the summer months with frequent precipitation and fog. During the winter months the streams tend to dry up, but there was still fog that covered the mountains on warmer days. A further influence of fog on high altitude frogs is the climate-linked contaminant pulse hypothesis. Fog and cloud water in montane regions collect atmospheric contaminants and these contaminants reach critical levels when conditions in the mountains are warm or dry (Pounds & Crump, 1994). These contaminants could have a negative effect on frogs by compromising their immune systems. This could make them more susceptible for disease. Furthermore, snow covers most of the mountain top during the winter months. These different conditions during winter and summer could have influenced the prevalence of infected frogs. During the cold winter months

frogs, were under severe stress due to the fact that the streams dried up, low temperatures and the clustering of tadpoles in streams and pools. This, in turn, could have increased their susceptibility to the disease.

Heavy rains had a marked influence on prevalence at the Mont-aux Sources and Royal Natal National Park sites. In the Drakensberg Mountains frequent flash floods occur that could last from one to 14 days. After November 2007, the prevalence decreased drastically up to March 2008 with a prevalence of 7% for January 2008 and March 2008 at the Mont-aux Sources sites. This was caused by heavy rains and floods that occurred during November 2007 and especially January 2008. Precipitation and prevalence were negatively correlated at the two sampling areas. Kriger and Hero (2006) found a negative relationship between precipitation and disease prevalence. Boykin and McDaniel (2008) found that the differences in stream flows have the potential to influence the infection and growth of the amphibian chytrid fungus. Prevalence as found in the current study could then be explained in two ways: (1) either the floods flushed away most of the infected tadpoles or (2) the quantity of water in the rivers was such that the tadpoles and zoospores were distributed according to a very low density (dilution effect). Prevalence increased again after heavy rains subsided at the Mont-aux Sources area. This could have been the case because reduced flow rates permitted ample opportunities for disease transmission. Precipitation occurred during some of these months, and affected prevalence at both the Mont-aux Sources and Royal Natal National Park sites. One could therefore conclude that heavy rainfall has a marked influence on infection prevalence, and temperature also had an influence. Nonetheless, the effect of temperature in summer was overshadowed by the effect of heavy rainfall.

4.2 Threat assessment of *Batrachochytrium dendrobatidis*

Despite significant infections among *H. natalensis* tadpoles, no mortalities were observed in metamorphosed frogs. It appears as if this species does not become diseased by chytridiomycosis. There are many examples of species resistant to chytridiomycosis. Retallick *et al.* (2004) conducted experiments on *Taudactylus eungellensis* and found that this species could survive while infected with the pathogen. The same is true for *Xenopus laevis,* which is known to survive and to be a reservoir host for *B. dendrobatidis* (Weldon *et al.*, 2004). Blaustein *et al.* (2005) also found that the bull frog (*Rana catesbeiana*) is susceptible to the pathogen, but does not die from being infected. Mortalities, were however, found in *A. vertebralis.* Dead amphibians in general are a rare occurrence in the field as carcases are frequently picked up by scavengers or decompose rapidly. Although the observed die-offs did not involve large numbers of frogs, and only 40 dead frogs were found during the course of a year, the mortalities still support the observation that this is a fairly

regular occurrence. A decline in the population was, however, not observed. Mortalities of *A. vertebralis* were encountered throughout the year. Die-offs did not occur in a seasonal pattern. Some dead juveniles that were collected were screened for chytrid and were found to have been infected. This suggests that the mortalities were caused by the pathogen. Interestingly, mortalities were not found at all the sites, but were usually found at the same sites on subsequent visits.

B. dendrobatidis survives on the host in cold conditions. The clustering of tadpoles during the cold months could have contributed to sustained transmission between individuals at a low rate despite sub-optimal conditions. This would explain the increase in prevalence at the onset of spring. Herrera *et al.* (2005) found the same pattern in Argentina, where they detected an outbreak in the middle of winter (mid-July) while the annual temperatures were at their lowest. Their finding is contrary to other cases described by Daszak *et al.* (1999) and Bradley *et al.* (2002), where outbreaks were observed during the late winter and early spring when temperatures were rising.

Amietia vertebralis is susceptible to the disease and die-offs have occurred in adult and juveniles of this species. Amphibian population declines have also been noted to be the most significant in species that have a strong association with streams (Williams & Hero; 1998, McDonald & Alford, 1999;. Lips *et al.,* 2003; Hero *et al.,* 2005). Although only die-offs have been found, it is also possible that the occurrence of population declines in *A. vertebralis* could occur at a later stage because it fits all the profiles for high altitude amphibian species that have experienced population declines.

There were some tadpoles that seemed to be feeding off of the carcases of the dead frogs. These dead frogs could have served as reservoirs for chytridiomycosis and zoospores could also have been released into the water. Due to the fact that *A. vertebralis* occurred at high altitudes and was very susceptible to the fungus, the possibility for a decline exists. Herrera *et al.* (2005) conducted a study on *Leptodactylus ocellatus* in Argentina and found dead amphibians during mid-winter (July), when annual temperatures were at their lowest. This was contrary to other documented cases where (Daszak *et al.,* 1999; Bradley *et al.,* 2002) outbreaks have occurred in late winter and early spring when temperatures were rising. The majority of declines and extinctions of amphibian populations all across the world have been focused in montane regions (Bradford, 1999; Richards *et al.,* 1993; Lips, 1998; Bosch *et al.,* 2001; Muths *et al.,* 2003; La Marca *et al.,* 2005; Rachowicz *et al.,* 2006) and approximately 85% of the world's frog's species under threat occurred at high altitudes (Hero & Morrison, 2004).

In the 2007 data, die-offs seemed to coincide with the breeding season of *A. vertebralis* from September through to March. As breeding commences, the frogs tend to lose condition as a lot of energy is directed towards finding a mate and mating, and less on feeding. Frogs in amplexus are in very close contact with each other, and this subsequently resulted in the transmission of the pathogen from one individual to another because of frog to frog contact which is a recognised mode of chytrid transmission (Berger, 1999a; Daszak 1999). Since the conditions of the reproducing frogs are already to some extent compromised, they were probably exposed to high doses of the pathogen. During the breeding season the prevalence was also extremely high. Prevalence reached a peak in January 2008, which was almost at the end of the breeding season. This prevalence indicates that heightened transmission of the pathogen occurred through the breeding behaviour of this species and more individuals were infected during the later stages of the breeding season.

The warm months could also have influenced the prevalence of *B. dendrobatidis* directly due to the fact that the temperature fell in the preferred temperature ranges for amphibian chytrid growth. Berger *et al.* (1994) and Daszak *et al.* (2003) suggested that lower temperatures benefit the amphibian chytrid, but Pounds *et al.* (2006) suggested that this might be an oversimplification of the pathogen's response to different climates. Lower temperatures could, nonetheless, have been the reason for the fluctuation in prevalence at the Mont-aux Sources area. The timing of the die-offs could be explained by the optimum growth of *B. dendrobatidis.* Tropical regions such as Australia have a warmer climate than the top of the Drakensberg Mountains. Temperature fluctuations for the two different regions (tropical and temperate) are indicated graphically (Figure 41). The ideal growth temperatures for *B. dendrobatidis* are indicated with horizontal lines. During summer months in temperate regions, such as the Drakensberg, the average day temperatures reach 28°C and 18°C at night. These temperatures fall within the optimum growth zone of *B. dendrobatidis.* During the winter months in temperate regions, the temperatures dropped well below the optimum zone for *B. dendrobatidis.* In contrast, summer temperatures in the tropics reach 35°C and warmer. These temperatures are too high for *B. dendrobatidis* and thus suppress its growth during the warm season. The winter temperatures for tropic regions fall within the optimum growth zone for *B. dendrobatidis* (Retallick *et al.,* 2004). This optimum temperature-linked die-off hypothesis could explain why the tropics experience die-offs in winter, and temperate regions experience die-offs during summer.

