Seasonal pattern of chytridiomycosis in common river frog (*Amietia angolensis*) tadpoles in the South African Grassland Biome

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Environmental parameters such as temperature and rainfall influence the biology of amphibians and are likely to similarly influence the growth and prevalence of associated pathogens. Amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), causes an infectious disease, chytridiomycosis, in amphibians worldwide. Field studies on post-metamorphic anurans from tropical Australia have correlated increased prevalence with cool winter temperatures, but similar studies are lacking from Africa. We monitored the seasonality of amphibian chytrid in the Highveld of South Africa through microscopic examination of common river frog (*Amietia angolensis*) tadpoles over 12 months. Within the study area Bd was found to be widespread, but largely limited to riverine systems. The seasonal infection pattern was inconsistent with the findings of past studies, which showed that prevalence usually peaks during the cooler months of the year. This study indicates that infection levels increased during spring in the Grassland Biome, when temperatures favoured optimum thermal growth of the fungus and when streams reached minimum flow levels.

Key words: conservation, Vredefort Dome, UNESCO, stream flow.

INTRODUCTION

Chytridiomycosis is an infectious disease of amphibians responsible for widespread morbidity and mortality in susceptible species, sometimes leading to population declines (Berger *et al.* 1998; Lips 1999; Bosch *et al.* 2001; Rachowicz *et al.* 2006). The importance of the etiological agent of chytridiomycosis, *Batrachochytrium dendrobatidis* (Bd), is highlighted by the fact that it is one of only two notifiable amphibian pathogens (the other being ranavirus) that are listed by the World Animal Health Organization. The disease is known to have a devastating impact on amphibian populations in many parts of the world, however, in some regions, for example, eastern U.S.A. and South Africa, the occurrence of Bd is not associated with disease, but acts as an endemic infection (Weldon *et al.* 2004; Longcore *et al.* 2007; Rothermel *et al.* 2008). Epidemiological data from field studies on Bd (Lips *et al.* 2008) and population genetics from a global selection of Bd strains (James *et al.* 2009) provide evidence for a newly introduced invasive pathogen. Once established in a population or region, subsequent chytridiomycosis outbreaks demonstrate a strong seasonal pattern, with increased prevalence of infection correlated with winter months (e.g. Berger *et al.* 2004; Retallick *et al.* 2004; Kriger & Hero 2007). These field events are consistent with the physiological thermal determinants of Bd observed in culture under laboratory conditions. The thermal growth range for Bd is 4–28°C and although the organism can survive freezing, temperatures above 29°C are lethal, and temperatures above 22°C result in reduced pathogenicity (Longcore *et al.* 1999; Piotrowski *et al.* 2004; Andre *et al.* 2008).

The Vredefort Dome, situated in the Grassland Biome (Mucina & Rutherford 2006), is inhabited by 13 species of anurans, none of which is listed as Threatened (Conradie *et al.* 2008). In 2005, the Vredefort Dome was added to the list of UNESCO World Heritage Sites for its unique geologic interest as the world's oldest and largest meteorite impact structure (Fleminger 2008). The vegetation type is Rocky Highveld Grassland (Bredenkamp & van Rooyen 1996). This is a transition type between typical grassland and bushveld. Habitats within

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this veld type include rocky mountains, hills, ridges and plains of quartzite (Bredenkamp & Van Rooyen 1996). Because of the conservation significance of the area and a lack of disease surveillance in protected areas in South Africa, we investigated the occurrence of Bd in the Vredefort Dome. Our focal species, the common river frog (*Amietia angolensis*) is the most abundant species in the area (Conradie *et al.* 2008). It is a widespread eastern and southern African species from the Grassland and Savanna biomes (Poynton 1964). Owing to its wide distribution, inclusion within many protected areas, and the ability to tolerate some habitat disturbance, this species is not threatened (Channing 2004). Surveys of 12 geographically distinct localities in South Africa conducted during the period 2004–2008 have shown that *A. angolensis* from half of these localities were infected with Bd (Weldon unpublished data). Infection prevalence can be high in populations (up to 60%) and both tadpoles and adults are known to be infected, however infection has never been linked with moribund or dead individuals in this species.

