

Infection intensity and sampling locality affect *Batrachochytrium dendrobatidis* distribution among body regions on green-eyed tree frogs *Litoria genimaculata*

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ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis, which has caused devastating amphibian population declines. Little is known about the biology of *Bd* on hosts, and techniques for diagnosing it on living and preserved animals are still evolving. We investigated the spatial distribution of *Bd* on the integument of naturally infected Australian hylid frogs *Litoria genimaculata* at 4 rain forest localities in northern Queensland, Australia. We collected 555 samples by swabbing 111 individuals on 5 regions of the body (back, abdomen, legs, forefeet and hindfeet). Numbers of zoospore equivalents on each body region were quantified using a real-time TaqMan PCR assay. The intensity of infection differed significantly among body regions and this pattern of differences differed among sampling localities. The lightest infections were usually centered on the abdomen, while heavier infections were concentrated on the legs and feet. The back was always either lightly infected or uninfected. Many frogs with light infections had positive PCR results only for the abdomen or the legs. We compared swabs taken from the legs and abdomen and found that they provided similar sensitivity to detect infections, but using both regions together led to greater sensitivity than either region alone. Because swabbing may transfer zoospores from infected to uninfected regions within individuals, we suggest that the best procedure for all species is to employ separate swabs for each body region. If that cannot be done, swabbing patterns that minimize potential harm should be determined for each species, and possibly each class of individuals (e.g. males, females, juveniles) within species, by examining the distribution of infection among body parts in naturally infected individuals.

KEY WORDS: *Batrachochytrium dendrobatidis* · *Litoria genimaculata* · Chytridiomycosis · Disease development · Diagnostic PCR

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INTRODUCTION

In 2004, the Global Amphibian Assessment estimated that of the 5743 species of described amphibians, populations of 2468 species were declining (Stuart et al. 2004). Habitat loss, climate change (temperature, rainfall, UV-B radiation), exotic species, exploitation, pollution and synergistic interactions between these factors have probably contributed to many amphibian declines (Alford & Richards 1999). Enigmatic declines (Stuart et al. 2004), occurring in

areas with no known anthropogenic influence, however, have raised concerns of an alternative threat to amphibians. A strong candidate as the cause of many enigmatic declines is chytridiomycosis, an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998, Longcore et al. 1999). Lips et al. (2006) demonstrated that chytridiomycosis was the proximate cause of the decline of an entire amphibian assemblage, in a manner consistent with many of the enigmatic declines identified by Stuart et al. (2004).

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The life cycle of *Bd* includes 2 stages (Longcore et al. 1999, Berger et al. 2000); no resting or sexual stages have been identified with certainty, although genetic recombination may occur (Morgan et al. 2007), and DiRosa et al. (2007) reported an unusual avirulent unicellular form in histological samples from *Rana lessonae*. The infectious stage is the flagellated zoospores (1 to 5 μm in diameter), which encyst on the skin surface and enter the cells of the stratum granulosum via germination tubes. They then form a thallus that produces a zoosporangium (10 to 40 μm in diameter). Immature zoosporangia occur within the deeper cells of the stratum granulosum, whereas mature zoosporangia occur in the stratum corneum (Berger et al. 2005b). As it matures, the zoosporangium forms at least one discharge tube, which eventually opens to the skin surface, releasing zoospores. These may reinfect the same host or disperse. Piotrowski et al. (2004) found (*in vitro*) that more than 95% of zoospores stop moving within 24 h, and on average zoospores swam <2 cm before encysting. This short-lived motile period may mean that many zoospores reinfect the host from which they originate.

In culture *Bd* thrives at 23°C, and typically dies at temperatures >29°C (Longcore et al. 1999) or after 1 h of desiccation (Berger 2001). In addition to growing in amphibian skin, *Bd* may also survive as a saprobe in moist soil (Longcore et al. 1999, Johnson & Speare 2005), or on bird feathers or other substrates (Johnson & Speare 2005). Johnson & Speare (2005) found that *Bd* cultures could be re-isolated for at least 3 mo after inoculation into sterile, moist river sand with no added nutrients, suggesting that contact between hosts and substrates may serve as a route of infection after zoospores lose their motility. Chemotaxis and water flow are likely to be the main methods of dissemination (Johnson & Speare 2003), and release of zoospores from zoosporangia may not be possible without sufficient moisture (Berger et al. 2005b).

The mechanism by which *Bd* infections cause host mortality has not been conclusively established. Carey et al. (2006) reported that the timing of death in experimentally infected *Bufo boreas* was consistent with the hypothesis that *Bd* populations grow exponentially following initial infection, with mortality occurring when a threshold population level is reached. Voyles et al. (2007) demonstrated that frogs with very intense *Bd* infections develop ionic imbalances that are probably sufficient to cause mortality, and suggested that these appear to be caused by loss of the normal osmoregulatory functions of the skin.

From its discovery until 2004, the primary method of diagnosing *Bd* infections was histological examination of thin skin sections (usually 5 μm), which means diagnosis is based on a very small fraction of the total skin

surface. The distribution of *Bd* on the body surface, particularly in early infections, is likely to be patchy (Puschendorf & Bolaños 2006). Nonlethal histological screening of frogs in the wild has usually relied on examination of toe clips (Berger et al. 1998, Lips 1998, Woodhams & Alford 2005). The combination of these factors produces a high rate of false negatives (Hyatt et al. 2007), at rates which could depend on the distribution of infection among body parts.