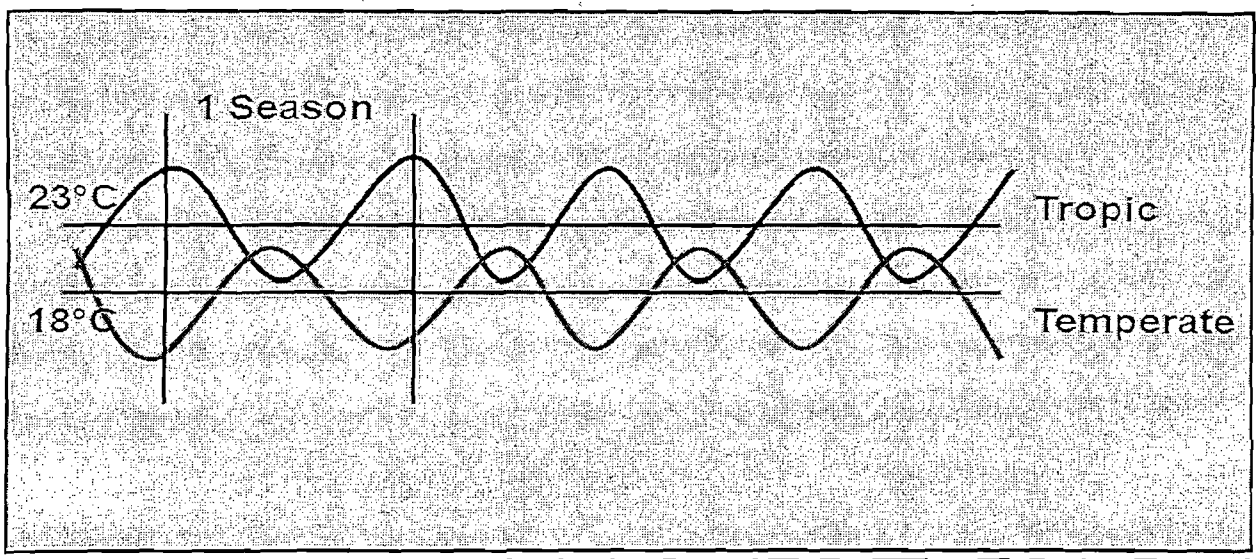

Figure 41: Comparison of temperature fluctuations between temperate regions and tropic regions to indicate the overlay of optimum growth temperatures for *Batrachochytrium dendrobatidis*.

During the colder months of May 2007 and 2008 to July 2007 and 2008, prevalence decreased. The low prevalence could also have been due to the low temperatures that restricted the growth and transmission of the pathogen. As the temperatures began to rise from September onwards, the prevalence increased again. In September 2006 and 2007, the prevalence was high directly after the cold winters. As the temperature increased to the optimum growth zone for amphibian chytrid, the prevalence started to increase. This could have been an indication that the fungus might not completely be removed and died off during the cold conditions, but it might experience a rest and lethargic phase. Infected tadpoles were found during the cold months, which indicate that the pathogen was present all year round.

At some sites dead *A. vertebralis* tadpoles were also found. This could mean that although the tadpoles survive infections, they could also be susceptible to the disease. Dead tadpoles were only found on some occasions and only a few were found every other month. Most of the time no dead tadpoles were found at all and all those tadpoles that were infected seemed completely healthy. Blaustein *et al.* (2005) provided experimental evidence that larval stages may also be susceptible to exposure of *B. dendrobatidis*. They found that larvae of some species died after exposure, while other species did not die and appeared to behave normally. It was found that tadpoles could have high infection prevalence and yet may not be susceptible to the amphibian chytrid pathogen, but in turn the tadpoles could exist in stream environments for several years with infection (Woodhams & Alford, 2005; Rachowicz *et al.*, 2006) and thus act as a vector that continuously releases zoospores into the streams.

Oral deformities and depigmentation of keratodonts and rostrodonts were found in the tadpoles of *A. vertebralis* and *H. natalensis.* Chytridiomycosis was the cause of the deformities and depigmentation of the keratodonts and rostrodonts. The mouthparts of *H. natalensis* had brown pigmented markings between the keratodonts rows and this was caused by the amphibian chytrid. *Batrachochytrium dendrobatidis* tended to infect only the mouthparts of tadpoles that were heavily pigmented (Berger *et al.,* 1998; Pessier *et al.,* 1999; Marantelli *et al.,* 2004; Altig & McDiarmid, 1999). Tadpoles that were infected appeared healthy and did not seem to be negatively affected at all. Because sporangia parasitized on the keratinised tissue, the loss of keratinised mouthparts has been linked to infections with *B. dendrobatidis* (Drake *et al.,* 2007). Although depigmentation could not be used as an accurate indicator of chytridiomycosis, this pathogen did cause depigmentation in some cases (Knapp & Morgan, 2006), although Smith *et al.* (2007) found that in *H. natalensis,* there were clearly defined areas of brown pigmentation where chytridiomycosis occurred. They found that the identification of brown foci by using a hand lens was highly reliable. The effects of *B. dendrobatidis* on tadpoles have included the decrease in body mass at pre-metamorphosis, impaired grazing and reduced growth and slower development (Rowe *et al.,* 1996; Parris, 2004).

4.3 Disease dynamics within *Amietia vertebralis*

In the Drakensberg Mountains at the Mont-aux Sources sites, the prevalence differed according to the site location. Sites that were located at the head waters of the river tended to show a lower prevalence than downstream sites. Ribbon Falls Upper and Vemvame Upper were upstream sites in the Mont-aux Sources area and had lower prevalences. When upper and lower sites were compared, it was established that tadpoles from the lower sites were more infected than those from the upper sites. Smith *et al.* (2006) found a correlation between tadpole size and level of infection. Larger and more developed tadpoles were found to be more susceptible. This could have been because the older tadpoles had longer times of possible exposure to zoospores or direct contact with infected individuals. In the present study I also found a positive correlation between tadpole development and infection status. This could also have affected the transmission of chytridiomycosis. The presence of more developed tadpoles would enhance the likelihood of being infected (Smith *et al.,* 2007). The mean Gosner stage for infected tadpoles was 36 and for uninfected tadpoles 32. All ten different Mont-aux Sources sites that were monitored had infected tadpoles and thus 100 % occupancy for *B. dendrobatidis.* Although there was statistical significance between the sites, it was not practically significant and had a small effect in the field.

Upper and lower sites in the Ribbon Falls River showed a larger correlation between the development and infection of the tadpoles than the upper and lower sites in the Vemvame River. The sites located in the Ribbon Falls River had an elevation difference between them of roughly 100 m, whereas the sites located in the Vemvame River had an elevation difference of roughly 20 m. The difference in elevation could possibly explain the observed difference in tadpole distribution and infection status.