The tadpoles of *A. angolensis* have a prolonged development that may take up to two years to complete (Channing 2004). Tadpoles with prolonged development provide an ideal model to monitor seasonal patterns in anurans related to their ecology or host–pathogen interactions. For instance, a species with extended larval development can be used to determine periodicity and synchrony of infection over a unique temporal and geographical span. Since it is generally agreed that amphibian larvae can act as reservoirs for Bd (e.g. Berger *et al.* 1998; Rachowicz & Vredenburg 2004), prolonged host availability during periods of unfavorable conditions when other amphibians are not active or present may benefit disease persistence in the system. Thus coupling epidemiology with extended larval development can be a useful approach for better understanding disease ecology over time. For instance, tadpole size and infection status can be used to indicate the effect of pond occupancy length on disease prevalence. These data can then be applied to conservation actions in the control of infectious disease.

Infection in tadpoles is limited to the keratinized mouthparts and this may lead to the depigmentation, a feature that has often been used for non-lethal diagnosis (Lips 1999; Fellers *et al.* 2001; Rachowicz & Vredenburg 2004). However, there are instances when the use of the direct microscopic method (examination of excised mouthparts) is preferred over the non-lethal indirect method, such as when false positives or false negatives could result in the incorrect classification of populations as infected or uninfected, especially when Bd is absent or present at low prevalence (Smith & Weldon 2007).

The objectives of this study were (i) to determine the status of Bd infection in *A. angolensis* in the Grassland Biome in terms of distribution and habitat type (ii) to subsequently monitor the seasonal pattern of Bd infection in *A.* angolensis tadpoles and (iii) to investigate whether body size was related to Bd infection prevalence and burden intensity within a pond. In this study, we present the first seasonal variation data of Bd infection in tadpoles of a South African frog.

MATERIALS & METHODS

We randomly selected a number of sites that spanned the width of the Vredefort Dome and included a variety of habitat types in which *Amietia angolensis* occurs (Fig. 1). Tadpoles of *A. angolensis* were collected once a month by dip-netting for the period March 2005 to February 2006. Dip-netting was standardized at 15 minutes per site.

Tadpoles were euthanased with a tricaine methanesulfonate (MS222, Sigma®) solution. Total length of tadpoles was measured with a Mitutoyo vernier calliper (0.05 mm precision) and tadpoles were staged according to Gosner (1960). Mouthparts were excised, prepared on slides and microscopically examined for the presence of Bd. Specimens were deposited in the amphibian collection of the African Amphibian Conservation Research Group (AACRG) hosted at the North-West University, Potchefstroom.

Sporangia density was calculated for each infected tadpole using the method described in Weldon & Du Preez (2006), but modified for use on tadpoles. Briefly, sporangia on infected mouthparts were counted for every field on the longest *X*-axis and *Y*-axis. The position of the *X*-axis was moved to overlap with the lower jaw sheath. The diameter of the microscope field of the ×40 objective was measured with a slide graticule and the area of the microscope field was calculated. The mean number of sporangia observed per field view was calculated and converted to sporangia/mm^2.

All statistical analyses were performed using NCSS 2004 and Sigmaplot 8.02 software. Linear regression analysis was performed to determine