In a thorough histological examination of 24 apparently healthy *Eleutherodactylus fitzingeri* from Costa Rica, Puschendorf & Bolaños (2006) found that of 12 different body regions tested, the first digit (next to the thumb) and the pelvic patch were the most reliable areas for locating *Bd*, and that the anterior abdomen and the gular areas were often negative in animals that were positive elsewhere. In another histological study, the spatial distribution of *Bd* was examined on 10 heavily infected *Litoria caerulea*, but no significant differences in the density of zoosporangia were found among the ventral sides of the head, legs and abdomen (Berger et al. 2005a). Marantelli et al. (2004) found zoosporangia in the hindfeet and tails of metamorphosing *Mixophyes fasciolatus*. Longcore et al. (2007) histologically examined sections of skin from the toe webbing, tibial region, and pelvic region of road-killed frogs of 7 species from Maine, USA, and found that in most species the toe webbing was more likely to be infected than the tibial or pelvic regions. They did not, however, simultaneously examine distribution of the fungus among body regions and overall infection intensity.

Diagnostic PCR-based assays (Annis et al. 2004, Boyle et al. 2004) appear to be a more sensitive means of detecting *Bd* infections. Collecting samples for PCR using sterile cotton swabs makes it possible to sample a much greater proportion of the animal's surface. This approach is therefore likely to be more sensitive than histology for detecting low-intensity infections. It also makes it possible to non-invasively examine the spatial distribution of *Bd* on naturally infected individuals in the wild, which may aid in improving diagnostic accuracy. Understanding the distribution of *Bd* on animals in nature could also provide insight into the biology of the disease, including how the fungus is transmitted and how the disease develops and spreads on individuals.

Our aims were the following: (1) to examine the spatial distribution of *Bd* on the integument of naturally infected frogs, and determine whether this distribution provides information on probable routes of infection and patterns of disease development, and (2) to contribute to the development of evidence-based methods for swabbing amphibians for the diagnosis of *Bd* infection by PCR analysis.

To address the first aim, we measured the numbers of zoospore equivalents detected on swab samples taken from 5 regions of the bodies of adult, male green-eyed tree frogs *Litoria genimaculata*. Samples were taken from frogs in natural populations in upland rain forests in northeastern Australia during the cool, dry season in 2005. This species experienced short-term population declines during 1990 to 1994, while many other species declined to local extinction, and has since recovered in areas where sympatric species have not (Richards & Alford 2005). It is susceptible to chytridiomycosis, but currently occurs in good numbers at most sites in the Australian Wet Tropics (Woodhams & Alford 2005). We analyzed the data to compare intensities of infection on different body regions of individual frogs, and to determine whether patterns of differences in infection intensity among body regions varied with the geographical source of the host populations. We also classified infections into 4 rank groups, ranging from very light to heavy, and examined how patterns of differences in intensity among body regions were related to overall infection level. We tested 5 null hypotheses: (1) the intensity of chytrid infections is the same across body regions, (2) patterns of differences among body regions do not differ among frog populations, (3) the amount of *Bd* DNA detected is independent of surface area of the body region examined, (4) the overall intensity of infection does not affect patterns of differences of intensity of infection among body regions, (5) sampling only the legs or only the abdomen would not reduce the probability of detecting *Bd* DNA from an infected frog.

We addressed the second aim by proposing a standardized method for swabbing *Litoria genimaculata* to diagnose *Bd* infection using PCR. This method targets the regions of the integument most likely to be infected, while taking precautions to avoid facilitating reinfection or spreading the pathogen on the host. We

also suggest an approach for the development and application of standardized methods for other species that will minimize possible harmful effects.

MATERIALS AND METHODS

Sampling. We sampled adult male *Litoria genimaculata* at 2 high (≥ 800 m) and 2 low (< 400 m) elevation tropical rainforest streams in northern Queensland, Australia, during the dry season (late August to late September 2005), when the prevalence of *Bd* is likely to be high (Woodhams & Alford 2005). Site locations, elevations, and mean weather conditions at the times of the surveys can be seen in Table 1.

We searched for frogs along 400 m stream transects on 2 consecutive nights at each site. At Kirrama Bridge 1, Kirrama Bridge 11, and Birthday Creek, less than half of the transect was sampled on the first night, and the second night's sampling commenced 20 m past the point at which the first night's ceased, making it very unlikely that any frogs could be sampled more than once (*Litoria genimaculata* very rarely move more than a few meters between streamside perches on successive nights; Rowley & Alford 2007). At Frenchman Creek, only 2 frogs were located in 400 m on the first night. To avoid resampling these individuals, their capture locations were flagged, and individuals were not searched for within 5 m of those flagged locations on the second night, when many more frogs were active. Frogs were captured by hand using plastic bags, then sexed, weighed, measured, and swabbed using 5 separate swabs, one for each designated body region. A new pair of latex gloves was worn to handle each individual frog. All equipment and footwear were disinfected between sites. Sampling followed the hygiene protocol for the control of diseases in frogs (NSW National Parks and

Table 1. *Litoria genimaculata*. Details of sampling localities, with environmental variables and infection prevalence (percentage positive) and intensity (number of zoospore equivalents on positive individuals). Prevalence and intensity data include results for all frogs sampled, including those that initially tested negative on the legs and abdomen. Lat/Long: Latitude and longitude