Two other sites located at the head waters in rivers in the Mont-aux Sources, Nampolice and Tugelahead area also showed a low prevalence. The lower infection levels observed at these sites supports the observed pattern of lower infection levels at the headwaters. Therefore the infection levels at the different locations in these rivers differed because of flow dynamics resulting in a downstream movement of zoospores and tadpoles. Thus tadpoles at the lower sites encounter a bigger dose of zoospores than those at the upstream sites and this affected the infection of the tadpoles. Carey *et al.* (2006) found that the dose of *B. dendrobatidis* zoospores that a frog encounters can affect the infection of the frog. High doses of *B. dendrobatidis* zoospores results in more severe infections and shorter average survival in *Anaxyrus boreas.* This phenomenon could explain the die-offs of *A. vertebralis* at downstream sites.

Tadpoles of *A. vertebralis* were frequently in contact with each other, especially during the colder months when tadpoles clustered together in the streams that dried up and the rivers that froze. The dose of zoospores that was transmitted between the tadpoles that were clustered together had an influence on the infection levels. This could have caused the zoospores to be transmitted along the river when it rained. The clustering of tadpoles would result in higher concentrations of zoospores per volume water that might have caused the infected individuals to succumb to chytridiomycosis. Feeding of tadpoles on the carcasses of dead frogs could also influence the concentration of zoospores. The pathogen could have used the congregation of tadpoles as reservoirs because the tadpoles were not affected by the pathogen. If frogs were also dependant on these dried-up streams and ponds, then continuous transmission could have taken place between adult frogs and tadpoles. When frogs are infected with *B. dendrobatidis,* the intensity of the infection must reach a certain threshold of zoosporangia before the infected frogs succumbed to chytridiomycosis and larger inocula would tend to reach that threshold faster (Carey *et al.,* 2006). It was not obvious which of the observed behaviours would expose them to highest concentrations of zoospores, but direct contact was most likely the most obvious explanation resulting in more transmission between individuals (Rowley & Alford, 2007).

4.4 Breeding behavior of *Amietia vertebralis*

The breeding season for *A. vertebralis* has been reported to be September through to March (Channing 1979), but in this study, adults were found breeding almost throughout the year showing that this species is an opportunistic breeder with a prolonged breeding season. Its reproductive behaviour thus would have a direct effect on opportunities for transmission of *B. dendrobatidis*.

Phofung river frogs tended to breed upstream and as tadpoles developed, they migrated or were flushed downstream and developed to larger tadpoles at lower sites. Zoospores could have been flushed downstream which contributed to a higher infection of tadpoles downstream. This effect was more pronounced at Ribbon Falls River than at the Vemvame River. Ribbon Falls had a large number of little waterfalls and rapids that also prohibited the tadpoles from swimming back upstream, whereas the Vemvame River did not have these obstructions, thus allowing the tadpoles to freely move up and down the river.

Temperature plays a determining role on anuran life, determining larval body size and length of the larval period (Reques & Tejedo, 1995; Smith-Gill & Berven, 1979), and this is, in turn, related to the survival and reproductive success of tadpoles (Berven, 1982; Berven & Gill, 1983; Berven *et al.,* 1979; Collins, 1979; Crump, 1989; Harkey & Semlitsch, 1988; Smith, 1987). Growth parameters were not always good predictors of timing of metamorphosis because growth and differentiation were affected differently by the different environmental factors such as temperature and population density. Growth rate was also related to the duration of larval development (McDiarmid, & Altig, 1999); because conditions that were favourable for differentiation were also favourable for growth (Smith-Gill & Berven, 1979). Body length of *A. vertebralis* tadpoles was found to have a positive correlation with its development. A distinct correlation between body size and larval development was observed. The standard deviation for each Gosner stage was very small and this indicated that very little variation in body length existed within the life stages. Specific clutches of different tadpole stages would be observed during the year if a frog species have a distinct and short breeding pattern. Due to the fact that *A. vertebralis'* breeding was stated in literature to be from September to March (Channing, 1979), the tadpoles collected indicated an extended breeding season.

The data point to three possible breeding strategies:

1) *Amietia vertebralis* has a longer breeding season, unlike that which was reported in the literature. Amplecting pairs were found outside their normal breeding season, as early as June. 2) That *A. vertebralis* are opportunistic breeders and only tended to breed whenever favourable conditions

initiate spawning. Favourable conditions could also have been conditions that were not too cold. For example, the amplecting pair was found in July, which is during winter, but the rivers were not frozen and the temperatures were not as cold. 3) Tadpoles breed from September through to March, but the tadpoles have a long development and take a long time to complete their metamorphoses. Tadpoles were present all year round - even in the harsh and cold winter months. Most of the different field stage groupings were present throughout the year. Developmental stages 42-46 were not encountered throughout the year (although we did not specifically search for them) and this could be explained by the fact that these tadpoles and juvenile frogs were hiding during day time in crevices and under the river banks and rocks. Tadpoles at other stages did not usually hide, although some tadpoles tended to hide under small rocks when they observed us. These rocks were turned over and the tadpoles could easily be collected with dip nets.

Stages 42-46 were crucial stages where the tadpoles developed faster and went through the final stages of metamorphosis. The pathogenic fungus *B. dendrobatidis* did infect tadpoles during these stages as well (Smith *et al.*, 2006) which resulted in mortality. This could also have been the cause of these stages not being as abundant as earlier stages. The other stages were present all year round and no multiple clutches of tadpoles were observed.

The cryptic colour patterns of adult *A. vertebralis* enabled them to breed during the daytime in the slow flowing streams and pools. The fact that amplexus takes place under water also provides protection from terrestrial predators. Tadpoles at certain stages did not hide when we approached the streams and pools; they kept still and blended with their environment - which makes it difficult for predators to spot them. Some adult frogs were spotted in amplexus as late as June. This was far outside the known breeding season for this species. June is during the middle of winter and the temperatures were very low during this month. It was unexpected to observe breeding at this time of the year as water levels are generally low in winter and temperatures are harsh, making it difficult for young tadpoles and eggs to survive. Another factor that made this behaviour strange was the fact that it was easy for the few predators that are present during that time to feed on the eggs and small tadpoles during this time of year because of the shallow and small, dried-up pools where the eggs were laid. For example, eggs were found at the beginning of September 2007 in a shallow pool. These observations indicates that this species breeds right through the year or that this species can be considered an opportunistic breeder.

Further research must still be done on this topic concerning the physiological development of tadpoles over time. A grow out study of the tadpoles of *A. vertebralis* under controlled laboratory

conditions to determine rate of tadpole development will provide valuable information. Monitoring of *A. vertebralis* should be continued on a long-term basis to determine its exact breeding timeframe. Another aspect that needs attention is which section of a river the frogs prefer to breed at, or whether they seem not to prefer specific sections. Different rivers must be selected for this part of the research, and monitoring must be done along the entire stretch of river. The time at which this species is active - day or night - must be clarified, as well as when and in what manner the frogs call (whether they call under water at rapids or on the river banks), and where egg clutches are laid.