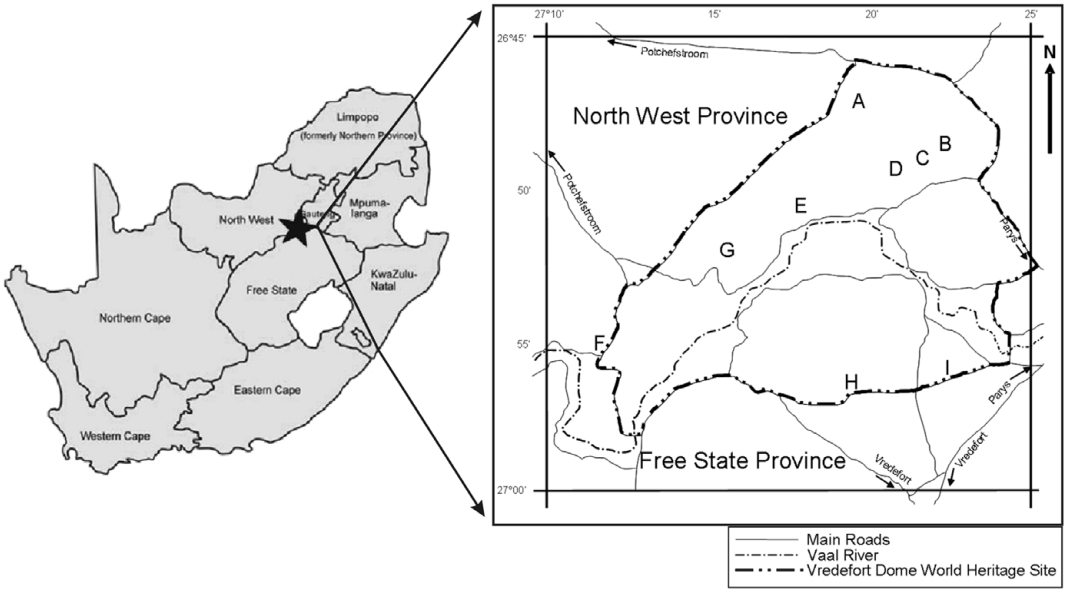

Fig. 1. Map showing the Vredefort Dome World Heritage Site and its location in South Africa (letters indicate sampling sites).

whether there was any correlation between tadpole body size and infection status, as well as between sporangium density and tadpole development and body size. A contingency table analysis was performed on the infection data by site to determine whether infected individuals were evenly distributed throughout the sites. For this purpose we divided the selected sites into endorheic (interior drainage basins) and riverine systems.

RESULTS

Amietia angolensis tadpoles were present at all nine sites during the survey period, but were not consistently found at all the sites throughout the year. However, the species was present throughout the year in the Vredefort Dome when considering the combined occupancy data for all sites. Tadpoles were not abundant at any of these sites (total $n = 235$), resulting in mostly small sample sizes (Table 1). Bd was only detected in *A. angolensis* tadpoles at three of the nine sites. Evenness of infection could not be determined reliably for each site for the months that Bd was detected because of low numbers of infected individuals. Comparison of infection data between sites of endorheic and riverine systems did, however, indicate a significant difference ($\chi^2 = 7.84$, $P = 0.0051$), with riverine systems being much more likely to harbour Bd; 23.4% of individuals collected were infected whereas none of the individuals from the endorheic sites were infected (Table 2).

Bd was only detected during the drier months of the year when cool to warmer temperatures

Table 1. Site details and breakdown of infection data among sites.

Site name	Site description	GPS coordinates	Bd infected/*n*
Mooinooiensfontein (A)	Earth-walled dam	26.80°S 27.32°E	0/2
Berhaka (B)	Artificial stream	26.82°S 27.38°E	1/19
Bluegumwoods Dam (C)	Earth-walled dam	26.83°S 27.38°E	0/12
Bluegumwoods River (D)	Perennial stream	26.83°S 27.36°E	0/16
Tabela Thabeng (E)	Perennial stream	26.86°S 27.24°E	0/25
Elgro bridge (F)	Perennial river	26.93°S 27.19°E	11/45
Waterfall (G)	Mountain torrent	26.92°S 27.24°E	6/85
Dampoort I (H)	Natural pool	26.95°S 27.32°E	0/21
Dampoort II (I)	Natural pool	26.95°S 27.35°E	0/10