Site	Lat/Long	Elevation (m)	Air (°C)	Water (°C)	Relative humidity (%)	No. of frogs		No. of zoospore equivalents			
						Sampled	Positive (%)	Mean \pm SD	Min.	Median	Max.
Kirrama, Bridge 1	18° 12' 11" S, 145° 53' 00" E	100	18	19	100	34	3 (9)	190.5 \pm 328.7	0.5	1.0	570.0
Kirrama, Bridge 11	18° 12' 55" S, 145° 47' 48" E	850	15	16.8	94	23	19 (83)	160.2 \pm 327.3	1.0	4.5	1073.0
Frenchman Creek	17° 18' 32" S, 145° 55' 16" E	40	19	19.8	95	17	15 (88)	22.6 \pm 38.5	1.0	7.0	135.0
Birthday Creek	18° 58' 54" S, 146° 10' 02" E	800	16.5	19.6	95	37	23 (62)	731.5 \pm 1694.4	1.5	221.5	8035.0

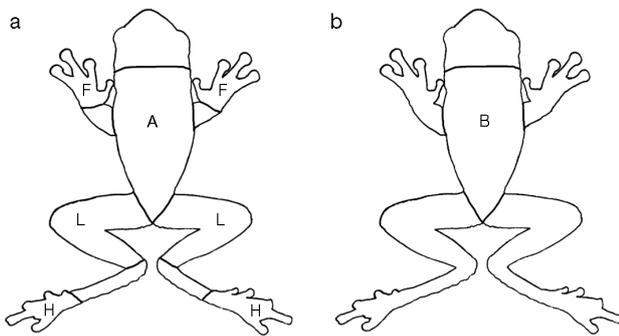


Fig. 1. *Litoria genimaculata*. (a) Ventral and (b) dorsal surfaces swabbed (A = abdomen, L = legs, H = hindfeet, F = forefeet, B = back)

Wildlife Service 2001) to avoid cross-contamination of infected individuals or sites.

Swabbing technique. To determine the distribution of the fungus among body regions, we sampled each individual with 5 separate swabs (MW100; Medical Wire & Equipment). An assistant held the frog during swabbing to ensure samples of different body regions were not cross-contaminated. Swabbing was performed as similarly as possible on all individuals, to reduce variation in measured infection intensity that might be caused by differences in technique, as follows (Fig. 1 shows an outline of a male *Litoria genimaculata* and illustrates the locations and relative sizes of the areas sampled): (1) Initially the back was swabbed, moving in from the posterior terminus of the body towards the head as many times as required to cover the entire back. This was done twice to ensure an adequate sample. (2) The ventral surface of the abdomen was swabbed in the same way until the entire area was covered twice. (3) The legs were sampled by swabbing the ventral surface of the thighs and calves in a proximal to distal direction enough times to cover the full ventral surfaces twice. (4) The ventral surfaces of both the forefeet were swabbed twice using a motion that encouraged the frogs to grip the swab to ensure good contact. If the swab was not well gripped during the first 2 passes, 1 to 2 additional passes were made. (5) The ventral surfaces of both of the hindfeet were swabbed twice each, by pressing them between the swab and the glove of the handler.

Sample selection. Because each PCR assay is expensive, and many of the individuals sampled in the field might have been uninfected, we initially ran diagnostic PCR tests only on swabs from the legs and abdomen of each individual to determine which were infected with *Bd*. The remaining 3 swabs from each individual that was positive in either region were tested separately. Finally, the samples from the forefeet and hindfeet of each individual that tested negative on both the

legs and abdomen were combined during the extraction phase and tested together to determine whether any animals that had tested negative on both the legs and abdomen were infected in the feet. Samples from the backs of individuals that were negative on the legs and abdomen were not processed because the rates of back infection found on individuals positive in either of these areas made it apparent that this area has the lowest prevalence and intensity of infection, often remaining uninfected until all other areas are positive. We incorporated the possible implications of this when considering our results.

Real-time TaqMan PCR assay. Extraction: PCR procedures for the extraction and amplification of *Batrachochytrium dendrobatidis* DNA were adapted from Boyle et al. (2004). PrepMan Ultra was used to prepare extractions of nucleic acids from swabs. Each swab was added to a 1.5 ml Eppendorf tube containing 30 to 40 mg 0.5 mm zirconium/silica beads (Daintree Scientific), to which 50 μ l of PrepMan Ultra were added. One negative and one positive control swab were included per run. DNA from laboratory cultured *Bd* was used as the positive control. Swabs were homogenized for 45 s using BeadBeater (Biospec) and were then centrifuged for 30 s at maximum speed to recover all material from the bottom of the tube. Homogenization and centrifugation were then repeated. The homogenized swabs were then heated in a dry heat block at 100°C for 10 min, cooled for 2 min at room temperature, then centrifuged at maximum speed in a microfuge for 3 min. Between 20 to 40 μ l of supernatant were collected and stored in columns of a microtitre plate (Sarstedt) at –80°C.

PCR assay. The extract was diluted 1/10 in molecular biology grade water (Sigma) into even columns of the plate. Assays were conducted using a Corbett RotorGene 3000 model. A total of 25 μ l (1 \times TaqMan Master Mix [Applied Biosystems], 900 nM PCR primer ITS1-3 Chytr and 900 nM PCR primer 5.8S Chytr, 250 nM ChytrMGB2 probe, and 5 μ l of DNA diluted 10⁻¹ in water) was added to each well and prepared in triplicate. Assay standards (prepared with dilutions of standard extract provided by Australian Animal Health Laboratory) were included in each run and used to create a standard curve. A control with distilled water (no DNA template) was also included and added after all samples and standards were made in triplicate.

Amplification conditions for the Corbett RotorGene 3000 were as follows: initial heating of 2 min at 50°C, and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The threshold cycle (C_t) values for each test and control reaction were determined using a ΔR_n of 0.10.