4.5 *Batrachochytrium dendrobatidis* infection across an altitudinal transect

The amphibian chytrid was found to be widely distributed all along the altitudinal transect that stretched from KwaZulu Natal through the Free State and in to the North-West Province. Evidence was found that infections decreased as altitude increased. Prevalence was the highest at the sites with lower altitudes. Vernon Crookes Nature Reserve, Boston and Winterton were the sites with the highest prevalence, whereas Potchefstroom was the site with the lowest prevalence (there were no infected frogs found in Potchefstroom). Although the prevalence at the lower elevation sites was higher, no die-offs were observed.

Prevalence differed according to season, but the chytrid was present during every season. During the October sampling, the temperatures reached favourable conditions for amphibian chytrid all along the transect. The sites at the coast that were humid had the highest prevalence and as sampling of sites progressed inland, infection was still present but not as high as at the lowland sites. During January the climate at the coastal sites were very hot and humid reaching temperatures above 30°C – these temperatures dampened the rate of infection at the sites but did not clear the infections. The inland sites also reached very high temperatures and this could have influenced the prevalence at these sites. The prevalence was fairly constant across all of the sites along the transect with prevalence of roughly 15%. There were sites that had no infected frogs. This could have been due to low sampling sizes. In May 2008, prevalence increased rapidly across the transect and the highest prevalences were observed. No frogs were found at Koppies, Potchefstroom and Swartruggens during May 2008, because anuran activity in this region is restricted to the rainy season. May was the start of winter and as temperature started to fall, prevalence increased. The temperature was suitable for chytrid growth and reproduction at all of our sites regardless of the altitudes throughout the season, although May was the coldest month.

The conditions in KwaZulu-Natal were more favorable to *B. dendrobatidis* due to the fact that the temperature gradients fell in the preferred zone for chytrid growth more often. At these sites,

frogs were as likely to be infected with *B. dendrobatidis* as were high altitude frogs. The low altitude frogs also carried infections as intense as the frogs at high altitudes. Kriger *et al.* (2007) conducted a study and found that a decrease in the intensity of chytrid infections in frogs occurred at low altitudes in regions close to the equator. This suggested that temperatures at sites at low altitudes were too hot to sustain high infections of this pathogen. Although these regions were too hot, it could be said that at high altitudes in these regions, there might have been a significant increase in infection levels where it was cool enough for the pathogen to thrive (Kriger & Hero, 2008).

The longer chytrid was present in frog populations at high altitudes the more likely it is that the temperature would have reached conditions suitable for disease outbreaks. As temperatures dropped, but were not very harsh, more time was allowed for uninfected frogs to become infected and even worsen the effects of chytridiomycosis on frogs. However, it is believed that chytrid has been present in South Africa for decades. It was stated that high altitude frogs were more likely to sustain their infections for a longer time periods than that of frogs that live at lower altitudes. Frogs that occurred at low altitudes could have cleared their infections in the warm summers (Kriger & Hero, 2006).

Precipitation occurred frequently in KwaZulu-Natal and the climate was very wet and humid, which favoured *B. dendrobatidis*. Cold winters in KwaZulu-Natal were also affected by occasional rain that kept the sites wet and more favorable for *B. dendrobatidis* in the winter as well (Nel & Sumner, 2007). Precipitation decreased towards the interior and drier conditions occurred so that the humidity decreased. The areas where the sites are situated were still wet, but some dams dried up during the May sampling in Koppies, Potchefstrrom and Swartruggens. This lowered infection levels.

Batrachochytrium dendrobatidis appeared to infect frogs at all altitudes in the subtropics; this suggesting that populations at any altitude were at risk of declining when the conditions were favourable for disease outbreaks (Kriger & Hero, 2008). Young *et al.* (2001) found that in Central America declines have occurred above 500 m (a.s.l.) and above 1000 m in the Andes Mountains. Evidence has shown that *B. dendrobatidis* prefers cooler temperatures. The fungus grows best and was most virulent below 25°C (Longcore *et al.*, 1999; Lamirande & Nichols, 2002; Berger *et al.*, 2004; Piotrowski *et al.*, 2004). Studies have also shown that infected wild populations showed an increase in chytrid prevalence in cooler months (Berger *et al.*, 2004; Retallick *et al.*, 2004; Ouellet *et al.*, 2005; Woodhams & Alford, 2005; Kriger & Hero, 2007), and also at cool latitudes (Kriger *et al.*, 2007). The temperature requirements that *B. dendrobatidis* grows and infects at, together with

the fact that the pathogen has been found in dead and dying frogs can be said to be synchronised with die-offs at high altitude regions all across the world (Berger *et al.,* 1998; Bosch *et al.,* 2001; Weldon & Du Preez, 2004; Lips *et al.,* 2006; Rachowicz *et al.,* 2006). Not only has *B. dendrobatidis* been found at high mountainous regions (Seimon *et al.,* 2007), but it has also been found close to sea level (Kriger *et al.,* 2007). Because chytrid has been found at sea level, it would be expected that as elevation increases, the prevalence or the intensity of infections would also increase. Little evidence was found to support this notion.

There were different factors present that influenced the infection levels of *B. dendrobatidis* in frog species such as temperature, precipitation and altitude. All of these factors did not influence prevalence in the same manner. Precipitation had a negative influence on infection levels in some cases, but it also influenced infection levels in a positive manner. When precipitation occurred constantly, prevalence seemed to increase or remain constant, but when floods occurred, prevalence decreased due to zoospores being washed away. Temperature had a positive influence on prevalence levels most of the time. Temperatures all along the transect fell within the optimum growth range for *B. dendrobatidis* and this assisted the pathogen in growing and infecting individuals. At high altitudes such as the Drakensberg Mountains, temperature played a less influential role in prevalence levels. Altitude did not influence infection levels as much. KwaZulu-Natal was the site with the highest prevalence throughout all of the seasons.

I was unable to identify a single climatic factor having the biggest influence on chytridiomycosis prevalence. All factors seemed to influence prevalence in different ways stressing the dynamic nature of this amphibian disease. This is an indication that *B. dendrobatidis* is not only widely occurring across South Africa, but it is also common among several species. Some species of amphibians however are more susceptible to become infected.

5 Conclusion and future research and improvements

It has been said that global warming could alter the geographical ranges of many pathogens (Epstein 2001, Harvell *et al.* 2002) including *Batrachochytrium dendrobatidis* and this sequentially could cause declines in amphibian species all across the world. This study showed that temperature could alter the effects of disease development and infection in anuran species. As temperature shifts –increases and decreases- it moves into the optimal growth zone for *B. dendrobatidis* in some areas that were outside of this range before (Berger *et al.* 2004). These temperature fluctuations could also have influenced the patterns of cloud formations in the Drakensberg Mountains. These agents could alter the thermal, light and moisture environments of many organisms. This could also have changed ecological interactions between anuran species and the amphibian chytrid pathogen (Pounds *et al.* 2006). Prevalence followed a pattern according to temperature but other factors, such as rainfall, influenced this pattern during some of the months in the year. Temperature may have influenced the infection of frog populations in the Drakensberg Mountains, but other factors that correlate with temperature seemed to have had an influence as well.