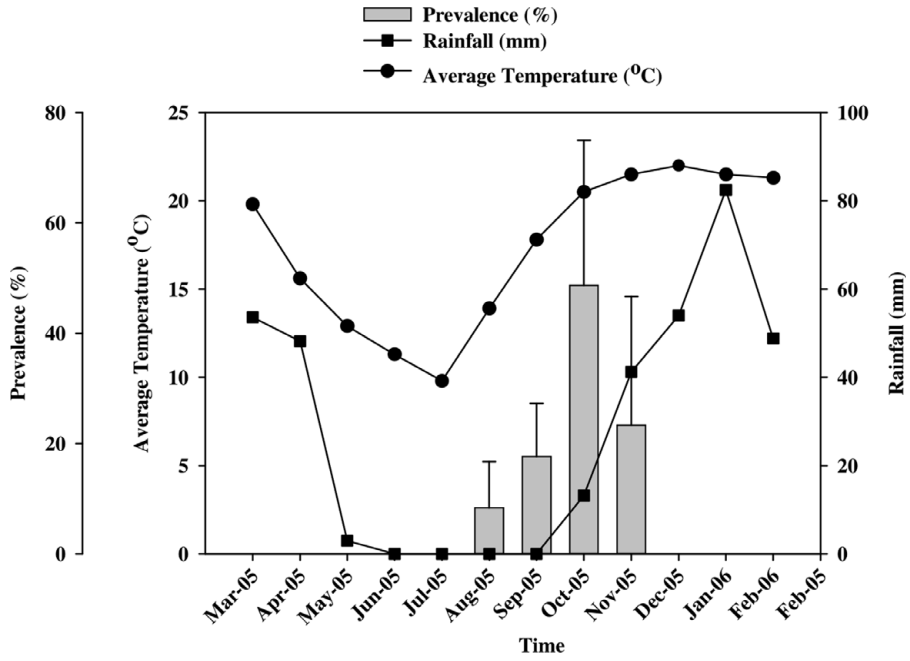

Fig. 2. Seasonal variation in the prevalence of *Batrachochytrium dendrobatidis* in tadpole *Amietia angolensis* across nine sites in the Vredefort Dome. Mean monthly rainfall and ambient temperature are indicated. See Table 1 for site specifications.

prevailed (spring to early summer; Fig. 2). Spring rains only commenced toward the latter half of the detection window for Bd and peaked during summer when Bd was no longer detected. Overall prevalence for infected sites steadily rose from 7.14% in August to a maximum of 49.6% in October before decreasing to below detection levels again in December.

Only tadpoles between Gosner stages 26 and 41 showed Bd infection (Table 3). Infection status showed a significant positive correlation with developmental stage ($P < 0.005$) and infected individuals were significantly larger than uninfected individuals ($P < 0.001$). Oral sporangia density varied considerably among infected tadpoles, from 32–1940/mm^2 (mean 446 ± 465/mm^2).

Table 2. Infection data grouped according to the hydrology of the sites.

Aquatic system	Infected (%)	Uninfected (%)	Total
Endorheic systems	0 (0%)	34 (100%)	34
Riverine systems	18 (23.4%)	59 (76.6%)	77
Total	18	93	111

Sporangium density showed a weak positive correlation with body length ($R^2 = 0.1282$, $P = 0.1582$; Fig. 3a), but did not show a significant correlation with developmental stage of tadpoles ($R^2 = 0.0187$, $P = 0.6006$; Fig. 3b).

Table 3. Body measurements and sporangium density data for *Amietia angolensis* tadpoles.