Correcting for relative areas of body regions. The body size of individuals measured at the time of swab-

bing ranged from 34.6 to 46.0 mm snout–vent length (SVL). To examine intensity of infection in relation to the relative area of each of the body region sampled, we measured the SVLs of a series of preserved adult male *Litoria genimaculata* spanning the same range of sizes. We then digitally photographed them against a light box to create silhouetted images. We used the program ImageTool v. 2a (Wilcox et al. 1995) to measure the area of each body region in cm² and regressed log(area) of each region on log(SVL). All of the regressions had slopes that were very close to 2.0, the slope expected if, within the limited size range of individuals we measured, all regions grew isometrically, and none differed significantly from 2.0. This indicates that the percentage of the total surface area contributed by each body region remains constant over the range of body sizes we sampled. We therefore determined the percentage of the total mean surface area sampled attributable to each body region (27.1, 26.5, 28.2, 8.0, and 10.1% for the abdomen, legs, back, forefeet, and hindfeet, respectively), and compared the percentage of the total zoospore equivalents found on each body region to these percentages.

Statistical analyses. The data were entered and organized using Microsoft Excel 2003 and analyzed using SPSS v. 12, except that multiple response permutation procedure and multiple response blocked procedure tests (MRPPs and MRBPs) were carried out using BLOSSOM version W2005.08.26 (Cade & Richards 2005). We log(n+1) transformed the PCR estimates of number of zoospore equivalents prior to analysis to reduce skewness and correlations between the mean and variance. To test the overall null hypothesis that there are no differences in intensity of infection among body regions, we calculated differences between the log-transformed intensities of infection for the legs and abdomen, abdomen and back, back and forefeet, and forefeet and hind feet for each individual, and tested these vectors of differences against the null hypothesis that on average they were equal to (0,0,0,0) using an MRBP test with hypothesis testing by 10 000 randomizations (Cade & Richards 2005); this is a distribution-free analogue of Hotelling's T^2 test for significant departure from a hypothesized mean vector. We performed a profile analysis (Morrison 1976) to test for differences in the distribution of intensity across body regions among sites. This analysis separately examined the total intensity of infection and patterns of differences in intensity of infection among body regions using MRPP tests with hypothesis testing by 10 000 randomizations (Cade & Richards 2005). Following significant overall tests, we performed separate profile analyses comparing each pair of sites using the same technique. We determined the significance of these tests using a

Bonferroni-adjusted alpha of 0.0167 to preserve the overall experimentwise alpha level at 0.05. We tested the null hypothesis that all body parts are infected with equal intensity by using an MRBP test of the null hypothesis that the percentage of zoospore equivalents on each body region was equal to the percentage contribution of that body region to the total area sampled. We also used an MRPP test to determine whether patterns of intensity of infection among body areas differed among frogs assigned to 4 groups based on their total infection intensities.

RESULTS

Overall prevalence and intensity of infection

Weather conditions at each site at the times of sampling were within the range for adequate but not maximal *Bd* growth (Table 1). Of the 111 individuals sampled, 58 were positive for *Bd* on the legs or abdomen, and therefore all other swabs for these individuals were analyzed separately. We performed PCR analyses on the combined forefeet and hindfeet swabs from the 53 individuals that were negative for *Bd* on both the abdomen and the legs; these revealed 2 additional positive individuals that were very lightly infected (1 and 4 zoospore equivalents, from Kirrama Bridge 1 and Kirrama Bridge 11, respectively). These individuals were not included in detailed statistical analyses because we had combined the samples from the forefeet and hindfeet, making comparisons among body regions impossible. There was no evidence for any inhibitory activity against the positive controls incorporated into the TaqMan master mix (Hyatt et al. 2007).

Only 3 of 34 frogs sampled at Bridge 1 in the Kirrama State Forest (9%) tested positive (Table 1). This low prevalence fits with patterns seen in other studies (e.g. Woodhams & Alford 2005), which have found that prevalence of *Bd* is usually lower at lowland sites. Data from this site were omitted from the detailed analyses of the distribution of *Bd* among body regions because of the small sample size. Prevalence at the remaining 3 sites were relatively high: 83% at Kirrama Bridge 11, 88% at Frenchman Creek, and 62% at Birthday Creek (Table 1). Intensity of infection, measured as number of zoospore equivalents totaled across body regions (Table 1), was relatively low at Bridge 1, Bridge 11, and Frenchman Creek (medians of 1, 4.5, and 7 zoospore equivalents, respectively) and substantially higher at Birthday Creek (median of 221.5 zoospore equivalents). However, at all sites some individuals carried very light infections and some carried relatively heavy infections.

Differences in infection intensity among body regions and sampling localities

Our MRBP test rejected the null hypothesis of equal intensity of infection among body regions ($\delta = 2.2125$, $p = 0.0001$). Both the total intensity of infection and the pattern of differences among body regions differed significantly among localities ($\delta = 2.843$, $p = 0.0001$ and $\delta = 2.307$, $p = 0.008$, respectively). Frogs at Bridge 11 and Frenchman Creek did not differ significantly in pattern of infection intensity among body regions ($\delta = 2.380$, $p = 0.5363$) or in the total intensity of infection ($\delta = 2.442$, $p = 0.1017$). Frogs at Bridge 11 and Birthday Creek differed significantly in pattern of intensity among body regions ($\delta = 2.285$, $p = 0.0055$) and in total intensity of infection ($\delta = 3.389$, $p = 0.0035$). Frogs at Frenchman Creek and Birthday Creek also differed significantly in their patterns of intensity among body regions ($\delta = 2.300$, $p = 0.0071$) and in total intensity of infection ($\delta = 2.504$, $p = 0.0001$).