Rainfall in most of southern Africa occurs during the warm summers. I found that rainfall had a negative correlation with infection prevalence. It seemed that heavy rainfall tended to reduce the pathogen within a population. During the months that rainfall was constant prevalence tended to increase. It therefore appears that floods or heavy rainfall is the main factor that influences infection levels in *Amietia vertebralis* populations. Tendencies at both areas, Mont-aux Source and Royal Natal, according to prevalence were shadowed by rainfall and the pattern that should have occurred when prevalence was correlated with temperature was not observed.

Altitude also did not seem to have an influence on *B. dendrobatidis* infection levels. I found infections of the amphibian chytrid along the entire altitudinal transect that stretched from the coast to central South Africa. Chytridiomycosis therefore infects any amphibian species within an altitudinal range of this extent. Although *B. dendrobatidis* was found at all the sites across the transect, mortalities were only found at high altitudes but without any observable population

declines. This is an indication that environmental conditions at high altitudes tend to favor chytridiomycosis outbreaks. The results also shows that *B. dendrobatidis* is widely spread across the country and can infect any frog.

More basic studies are needed on temperature and rainfall and the influence they have on prevalence of chytrid in anuran species. Research must also be done to evaluate the behavior of *A. vertebralis* species during cold and dry winter months. The need to better understand the nature and dynamics of chytrid-linked amphibian mortalities in the Drakensberg Mountains is critical because *A. vertebralis* is susceptible to the disease and declines in this species could happen at any moment. I also recommend that the monitoring of the high altitude populations in the Drakensberg Mountains continue in order to attain long-term data on the ecology and disease dynamics of *B. dendrobatidis*.

The breeding behavior of *A. vertebralis* was known to be from September until March. We observed amplecting pairs of *A. vertebralis* in mid winter (July) and tadpoles were present all year round. The data points to three possible breeding strategies, but the strategy that seems to be most relevant is the one where we purpose that *A. vertebralis* has a extended breeding season.Further monitoring of the breeding behavior of *A. vertebralis* adults must be done to establish the exact breeding season of this species. Tadpoles should also be taken through metamorphosis to determine the development time of the tadpoles under different environmental conditions.

References

Alexander M.A . & Eischeid J.K. 2001. Climate variability in regions of amphibian declines. *Conservation Biology,* 15: 930-942.

Alford R.A. & Richards S.J. 1999. Global amphibian declines: A problem in applied ecology. *Annual review of Ecology and Systematics,* 30: 133-165.

Altig R., & McDiarmid R.W. 1999. Body plan: Development and morphology- In R.W. McDiarmid and R. Altig (eds.) Tadpoles: the biology of anuran larvae. Chicago, Illinois: University of Chicago Press, pp. 24-51.

Ammann C.M., Fortunat J., Schimel, D.S., Otto-Bliesner, B.L. & Tomas, R.A. 2007. Solar influence on climate during the past millenium: Results from tansient simulations with the NCAR Climate System Model. *Proceedings of the National Academy of Sciences of the United States of America,* 104 (10): 3713-3718.

AmphibiaWeb: Information on amphibian biology and conservation. [web application]. 2009. Berkeley, California: AmphibiaWeb. Available: http://amphibiaweb.org/. (Accessed: Jul 8, 2009).

Aplin K. & Kirkpatrick P. 1999. Progress report on investigations into chytrid fungal outbreak in Western Australia. Perth: Western Australia Museum.

Barr D.J.S. 1990. Phylum Chytridiomycota. In Margulis L., Corliss J.O., Melkonian M. & Chapman D.J. (eds). Handbook of Protoctista. New York: Lubrecht and Kramer, pp. 454-466.

Bates M.F. & Haacke W.D. 2003. The frogs of Lesotho: Diversity and distibution. *Annals of the National Museum of Bloemfontein,* 19 (6): 101-157.

Beebee T.J.C., Denten J.S. & Buckley J. 1996. Factors affecting population densities of adult natterjack toads *Bufo calamita* in Britain. *Journal of Applied Ecology,* 33: 263-268.

Begon M., Bennett M., Bowers R.G., French N.P., Hazel S.M. & Turner J. 2002. A clarification of transmission terms in most micro-parasite models: numbers, densities and areas. *Epidemiology and Infection,* 129: 147-153.

Berger L., Hyatt A.D., Speare R., Longcore J.E. 2005. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis. Disease of Aquatic Organisms,* 68: 51-63.

Berger L., Spear R., Daszak P., Green D.E., Cunningham A.A., Goggin C.L., Slocombe R., Ragan M.A., Hyatt A.D., McDonald K.R., Hines H.B., Lips K.R., Marantelli G. & Parkes H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central Ameriica. *Proceedings of the National Academy of Sciences, USA,* 95:9031-9036.

Berger L., Speare R., Hines H.B., Marantelli G., Hyatt A.D., McDonald K.R., Skerratt L.F.,Olsen V., Clarke J.M., Gillespie G., Mahony M., Sheppard N., Williams C. & Tyler M.J. 2004. Effect of season and temperature on mortality on amphibians due to chytridiomycosis. *Australian Veterinary Journal*, 82: 434-439.

Berger L., Speare R. & Hyatt A.D. 1999a. Chytrid fungi and amphibian declines: overview, implications and future directions. In Campbell A. (ed.) Declines and disappearances of Australian frogs. Canberra: Environment Australia,. pp. 23-33.

Berger L., Speare R. & Kent A. 1999b. Diagnosis of chytridiomycosis in amphibians by histologic examination. Available at www.jcu.edu.au/school/phtm/PHTM/frogs/histo/chhisto.htm, November 1999.

Berven K.A. 1982. The genetic basis of altitudinal variation in the wood frog *Rana sylvatica.* 1. An experimental analysis of life history traits. *Evolution,* 36: 962-983.

Bervern K.A. & Gill D.E. 1983. Interpreting geographic variation in life history traits. *Integrative and Comparative Biology*, 23: 85-97.

Berven K. A., Gill D.E. & Smith-Gill S. J. 1979. Countergradient selection in the green frog, *Rana clamitans. Evolution*, 33:609–623.

Blaustein A.R., Romansic J.M., Kiesecker J.M. & Hatch A.C. 2003. Ultraviolate radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions,* 9: 123-140.

Blaustein A.R., Wake D.B. & Sousa W.P. 1994. Amphibian declines: Judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology*, 8(1): 60-71.

Bosch J., Martinez-Solano I. & Garcia-Parris M. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Conservation Biology*, 97: 331-337.

Boykin K.G. & McDaniel K.C. 2008. Simulated potential effects of ecological factors on a hypothetical population of Chiricahua leopard frog *(Rana chiricahuensis). Ecological Modelling*, 218: 175-181.

Boyle D.G., Boyle D.B., Olsen V., Morgan J.A.T. & Hyatt A.D. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis)* in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms,* 60: 141-148.

Bradford D.F. 1991. Mass mortality and extinction in a high elevation population of *Rana muscosa. Journal of Herpetology,* 25: 174-177.

Bradley G.A., Rosen P.C., Sredl M.J., Jones T.R. & Longcore J.E. 2002. Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, 38: 206-212.

Bridges C.M. & Semlitsch R.D. 2000. Variation in pesticide tolerance of tadpoles among and within species of ranidae and patterns of amphibian decline. *Conservation Biology*, 14: 1490-1499.

Bristow D. 2007. Best walks of the Drakensberg. Cape Town: Struik Publishers.