Measurement	Range		Mean (± S.D.)	
	Infected	Uninfected	Infected	Uninfected
Gosner stage	26–41	23–45	37 ± 5	32 ± 6
Total length (mm)	37–88	12–87	67 ± 12	45 ± 16
Sporangia/field	5–322	0	74 ± 77	0
Sporangia/mm^2	32–1940	0	446 ± 465	0

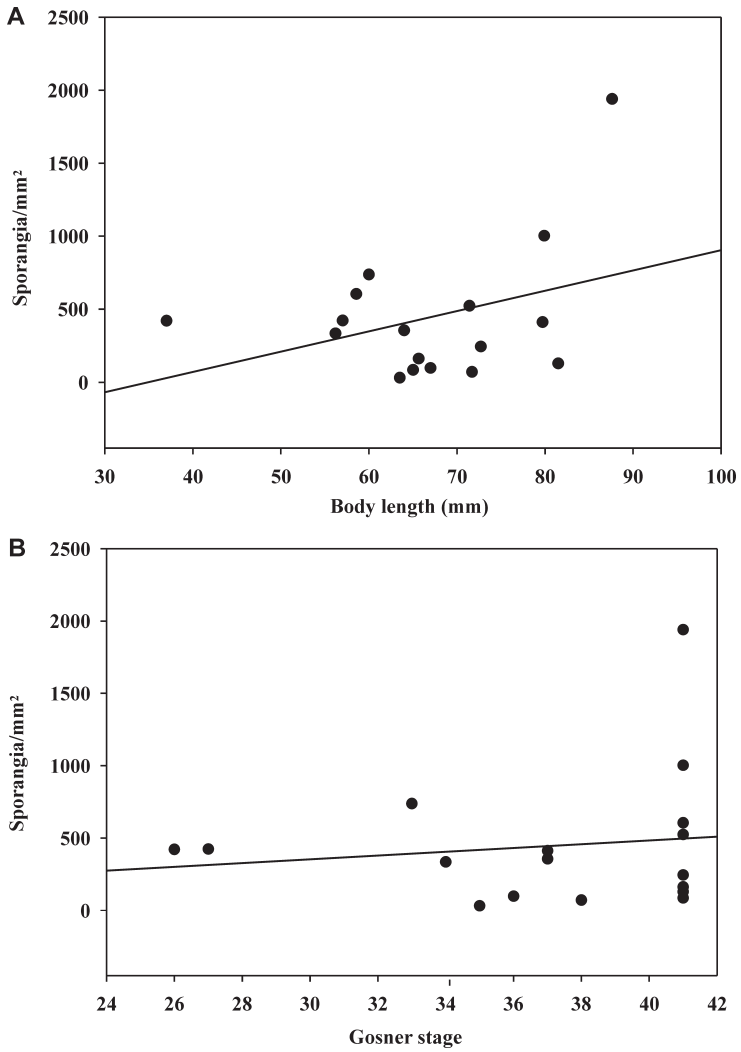

Fig. 3. Relationship between sporangium density of *Batrachochytrium dendrobatidis* detected on *Amietia angolensis* tadpoles and (**a**) tadpole body length (R^2 = 0.1282, P = 0.1582) and (**b**) tadpole Gosner stage of development (R^2 = 0.0187, P = 0.6006).

DISCUSSION

This study shows that *A. angolensis* is widespread in the Vredefort Dome and that it carries Bd infection, but that the distribution of infected populations appears to be influenced by the hydrology of the habitat. All of the sites where Bd was detected had the same physical characteristics: clear perennial riverine systems associated with the outcrops that form the remnant crater of the Vredefort Dome. No infected individuals were collected at any of the larger more permanent endorheic water bodies. The amount of rainfall and rainfall pattern in the Grassland Biome contributes to the formation of predominantly seasonal pans and wetlands and fewer perennial water bodies. *Amietia angolensis* is semi-aquatic and active throughout the year (Channing 1979) and is therefore only present at perennial water bodies during the dry season. As such, these perennial habitats provide an ecological niche that is sustainable for Bd in the Grassland Biome throughout the year.