Fig. 2 illustrates the patterns of infection for the 5 body regions at the 3 localities with relatively high prevalences of *Bd*. Most individuals at both Bridge 11 and Frenchman Creek had fairly light infections. On average, at these localities infection was most intense on the legs and the abdomen, followed in decreasing order by the hindfeet, forefeet and back. At Birthday Creek, where most individuals had more intense infections, intensity tended to be highest on the legs, followed in decreasing order by the hindfeet, the abdomen, the forefeet, and the back.

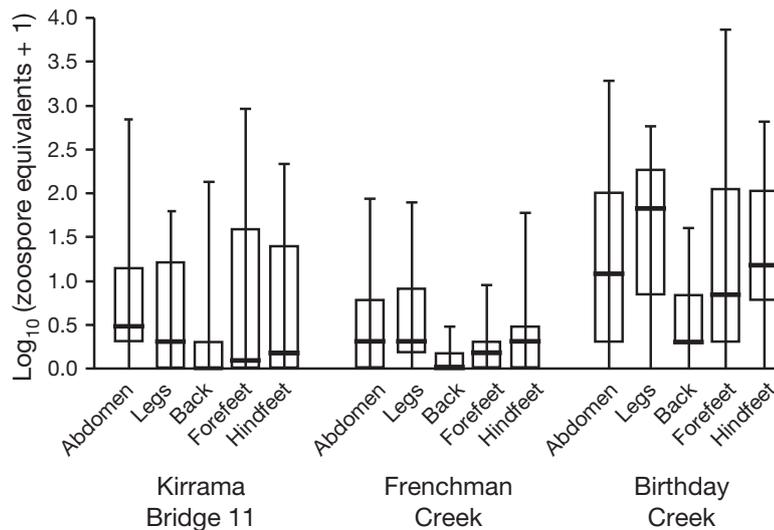


Fig. 2. *Litoria genimaculata*. Distributions of infection intensity (number of zoospores equivalents) for each body region at the 3 sites used in analyses, Kirrama Bridge 11 (850 m), Frenchman Creek (40 m), and Birthday Creek (800 m). Heavy horizontal bars represent medians, upper and lower limits of boxes are 75th and 25th percentiles, respectively, and vertical bars represent ranges

Effects of overall infection intensity on distribution among body regions

Overall, the percentage of total intensity of infection occurring on different body regions differed significantly from that expected if all regions of the body were infected in proportion to their surface area (MRBP, $\delta = 39.076$, $p = 0.00005$). To determine whether the distribution of *Bd* among regions of the body is related to the overall intensity of infection, for the 56 frogs included in our locality analyses we totaled the estimated zoospore equivalents across all body regions, ranked frogs by this total, and divided them into 4 groups. Each group contained 14 individuals (Table 2). The very light rank group included frogs that totaled 1 to 3 zoospore equivalents. The light and moderate rank groups totaled 4 to 30 and 31 to 233 zoospore equivalents, respectively. The heavy rank group contained individuals that totaled 248 to 8035 zoospore equivalents. An MRPP test rejected the null hypothesis that the patterns of *Bd* intensity among body regions did not differ among these 4 groups ($\delta = 2.016$, $p = 0.0001$).

Fig. 3 illustrates the distribution of zoospore equivalents on body regions of frogs in each of the 4 infection rank groups. The abdomen accounted for a median of 58% of the total zoospore equivalents estimated from individuals in the very light group; this is over twice the proportion expected based on the relative surface area of the abdomen. The relative intensity of infections in the abdomen decreased as total infection intensity increased, until in the heavy rank group, more than 75% of individuals had a lower proportion of zoospore equivalents on the abdomen than would be expected based on its relative size. The median percentage of zoospore equivalents on the legs in the very light infection rank group was 0, although more than 25% of individuals had a greater proportion of the total number of zoospore equivalents on their legs than would be expected based on their relative size. The relative intensity of infection in the legs increased as total intensity increased in the light and moderate groups, then decreased to a median value of roughly half that expected from the relative size of the legs in the heavy group. The back was under-represented in all infection rank groups; only 1 of the 56 individuals examined had a greater percentage of the total number of zoospore equivalents in the sample from the back than expected based on the rel-

Table 2. *Litoria genimaculata*. Summary of positive PCR results by body region for 14 individuals in each infection rank group

Infection rank	Body region	No. of positives	No. that were also positive on:					No. negative in all other regions
			Legs	Abdomen	Back	Forefeet	Hindfeet	
Very light	Legs	6	–	1	1	3	2	3
	Abdomen	9	1	–	1	2	2	6
	Back	1	1	1	–	1	1	0
	Forefeet	4	3	2	1	–	2	0
	Hindfeet	3	2	2	1	2	–	0
Light	Legs	12	–	11	7	9	10	0
	Abdomen	13	11	–	8	10	10	1
	Back	8	7	8	–	7	8	0
	Forefeet	10	9	10	7	–	9	0
	Hindfeet	11	10	10	8	9	–	0
Moderate	Legs	13	–	10	10	11	12	0
	Abdomen	11	10	–	9	10	11	0
	Back	11	10	9	–	9	10	0
	Forefeet	12	11	10	9	–	12	0
	Hindfeet	13	12	11	10	12	–	0
Heavy	Legs	14	–	14	12	14	14	0
	Abdomen	14	14	–	12	14	14	0
	Back	12	12	12	–	12	12	0
	Forefeet	14	14	14	12	–	14	0
	Hindfeet	14	14	14	12	14	–	0

ative size of this body area, and most had substantially less than expected. The patterns exhibited by the forefeet and hindfeet were similar across infection rank groups. The medians for both were 0 in the very light group, with slightly more individuals showing percentages of total zoospore equivalents >0 in samples from the forefeet. In the light infection rank group, median percentage of total zoospore equivalents was close to that expected from the relative sizes of the regions for both forefeet and hindfeet, and more than 75% of individuals returned positives for each. Relative intensity of infection in both forefeet and hindfeet decreased slightly in the moderate group, probably reflecting sampling variation. In the heavy infection rank group, the relative intensity of infection in the forefeet and hindfeet increased substantially, leading to median percentages of zoospore equivalents approximately twice as great as expected from the size of the body regions.