Burger J. & Snodgrass J.W. 2000. Oral deformities in several species of frogs from the Savannah River site, USA.. *Environmental Toxicology and Chemistry,* 19: 2519-2524.

Burrows P.A., Joglar R.L. & Green D.E. 2004. Potential causes for amphibian declines in Puerto Rico. *Herpetologica,* 60(20): 141-154.

Burton T.M. & Likens G.E. 1975. Salamander populations and biomassin the Hubbard Brook experimental forest, New Hampshire. *Copeia,* 1975: 541-546.

Carey C. & Alexander, M.A. 2003. Climate change and amphibian declines: Is there a link? *Diversity and Distributions*, 9 (2): 111–121.

Carey C., Bruzgul J.E., Livo L.J., Walling M.L., Kuehl K.A., Dixon B.F., Pessier A.P., Alford R.A. & Rodger K.B. 2006. Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth,* 3: 5-21.

Carey C., Cohen N. & Rollins-Smith L. 1999. Amphibian declines an immunological perspective. *Developmental & Comparative Immunology,* 23: 459-472.

Carruthers V.C. 2001. Frogs and frogging in South Africa. Cape Town: Struik Publishers.

Channing A. 1979. Ecological and systematic relationships of *Rana* and *Strongylopus* in southern Natal. *Annals of the Natal Museum,* 23: 797-831.

Cohen J. 1988. Statistical power analysis for behavioural sciences. 2nd edition. Hillsdale, NJ: Erlbaum.

Collins J.P. 1979. Intrapopulation variation in the body size at metamorphosis and timing of metamorphosis in the bullfrog, *Rana catesbeiana. Ecology,* 60: 738-749.

Collins J.P., Brunner, J.L., Miera, V., Parris, M.J., Schoch, D.M. & Storfer, A. 2003. Ecology and evolution of infectious disease. In: Semlitsch RD (ed) Amphibian conservation. Washington, DC: Smithsonian Institution, pp 137–151.

Crump M.L. 1989 Effect of habitat drying on developmental time and size at metamorphosis in *Hyla pseudopuma. Copeia,* 1989: 794-797.

Czechura G.V. & Ingram G.J. 1990. *Taudactylus diurnus* and the case of the disappearing frogs. *Memoirs of the Qld Museum*, 29(2): 361-365.

Daszak P., Berger L., Cunningham A.A., Hyatt A.D., Green D.E., & Spear R. 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases,* 5: 735-748.

Daszak P, Cunningham A.A. & Hyatt A.D. 2003. Infectious disease and amphibian population declines. *Diversity & Distributions*, 9: 141-150.

Dickman M. 1968. The effect of grazing by tadpoles on the structure of a periphyton community. *Ecology*, 49: 1188-1190.

Drake D.L., Altig R. Grace J.B. & Walls S.C. 2007. Occurrence of oral deformaties in larval anurans. *Copeia,* 2: 449-458.

Drost C.A. & Fellers G.M. 1996. Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. *Conservation Biology*, 10(2): 141-425.

Duellman W.E. & Trueb L. 1986. Biology of Amphibians. New York: McGraw-Hill.

Du Preez L.H. & Carruthers V.C. (In press) Complete guide to the frogs of southern Africa. Cape Town: Random House. Struik Publishers.

Ellis S.M. & Steyn H.S. 2003. Practical significance (effect sizes) versus or in combination with statistical significance (p-values). *Management Dynamics,* 12(4): 51-53.

Epstein P.R. 2001. Climate change and emerging infectious diseases. *Microbes Infect.* 3: 747-754.

Fellers G.M., Green D.E. & Longcore J.E. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia,* 4: 945-953.

Frost D.R. 2009. Amphibian species of the world: an online reference. Version 5.3 (12 February 2009). Electronic database accessible at http://research.amnh.org/herpetology/amphibia/index.php. American Museum of Natural History, New York, USA..

Gosner K.L. 1960. A simplified table for staging anuran embryos and larvae. *Herpetologica*, 1666: 183-190.

Green D.M. 2005. Biology of amphibian declines. In: Lannoo, M. (ed.), Amphibian declines: The conservation status of United States species. Berkeley, CA: University of California Press, pp.28-33.

Harkey G.A. & Semlitsch R.D. 1988. Effects of temperature on growth, development, and color polymorphism in the ornate chorus frog *Pseudacris ornate. Copeia,* 1988: 1001-1007.

Harvell C.D., Mitchell C.E., Ward J.R., Altizer S., Dobson A.P., Ostfeld R.S. and Samuel M.D. 2002. Climate warming and disease risk for terrestrial and marine biota. *Science* 296: 2158-2162.

Hayes M.P. & Jennings M.R. 1986. Decline of ranid frogs species in Western North America: Are bullfrogs (*Rana catesbiana*) responsible? *Journal of Herpetology,* 20: 490-509.

Hayes T.B., Wu T.H. & Gill T.N. 1997. DDT-like effects as a result of corticosterone treatment in an anuran amphibian: Is DDT a corticoid mimic or a stressor? *Environmental Toxicological Chemistry*, 16: 1948-1953.

Hewitt J. 1926. Descriptions of some new species of batrachians and lizards from South Africa. *Annals of Natal Museum*, 5: 435-448, pl 25, 2 text figs.

Hegerl G.C., Zwiers F.W., Braconnot P., Gillett N.P., Luo Y., Marengo Orsini J.A., Nicholls N., Penner J.E. & Stott P.A. 2007. Understanding and attributing climate change. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.). Cambridge, UK: Cambridge University Press..

Herrera R.A., Steciow M.M. & Natale G.S. 2005. Chytrid fungus parasitizing the wild amphibian *Leptodactylus ocellatus* (Anura: Leptodactylidae) in Argentina. *Diseases of Aquatic Organisms,* 64: 247-252.

Hero J.M. & Morrison C. 2004. Frog declines in Australia: global implications. *Journal of Herpetology*, 14: 175-186.

Hero J.M., Williams S.E. & Magnusson W.E. 2005 Ecological traits of declining amphibians in upland areas of eastern Australia. *Journal of Zoology,* 267: 221-232.

Hewitt J. 1913. Description of *Heleophryne natalensis*, a new batrachianfrom Natal; and notes on several South African batrachians and reptiles. Annals of Natal Museum 2: 475-484.

Hogan, N.S., Duarte, P., Wade, M.G., Lean, D.R.S. & Trudeau, V.L. 2008. Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (*Rana pipiens*): Identifying critically vulnerable periods of development. *General and Comparative Endocrinology,* 156: 515-523.

Hyatt A.D., Boyle D.G., Olsen V., Boyle D.B., Berger L., Obendorf D., Dalton A., Kriger K., Hero M., Hines H., Phillott R., Campbell R., Marantelli G., Gleason F. & Colling A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 73: 175-192.

Johnson M., Berger L., Phillips L. & Speare R. 2003. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid, *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 57: 255-260.

Johnson M. & Speare R. 2003. Survival of *Batrachochytrium dendrobatidis* in water: Quarantine and disease control implications. *Emerging Infectious Diseases,* 9: 922-925.

Kiesecker J.M., Root, T.L., Green, D.M., Olsen, D.H., Belden, L.K. & Blaustein, A.R. 2001. Amphibian breeding and climate change. *Conservation Biology*, 17 (2): 626-627.