Bd infections were detected in *A. angolensis* populations in the Vredefort Dome area during the drier, cooler spring season preceding the wet, hot summer months, but were not detected during the cold winter season. These findings contrast with those of previous studies from tropical forest biomes, which indicated a rise in prevalence

during winter (Berger *et al.* 2004; Kriger & Hero 2007). In a temperate climate, such as that experienced in the Vredefort Dome area, winter temperatures drop below the optimum growth range of Bd (17–25°C, Piotrowski *et al.* 2004), but rise to within this range during spring. Mid-summer temperatures above 30°C will again inhibit Bd growth. A cool climate was found to be an important factor for the presence of chytridiomycosis in Australia as opposed to rainfall or altitude (Drew *et al.* 2006). However, Collins *et al.* (2003) stated that synchronization of optimal temperature and hydric cycles may influence chytridiomycosis growth. The appearance of Bd in *A. angolensis* preceded the peak rainy season, with prevalence increasing mid-way through the dry season, and was more closely linked to optimal thermal conditions for the fungus. Our results indicate that stream flow rate (as a measure of rainfall) is a better indicator of Bd infection in the Grassland Biome. Although quantitative data on flow rates are lacking, it was observed that streams had reached minimum flow levels at the time when Bd prevalence peaked, and approached peak flow in summer at the time when Bd was not detected. The effect of water flow rate on Bd infection rate in wild amphibian populations has not been investigated before. Population modelling showed that although Bd is the proximate cause of many amphibian declines, the added effect of both minimum flow and peak flow can hasten population decline in chytridiomycosis-susceptible species by causing increased mortality (Boykin & McDaniel 2008). Less understood is how stream flow influences disease transmission between individuals of a population. Theoretically, transmission becomes ineffective at low host densities and the pathogen fails to persist (De Castro & Bolker 2005). In the Vredefort Dome area, peak flow could have resulted in reduced contact, and subsequent transmission, between infected and susceptible hosts which would account for the drop in infection during the wet summer season.

Possible explanations for the observed infection pattern of low prevalence for most of the year, with a short (approximately four months) of high Bd prevalence, could be explained by the design of the study. First, conventional theory of Bd seasonal infection patterns is based on disease response in post-metamorphic amphibians to environmental fluctuations. This study followed the progress of Bd infection in tadpoles, which were subjected to differing suites of interactions and environmental variables. Second, previous studies tended to focus on regions and susceptible species that experienced population declines. Based on the infection data and host resistance (no observed mortalities among metamorphosed frogs), Bd in *A. angolensis* indicates a low prevalence, non-lethal endemic infection. Lastly, the sensitivity of the diagnostic technique used in this study limited the detectability of early infections, compared to the more sensitive quantitative PCR technique that enables the detection of lower infections (Kriger *et al.* 2006).

A sporangium density of 446/mm^2 for tadpoles is high in comparison with infection in sloughed skin of the closely related species *Amietia fuscigula,* (mean of 55/mm^2) (Weldon & Du Preez 2006). The high infection densities in *A. angolensis* tadpoles are explained by the concentration of infection in the keratinized mouth parts (Altig 2007), whereas the lower values in *A. fuscigula* reflect infection across ventral adult skin with highly variable regions of infection intensity. Deductions about differences between *A. angolensis* and *A. fuscigula* infection intensity and host susceptibility cannot be made here as they would be based on a comparison between different life stages of the host. The relationship between disease susceptibility and tolerable infection burden needs further investigation in *A. angolensis.* Growth specific infection in *A. angolensis* provides additional support to the observation by Smith *et al.* (2007) that more advanced developmental stages are more prone to higher Bd infection, thus illustrating a age-dependant infection in wild anuran tadpoles.

The confounding effect of temperature on the growth and persistence of Bd, best explains the seasonal infection pattern observed in *A. angolensis.* Whereas optimal thermal conditions in the tropics are usually present in winter, similar conditions at our study sites only persisted in spring in the South African Grassland Biome, thus demonstrating a seasonal shift in infection peak in a temperate region towards the warmer spring months. The hydric cycle of streams also favoured disease dynamics during these optimal thermal conditions through minimum flow rates and resulting high tadpole densities. This study further demonstrates that tadpoles can effectively be used to study seasonal dynamics of diseases in amphibians. A better understanding of seasonal infection patterns will be attained when surveys include data from both larval and post-metamorphic life stages.

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