Examining Fig. 3 by looking across body regions within infection rank groups, very light infections were con-

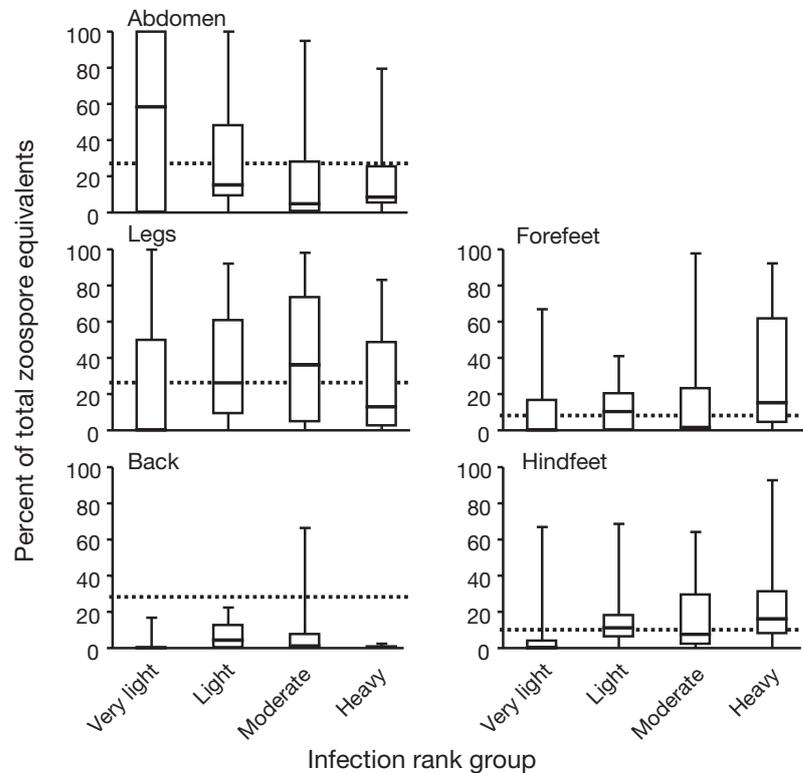


Fig. 3. *Litoria genimaculata*. Distributions of infection intensity (percentage of total zoospore equivalents) among individuals in 4 infection rank groups for each body region. Dotted horizontal lines indicate the mean percentage each body region contributes to the total area sampled, and delineate the null hypothesis of equal intensity of infection per unit area. Each rank group contained 14 individuals. Plot symbols defined as in Fig. 2

centrated on the abdomen, with some individuals also having relatively intense infections on the legs, and tended to be absent from the back, forefeet, and hindfeet. Light infections were concentrated on the legs, forefeet, and hindfeet; the median percentages of zoospore equivalents on all 3 of these body regions were similar to those expected from their relative sizes. Although the abdomens of most individuals with light infections returned positive PCR results, the percentage of total zoospore equivalents was lower on the abdomen than expected from its relative size. The median relative intensity of infection on the back reached a peak in this group, but remained low, with a median percentage of total zoospore equivalents approximately 25% as great as expected from the relative size of the back. The pattern for moderate infections was similar to that for light infections, with even lower relative intensity on the abdomen, and higher relative intensity on the legs. Finally, heavy infections had median relative intensities on the forefeet and hindfeet that were well above the levels expected from the relative sizes of these regions, relative intensities on the legs that were lower than those in moderate infections, and low and very low relative intensities on the abdomen and back, respectively.

Table 2 makes it possible to examine patterns of relationships of positive PCR results among body regions within individuals in each of the infection rank groups. Nine of the 14 individuals in the very light rank group were positive for only 1 body region, in each case either the abdomen or the legs. Individuals that were positive for more than one body region tended to be positive on either the forefeet or the hindfeet or both. In the light rank group, 11 of the 14 individuals were positive for both the legs and the abdomen, and most individuals that were positive for either of these regions were also positive for the forefeet, the hindfeet, or both. Only 1 individual was positive for only a single region, the abdomen. In the moderate and heavy rank groups, almost all individuals were positive on all body regions; even the back was consistently positive, although as noted in the discussion of Fig. 3, the percentage of the total estimated zoospore number for the back remained low in all infection rank groups. Including the results for the 2 individuals of the group of 53 that tested negative for both abdomen and legs would make little difference to this general pattern. When we tested the combined hindfeet and forefeet samples for those 53 individuals, only 2 of them were positive, with infections that would have placed them in the very light rank group (1 zoospore equivalent) and just into the light rank group (4 zoospore equivalents). These individuals were negative on all other body regions.