Knapp R.A. & Morgan J.A.T. 2006. Tadpole mouthpart depigmentation as an accurate indicator of Chytridiomycosis, an emerging disease of amphibians. *Copeia,* 2: 188-197.

Kriger K.M., Hines H., Hyatt A.D., Boyle D.G. & Hero J.M. 2006a. Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. *Diseases of Aquatic Organisms,* 71: 141-148.

Kriger K.M., Hero J.M. & Ashton K.J. 2006b. Cost efficiency in the detection of chytridiomycosis using PCR assay. *Diseases of Aquatic Organisms,* 71: 149-154.

Kriger K.M. & Hero, J.M. 2007. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions*, 13 (6): 781-788.

Kriger K.M., Preoglou F. & Hero J.M. 2007. Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. *Conservation Biology*, 21: 1280-1290.

Kriger K.M. & Hero, J.M. 2008. Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis)* infection in subtropical Australian frogs. *Australian Ecology*, 33: 1022-1032.

Lambiris A.J.L. 1987. Description of the tadpole of *Strongylopus hymenopus* (Boulenger, 1920) (Amphibia: Anura: Ranidae) and a key to described southern African tadpoles of the genus. *Annals of the Natal Museum,* 28 (2): 455-462.

Lambiris A.J.L. 1988. Frogs and Toads of the Natal Drakensberg. Pietermaritzburg. University of Natal Press.

Lamirande E.W., & Nichols D.K. 2002. Effects of host age on susceptibility to cutaneous chytridiomycosis in blue-and-yellow poison dart frogs (*Dendrobates tinctorius*). In *Proceedings of the Sixth International Symposium on the Pathology of Reptiles and Amphibians.* McKinnell R.G., Carlson D.L. (eds) pp. 3-13. Saint Paul, Minnesota.

La Marca E., Lips K., Lötters S., Puschendorf R., Ibafiez R., Rueda-Almonacid J.V., Schulte R., Marty C., Castro F., Manzanilla-Puppo J., Garcia-Pkrez J.E., Bolafios F., Chaves G., Pounds J.A., Toral E. & Young B.E. 2005. Catastrophic population declines and extinctions in neotropical Harlequin frogs (Bufonidae: Atelopus). *Biotropica,* 37: 190-201.

Lips K.R. 1998. Decline of a montane tropical amphibian fauna. *Conservation Biology,* 12: 106-117.

Lips K.R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. *Conservation Biology,* 13: 117-125.

Lips K.R., Reeve J.D. & Witters L.R. 2003. Ecological traits predicting amphibian population declines in Central America. *Conservation Biology,* 17: 1078-1088.

Longcore J.E., Pessier A.P. & Nichols D.K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. Nov., a chytrid pathogenic to amphibians. *Mycologia,* 91: 219-227.

Marantelli G., Berger L., Speare R. & Keegan L.. 2004. Distribution of *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology,* 10: 173-179.

Marsh D.M. 2001. Fluctuations in amphibian populations: a meta-analysis. *Conservation Biology,* 101: 327-335.

Mazzoni R., Cunningham A.C., Daszak P., Apolo A., Pedomo E. & Speranza G. 2003. Emerging pathogen of wild amphibians in frogs (*Rana catesbiana*) farmed for international trade. *Emerging Infectious Diseases,* 9: 995-998.

McDiarmid, R.W. & Altig R. 1999. Tadpoles: The biology of anuran larvae. Chicago: University of Chicago Press, pp. 279-288.

McDonald K.R. & Alford R.A. 1999. A review of declining frogs in Northern Queensland. In: Declines and disappearances of Australian frogs. Environment Australia, Canberra. Campbell A. (ed). pp 14-22.

McDonald K.R., Mendéz D., Muller R., Freeman A.B. & Speare R. 2005. Decline in the prevalence of chytridiomycosis in upland frog populations in North Queensland, Australia. *Pacific Conservation Biology,* 11: 114-120.

Minter L.R., Burger, M., Harrison, J.A., Braack, H.H., Bishop, P.J., Kloepfer, D., (eds.). 2004 Atlas and Red Data Book of the frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Washington DC: The Smithsonian Institution.

Morin P.J., Lawler S.P. & Johnson E.A. 1990. Ecology and breeding phenology of larval *Hyla andersonii:* The disadvantage of breeding late. *Ecology,* 71: 1590-1598.

Muths E., Corn P.S., Pessier A.P. & Green D.E. 2003. Evidence for disease related amphibian decline in Colorado. *Conservation Biology,*110: 357-365.

Mutschmann F., Berger L., Zwart P. & Gaedicke C. 2000. Chytridiomycosis on amphibians-first report from Europe. *Berliner und Munchener Tieraerztliche Wochenschrift,* 113: 380-383.

Nel W. & Sumner P.D. 2007. Intensity, energy and erosivity attributes of rainstorms in the KwaZulu-Natal Drakensberg, South Africa. *South African Journal of Science,* 103: 398-402.

Nel, W. and Sumner, P.D., 2008. Rainfall and temperature attributes on the Lesotho-Drakensberg escarpment edge, southern Africa. *Georgr. Ann.,* 90 A (1): 1-12.

Ouellet M., Mikaelian I., Pauli B.D., Rodrique J. & Green D.M. 2005. Historical evidence for widespread chytrid infection in North American amphibian populations. *Conservation Biology,* 19: 1431-1440.

Padgett-Flohr G.E. & Goble M.E. 2007. Evaluation of tadpole mouthpart depigmentation as a diagnostic test for infection by *Batrachochytrium dendrobatidis* for four California anurans. *Journal of Wildlife Diseases,* 43(4): 690-699.

Parker J.M., Mikaelian I., Hahn N. & Diggs H.E. 2002. Clinical diagnosis and treatment of epidermal chytridiomycosis in african clawed frogs (*Xenopus tropicalis).Complementary Medicine,* 52: 265-268.

Parris M.J. & Cornelius T.O. 2004. Fungal pathogen causes competative and developmental stress in larval amphibian communities. *Ecology*, 85: 3385-3395.

Pearman P.B. 1995. Correlates of amphibian diversity in an altered landscape of Amazonian Ecuador. *Conservation Biology,* 11: 1211-1225.

Pessier A.P., Nichols D.K., Longcore J.C. & Fuller M.S. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates spp.*) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation,* 11: 194-199.

Phillips K. 1990. Where have all the frogs and toads gone? *Bioscience*, 40: 422-424.

Piotrowski J.S., Annis S.L. & Longcore J.F. 2004. Physiology of Batrachochytrium dendrobatidis, a chytrid pathogen of amphibians. *Mycologia,* 96(1): 9-15.

Porter K.R. 1972. Herpetology, Philadelphia, Penn: Saunders.

Pounds J.A., Bustamante M.R., Coloma L.A., Consuegra J.A., Fogden M.P.L., Foster P.N., La Marca E., Masters K.L., Merino-viteri A., Puschendorf R., Ron S.R., Sanchez-Azofeifa G.A., Still C.J. & Young B.E. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, 439: 161-165.

Pounds J.A. & Crump M.L. 1994. Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. *Conservation Biology,* 8: 72-85.

Pounds J.A., Fogden M.P.L., & Campbell J.H. 1999. Biological response to climate change on a tropical mountain. *Nature,* 398: 611-615.

