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Tadpole Mouthpart Depigmentation as an Accurate Indicator of Chytridiomycosis, an Emerging Disease of Amphibians

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Chytridiomycosis is an emerging infectious disease of amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis*, and its role in causing population declines and species extinctions worldwide has created an urgent need for methods to detect it. Several reports indicate that in anurans chytridiomycosis can cause the depigmentation of tadpole mouthparts, but the accuracy of using depigmentation to determine disease status remains uncertain. Our objective was to determine for the Mountain Yellow-legged Frog (*Rana muscosa*) whether visual inspections of the extent of tadpole mouthpart depigmentation could be used to accurately categorize individual tadpoles or *R. muscosa* populations as *B. dendrobatidis*-positive or negative. This was accomplished by assessing the degree of mouthpart depigmentation in tadpoles of known disease status (based on PCR assays). The depigmentation of *R. muscosa* tadpole mouthparts was associated with the presence of *B. dendrobatidis*, and this association was particularly strong for upper jaw sheaths. Using a rule that classifies tadpoles with upper jaw sheaths that are 100% pigmented as uninfected and those with jaw sheaths that are <100% pigmented as infected resulted in the infection status of 86% of the tadpoles being correctly classified. By applying this rule to jaw sheath pigmentation scores averaged across all tadpoles inspected per site, we were able to correctly categorize the infection status of 92% of the study populations. Similar research on additional anurans is critically needed to determine how broadly applicable our results for *R. muscosa* are to other species.

CHYTRIDIOMYCOSIS is an emerging infectious disease of amphibians caused by *Batrachochytrium dendrobatidis*, a member of the fungal order Chytridiales. This pathogen was first described in the late 1990s (Berger et al., 1998; Longcore et al., 1999; Pessier et al., 1999) and by 2004 had been found infecting animals in two amphibian orders (Anura and Caudata), 14 families, and 93 species distributed on every continent except Asia and Antarctica (Speare and Berger, 2004; Rachowicz et al., 2005). Chytridiomycosis often causes mass mortality of anuran populations and has been implicated in numerous species declines and possible extinctions (e.g., Berger et al., 1998; Daszak et al., 2003; Lips et al., 2004). Although it remains unclear whether the rapid emergence of *B. dendrobatidis* is a result of recent spread (Morehouse et al., 2003; Weldon et al., 2004) or increased virulence (Carey, 2000), this disease is increasingly a global threat to the long-term persistence of anuran species (Daszak et al., 2000).

Amphibians are the only known host for *B. dendrobatidis*, which attacks keratinized cells that are found in the skin of adults and mouthparts of anuran larvae (Berger et al., 1998). Transmission occurs in water via a flagellated zoospore that encysts in the amphibian and creates a zoosporangium, from which new zoospores are subsequently released into the water (Longcore et al., 1999). Although chytridiomycosis causes mortality in post-metamorphic stages of numerous anuran species (Berger et al., 1998; Nichols et al., 2001), tadpoles generally carry the fungus without experiencing increased mortality (Rachowicz and Vredenburg, 2004; but see Blaustein et al., 2005).

Given the urgent need to understand more about the biology of *B. dendrobatidis* and its effects on amphibians, the development of methods to detect the presence of *B. dendrobatidis* is critically important. To date, these methods include cytology (Berger et al., 1998; Nichols et al., 2001), histology (Longcore et al., 1999; Fellers et al., 2001; Nichols et al., 2001), histochemistry (Berger et al., 2002), standard PCR (Annis et al., 2004), and real-time (quantitative) PCR (Boyle et al., 2004). All of these methods provide accurate means of screening amphibians for *B. dendrobatidis*, but also have important limitations. These screening techniques generally necessitate collecting specimens or tissue samples, require specialized laboratory equipment, and are time consuming and relatively expensive. These limitations are of particular concern when making collections is not practical (e.g., when working with endangered species) and when it is necessary to screen large numbers of animals in the field (for example, when the incidence of *B. dendrobatidis* is very low).

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The mouthparts (oral discs) of tadpoles normally have keratinized jaw sheaths (beaks) and toothrows that are heavily pigmented (black) and bilaterally symmetrical (McDiarmid and Altig, 1999), but *B. dendrobatidis* infection can cause the depigmentation of these structures (e.g., Lips, 1999; Fellers et al., 2001; Rachowicz and Vredenburg, 2004). This suggests the possibility of using visual inspections of tadpole mouthparts in the field to quickly determine the presence/absence of *B. dendrobatidis*. However, tadpole mouthparts can become depigmented for other reasons (e.g., cold temperature, chemical contaminants; Hayes et al., 1997; Rowe et al., 1998; Rachowicz, 2002), making uncertain the accuracy of mouthpart depigmentation as an indicator of chytridiomycosis.