DISCUSSION

Prevalence, intensity and sampling locality

Populations from 3 of the 4 localities we sampled had relatively high prevalences of infection (Table 1), indicating that *Litoria genimaculata* populations are now persisting in equilibrium with the disease and that it does not inevitably result in rapid mortality; this species may thus be serving as a reservoir host. Although prevalences at 3 of the 4 localities were high and similar, they differed from one another, and the fourth locality had a very low prevalence. There is no obvious environmental cause for these differences; measured environmental variables were similar at all localities at the times of sampling. It is likely that weather at these localities differs more than is indicated by our short-term measures when it is integrated over the full annual cycle; however, the pattern detected was not the expected one, in which higher prevalences occur at upland localities (Woodhams & Alford 2005). The typical upland–lowland differences in prevalence may result from different thermal regimes (Woodhams et al. 2003), and the recent history at Frenchman Creek may simply have not included temperatures elevated sufficiently to reduce the prevalence of *Bd*. Most individuals at 3 of the 4 localities had relatively low total intensities of infection, while most at the fourth, Birthday Creek, had relatively high total intensities; this is also likely to reflect the recent detailed history of the locality. The fact that at all localities some individuals had low and some had high total intensities indicates that although there appear to be effects of the environment and history that operate at the locality level, factors affecting individuals vary on a finer scale.

Distribution among body regions

Berger et al. (2005a) found no significant differences in the densities of zoosporangia on the ventral surfaces of the head, abdomen, legs, and toes in *Litoria caerulea* suffering from severe chytridiomycosis that were examined post-mortem using histology, but did find lower densities on the dorsal surfaces of the back and calves. Because they examined only frogs suffering from advanced symptoms of the disease, their results provide no insight into patterns of development of infection, or patterns of distribution of infection in asymptomatic frogs. Longcore et al. (2007) examined road-killed and deliberately collected frogs of 7 species collected in Maine, USA. They performed histological diagnoses on sections of skin from the webbing between the toes of the hindfoot, the pelvic area of the ventral abdomen, and (for some specimens) the ventral

surface of the tibial region of the hind leg. They were not able to examine all regions for all frogs due to varying degrees of damage to the animals prior to collection. They found that prevalence and intensity of infection varied among species and collection localities. Overall, they found that prevalences of infection on skin from toe webbings were somewhat higher than on skin from the pelvic region (15.5 vs. 9.8%), and that prevalences were lowest on skin from the tibial region (5.3%). These patterns differed among species. They classified the majority of infections as relatively light, but did not examine how intensity of infection was distributed among body regions within individuals. Puschendorf & Bolaños (2006) found that sections from the fingers, toes, and pelvic patch region of *Eleutherodactylus fitzingeri* returned positive histological diagnoses more often than sections from the gular region and abdomen; however, they did not categorize intensity of infection.

Our results from living frogs differ from both histological surveys, as total intensity of infection increased, and larger amounts of DNA were found on all ventral surfaces; however, even in heavily infected frogs body regions differed in intensity of infection. In heavy infections, the greatest median percentages of DNA were swabbed from the hindfeet and forefeet, followed by legs and abdomen. In light infections, the percentage of zoospore equivalents was greatest for the abdomen, which accounted for more than twice as many zoospore equivalents as expected from its relative size. The relative intensity of infection on the abdomen decreased as overall intensity increased, while the relative intensity on legs and feet increased (Fig. 3).

The relationships we found between the distribution of *Bd* DNA among body regions and overall intensity of infection could have 3 causes. They could reflect differences in opportunity for infection; body regions that are more likely to be exposed to infective zoospores from outside the individual should tend to become infected first, and be the first sites to develop heavier infections through local reinfection. They could reflect differences in the vulnerability of body regions to reinfection; if the skin surface of a body region presents a more favorable environment for local zoospore release and reinfection, for example if it tends to retain a film of moisture, that part might become more heavily infected earlier in the process. Finally, they could reflect differences among body regions in their innate resistance to *Bd*, perhaps in the form of localized concentrations of antimicrobial peptides, many of which are effective against *Bd* (Rollins-Smith et al. 2002, Woodhams et al. 2006a,b), or differences in the local structure of the outer layers of the skin, which could also affect vulnerability

(Berger et al. 2005a). When frogs are not moving, the ventral surfaces of their feet and legs are almost always in contact with the substrate, whereas their abdomen is only sometimes in such contact. Berger et al. (2005b) found that zoospores growing *in vitro* appeared to be attracted to established colonies and that thalli grow better in clusters. They also found that single zoospores placed on agar usually died. Contact with substrates may maintain a moist microenvironment, allowing high zoospore survival and reinfection rates, and may also increase local reinfection rates by reducing rates of dispersal of zoospores to the external environment.

The intensity of infection on the backs of even heavily infected individuals was always relatively low. Berger et al. (2005a) also found low intensities on the back. This may be due to the high number of serous glands containing anti-fungal peptides located in this region (Rollins-Smith et al. 2002, Berger et al. 2005a), or the relative dryness of the dorsal surface (Berger et al. 2005a), which might inhibit reinfection. *Litoria genimaculata* often spend extended periods on open perches away from water (Rowley & Alford 2007); the relatively low intensities of infection we found on their backs could result from dryness during these times, or from higher temperatures experienced when the dorsal skin is exposed to direct sunlight, either of these could slow reinfection by *Bd*.