Powell M.J. 1993. Looking at mycology with a Janus face: A glimpse of chytridiomycetes active in the environment. *Mycologia,* 85: 1-20.

Rachowicz L.J., Knapp R.A., Morgan J.A.T., Stice M.J., Vredenburg V.T., Parker J.M. & Briggs C.J. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology,* 87: 1671-1683.

Rachowicz L.J. & Vredenburg V.T. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms,* 61: 75-83.

Reques R. & Tejedo M. 1995. Negative correlation between length of larval period and metamorphic size in natural populations of Natterjack toads (Bufo calamita). *Journal of Herpetology*, 29(2): 311-314.

Retallick R.W.R. 2002. Using experimental translocations to learn about declines in Queensland's threatened frog populations. Implementation of Queensland's threatened frog recovery plans-experimental ecology. Atherton: Queensland Parks and Wildlife Service.

Retallick R.W.R., McCallum H. & Speare R. 2004. Endemic infection of the Amphibian Chytrid fungus in a frog community post-decline. *PLoS Biology,* 2: 1-7.

Richards S.J., McDonald K.R. & Alford R.A. 1993. Declines in populations of Australia's endemic tropical rainforest frogs. *Pacific Conservation Biology,* 1: 66-77.

Ron S.R. 2005. Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the new world. *Biotropica,* 37: 209-221.

Rowe C.L., Kinney O.M., Fiori A.P. & Congdon J.D. 1996. Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: effect on grazing ability and growth. *Freshwater Biology,* 36: 723-730.

Rowley J.J.L. & Alford R.A. 2007. Behavior of Australian rainforest stream frogs may effect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms*, 77: 1-9.

Scherer R.D., Muths E., Noon B.R. & Corn P.S. 2005. An evaluation of weather and disease as causes of decline in two populations of Boreal toads. *Ecological Applications,* 15(6): 2150-2160.

Seale D.B. 1980. Influence of amphibian larvae on primary production, nutrient flux, and competition in a pond ecosystem. *Ecology*, 61: 1531-1550.

Seimon T.A., Seimon A., Daszak P., Halloys S.R.P., Schloegel L.M., Aguilar C.A., Sowell P., Hyatt A.d., Konecky B. & Simmons J.E. 2007. Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Global Change Biology*, 13: 288-299.

Shoemaker V.H., Hillyard S.D., Jackson D.C., McClanahan L.L.,Withers P.C. & Wygoda M.L. 1992. Exchange of water, ions, and respiratory gases in terrestrial amphibians. In Feder M.E. & Burggren W.W. (eds.). Environmental physiology of the amphibian. Chicago: University of Chicago Press, pp. 125-150.

Skerratt L.S., Berger L., Speare R., Cashins S., McDonald K.R., Phillott A.D., Hines H.B. & Kenyon L.. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, DOI: 10.1007/s10393-007-0093-5.

Smith D.C. 1987. Adult recruitment in chorus frogs: Effects of size and date at metamorphosis. *Ecology.* 68: 344-350.

Smith K.G., Weldon C., Conradie, W. & du Preez L.H. 2006. Relationships among size, development and *Batrachochytrium dendrobatidis* infection in African tadpoles. *Diseases of Aquatic Organisms,* 74: 159-164.

Smith-Gill S.J. & Berven K.A. 1979. Predicting amphibian metamorphosis. *American Naturalist*, 113: 563–585.

Sparling D.W., Bishop C.A., Pauli B. & Money S. 2000 Epilogue: Lessons yet to be learned. In: Sparling D.W., Linder G. and Bishop C.A. (eds). Ecotoxicology of amphibians and reptiles. Pensacola, FL, USA: SETAC, pp. 811-822.

Speare R. 1994. Preliminary study on diseases in Australian Wet Tropics amphibians. Deaths of rainforest frogs at O'Keefe Creek, Big Tableland. Final report to Queensland Department of Environment and Heritage. Unpublished report, Queensland Department of Environment and Heritage.

Speare R. and Core Working Group of Getting the Jump on Amphibian Disease 2001. Nomination for listing of amphibian chytridiomycosis as a key threatening process under the environment Potection and Biodiversity Conservation Act 1999. In: Speare R. and Steering Committee of Getting the Jump on Amphibian Disease (eds.). Developing management *strategies to control amphibian diseases: decreasing the risk due to communicable* diseases. School of Public Health and Tropical Medicine Townsville: James Cook University, pp. 163-187.

Speare R. & Berger L. 2000. Global distribution of chytridiomycosis in amphibians. World Wide Web –http://www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm. 11 November 2000.

Speare R., McCallum H., Retallick R.W.R. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology*, 2(11): 351-356.

Steyn H.S., (jr.). 1999. PRAKTIESE BEDUIDENHEID: Die gebruik van effekgroottes. Wetenskaplike Bydraes, Reeks B: Natuurwetenskappe nr. 117. . Potchefstroom: PU vir CHO, Publikasiebeheerkomitee.

Steyn H.S., (jr.). 2000. Practical significance of the difference in means, *Journal of Industrial Psychology*, 26(3): 1-3.

Stuart S.N., Chanson J.S., Cox N.A., Young B.E., Rodrigues A.S.L., Fischmna P.L. & Waller R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science,* 306: 1783-1786.

Suchet R. 2006. A backpackers guide to Lesotho. Underberg:Russel Suchet.

Swinton J. 1998. Extinction times and phase transition for spatially structured closed epidemics. *Bulletin of Mathematical Biology,* 60: 215-230.

Tarrant J. Cunningham M.J. Du Preez L.H. 2008. Maluti Mystery: A systematic review of Amietia vertebralis (Hewitt, 1927) and Strongylopus hymenopus (Boulenger, 1920) (Anura: Pyxicephalidae). *Zootaxa* 1962: 33-48.

Tyler M.J. 1997. The action plan for Australian frogs. *Wildlife Australia,* Canberra.

Van Dijk E.D. 2008. Clades in *Heleophrynid* Salientia. *African Journal of Herpetology,* 57(1): 43-48.

Weldon C. 2002. Chytridiomycosis survey in South Africa. Froglog, 51: 1-2.

Weldon C. & Du Preez L.H. 2006. Quantitative measurement of *Batrachochytrium dendrobatidis* in amphibian skin. *Diseases of Aquatic Organisms,* 72: 153-161.

Weldon C., Du Preez L.H., Hyatt A.D., Muller R. & Speare R. 2004. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases,* 10: 2100-2150.

Wikipedia. http://en.wikipedia.org/wiki/Lesotho. Accessed September 2008.

Williams S.E. & Hero J-M. 1998. Rainforest frogs of the Australian Wet Tropics: guild classification and the ecological similarity of declining species. *Proceedings of the Royal Society, London.* 265: 597-602.

Woodhams D.C. & Alford R.A. 2005. The ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conservation Biology,* 19: 1449-1459.

Woodhams D.C., Alford R.A. & Marantelli G. 2003. Emerging disease of amphibians cured by elevated body temperature. *Disease of Aquatic Organisms*, 55: 65-67.

Wyman R.L. 1990. What's happening to the amphibians? *Conservation Biology*, 4: 350-352.

Young B.E., Lips K.R., Reaser J.K., Ibanez R. & ten others. 2001. Population declines and priorities for amphibian conservation in Latin America. *Conservation Biology,* 15: 1213–1223.