Effects of sampling localities on distribution among body regions

Patterns intensity of infection among body regions were significantly different between Birthday Creek and the other 2 localities with large enough samples for analysis. These differences among localities did not appear to be a product of differences in elevation, because Kirrama Bridge 11 and Frenchman Creek, which did not differ significantly, are at high and low elevations, respectively, whereas Birthday Creek, which differed from both of those localities, is at an elevation similar to Bridge 11. It is likely that the differences among localities in pattern of distribution on body regions were caused by a combination of the large differences among localities in intensity of infection (Table 1) with the relationship between intensity of infection and distribution of *Bd* among body regions. Most individuals at Bridge 11 and Frenchman Creek fell into our very light and light infection rank groups, which tended to be most heavily infected on the abdomen, followed by the legs (Fig. 3). Most individuals at Birthday Creek fell into our moderate and heavy infection rank groups, which tended to carry relatively high percentages of zoospore equivalents on their forefeet and hindfeet and

relatively lower (although still high in absolute terms, since these regions are much larger) percentages on the legs and abdomen (Fig. 3).

Patterns of development of infections

Our data are consistent with the hypothesis that the overall intensities of infection we measured are related to the pattern of development of infection in most individuals, rather than to patterns of loss of infection or a combination of patterns of development in some individuals and loss in others. Berger et al. (2005a) found that serous glands, which produce antimicrobial peptides (AMPs), are most dense in the dorsal skin of *Litoria caerulea*, and are also relatively dense in the ventral skin of the abdomen and legs, but are less dense in the ventral skin of the forefeet and hindfeet. This suggests that resistance to colonization and reinfection by *Bd* should be greatest in the dorsal skin, intermediate in the ventral skin of the legs and abdomen, and lowest in the ventral skin of the feet. Resistance to infection caused by AMPs, and therefore possible reduction in intensity or loss of infection, should occur most rapidly in the dorsal skin, followed by the ventral skin of the legs and abdomen, and finally by the ventral skin of the feet. The lightest infections should thus be concentrated in the feet. This pattern is not the most common one in our data. Some individuals may acquire infections via the forefeet or the hindfeet, or may be losing infections, but the great majority of individuals in the very light and light infection rank groups were most heavily infected in the abdomen and legs, suggesting that these individuals had infections in early stages of development and that the abdomen and legs are the most common sites of initial infection. Infections in the 2 most intense infection rank groups tended to be heaviest in the feet, suggesting that once infections reach these areas, their lower resistance may allow greater densities of *Bd* thalli to develop. It is also very likely that the back is not a common route of infection; all individuals but one had fewer zoospore equivalents on their backs than expected from the relative size of this region, and over two-thirds of individuals in the very light and light infection rank groups did not return positive results from their back samples. Because we did not test back samples for the 51 individuals who did not test positive on any of the legs, abdomen, forefeet, or hindfeet, it remains possible that we have missed a class of individuals who are infected only on their backs, but the pattern in the remainder of the data makes this seem very unlikely. The abdomen, followed by the legs, may be the most common sites of initial infection because they present relatively large areas for zoospores to

establish on, and are often pressed against the substrate, both when water is being absorbed through the pelvic patch and when frogs adopt a water-conserving posture to minimize their exposed surface area (Shoemaker et al. 1992). It is also possible that the nature of the skin in these regions, both of which include parts of the pelvic patch (Shoemaker et al. 1992) in *L. genimaculata*, facilitates invasion by *Bd* zoospores.

Implications for swabbing methodology

The increased use of PCR to identify *Bd* infection in amphibians suggests a need for care in the selection of patterns used in swabbing individuals. For results to be comparable between individual researchers and between studies, samples must be collected from a standard set of body regions, swabbed with equal effort. Many publications have described swabbing methods and patterns (e.g. Speare et al. 2005, Hyatt et al. 2007) that could be adopted as standards. However, adoption of a single universal pattern for swabbing several body regions could inadvertently harm the individuals that are sampled. Because populations of *Bd* increase on hosts through external reinfection, and zoospores may settle very close to the sites from which they are released (Berger et al. 2005b), swabbing may artificially increase the rate of dispersal of zoospores across hosts. This might increase the severity of infections on swabbed animals. To prevent this, swabbing could be limited to a single region of the body. If this were done, the area most likely to return a positive result should be sampled. However, our results indicate that swabbing any single body region can lead to a high proportion of false negatives in lightly infected frogs. A second approach, optimal from the points of view of sensitivity and the minimization of potential harm, is to use separate swabs for each body region. These could then be combined before diagnostic PCR is performed. However, the increased sample volume and time needed to collect and process samples required by this technique may make it impractical. A third alternative is to swab more than one region, in a pattern that minimizes the probability of transporting zoospores from more heavily infected regions to more lightly infected ones. In *Litoria genimaculata*, we suggest that for male *L. genimaculata* the ventral surfaces of the legs, the abdomen, and the gripping surfaces of the fore- and hindfeet should all be swabbed, with the feet first, followed by the legs and then the abdomen. This would minimize the probability of spreading the infection in lightly infected individuals. Patterns for other species are likely to differ; Longcore et al. (2007) found the feet to be infected at the greatest intensity in lightly infected *Rana* spp. There could also be differences

among males, females, and juveniles within species; Rowley & Alford (2007) found differences in behaviour between males and females that could lead to differences in the pattern of development of infections. This suggests that in the absence of species- and class-specific information, our second approach, the use of separate swabs for each body region should be followed, at least until sufficient data have accumulated to allow an evidence-based pattern to be specified.

It would be useful in future longitudinal studies in which individuals are repeatedly swabbed to swab major regions of the body separately, perhaps combining half of each sample for diagnosis of the presence of *Bd*, and then examining samples from each body region of infected individuals separately. This would clarify initial routes of infection and patterns of development of infections. It would also allow identification of any region of the body producing skin secretions that inhibited the diagnostic PCR (Hyatt et al. 2007). This information should aid in understanding how *Bd* is transmitted and why hosts vary in their susceptibility and could be used to develop species- and class-specific sampling orders for swabbing with single swabs.

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