Our study objective was to determine in the Mountain Yellow-legged Frog *Rana muscosa* whether mouthpart depigmentation is an accurate indicator of chytridiomycosis at the level of both individual tadpoles and populations. This was accomplished by comparing the degree of mouthpart depigmentation in tadpoles of known disease status (i.e., *B. dendrobatidis*-positive or negative, based on PCR). We chose *R. muscosa* as our study species because the general association between chytridiomycosis and tadpole mouthpart depigmentation in this species has already been described (Fellers et al., 2001; Rachowicz and Vredenburg, 2004).

Materials and Methods

Study species.—*Rana muscosa* is endemic to the Sierra Nevada of California and Nevada and to the Transverse Ranges in southern California, where historically it was abundant in lakes, ponds, and streams (Grinnell and Storer, 1924; Stebbins, 2003). Adults are highly aquatic, rarely moving more than a few meters from water (Bradford, 1984; Matthews and Pope, 1999), and tadpoles overwinter at least once prior to metamorphosing (Bradford, 1983). During the past century, *R. muscosa* has disappeared from at least 80% of its historic localities (Drost and Fellers, 1996). Although the best documented cause of this decline is the introduction of non-native fishes (Bradford, 1989; Knapp and Matthews, 2000; Vredenburg, 2004), recent studies also suggest the importance of chemical contaminants (Davidson, 2004) and chytridiomycosis (Rachowicz et al., in press).

Field mouthpart inspections.—During 2002, 2003, and 2004, we inspected mouthparts of *R. muscosa* tadpoles at 116 lakes and ponds in Sequoia, Kings Canyon, and Yosemite National Parks, and the John Muir Wilderness, California. Jaw sheaths and toothrows of 1–24 tadpoles per site (mean = 10) were inspected using a 10X hand lens. Chytridiomycosis-induced mouthpart depigmentation in *R. muscosa* typically starts as a small, conspicuous white gap in the normally black jaw sheaths and toothrows that subsequently expands until most or all black pigment is gone (Rachowicz and Vredenburg, 2004). Therefore, for each tadpole, we estimated the percent of the upper and lower jaw sheath lengths (as integer values) that were pigmented. We also categorized the pigmentation status of all toothrows combined as ''fully pigmented'' if toothrows were black with no obvious gaps, ''partially depigmented'' if toothrows were discolored (brown or gray instead of black) and/or contained obvious white gaps in the black pigment, or ''completely depigmented'' if toothrows contained no pigment at all. One of the inspected *R. muscosa* tadpoles from each site (selected randomly) was collected for subsequent PCR assays to determine *B. dendrobatidis* presence/absence. Collected tadpoles were euthanized with an overdose of buffered tricaine methane sulfonate and preserved in 95% ethanol. Following mouthpart inspections, tadpoles that were not collected were released at the site of capture.

We restricted the developmental stage of inspected tadpoles to those of Gosner stage 30–41 (Gosner, 1960) because (1) chytridiomycosis-induced depigmentation of *R. muscosa* jaw sheaths is typically not evident until tadpoles reach stage 30 (R. Knapp, unpubl. data), and (2) tadpole mouthparts are lost at the end of stage 41 as a result of metamorphosis (Gosner, 1960). To minimize the chances that observed jaw sheath depigmentation was a consequence of freezing temperatures (Rachowicz, 2002), all inspections were conducted during summer and at least two weeks after ice-out.

As part of a study of the effects of chytridiomycosis on *R. muscosa* population dynamics (Rachowicz et al., in press), we have been conducting visual encounter surveys (Knapp and Matthews, 2000) at ten *R. muscosa* populations three times per summer. Following each visual survey, we also conduct tadpole mouthpart inspections and collect specimens (one specimen per survey date) for PCR assays of *B. dendrobatidis* presence/absence. At one of these sites located in the John Muir Wilderness (Fresno County, California), we found no evidence of *B. dendrobatidis* on *R. muscosa* in 2002 but found *B. dendrobatidis* present in the population in 2003. In the current study, we used this site to describe the effect of the *B. dendrobatidis* outbreak on

tadpole mouthpart pigmentation. We refer to this site as the ''intensive survey site.''

Batrachochytrium dendrobatidis *DNA extraction and amplification.*—Entire tadpole mouthparts, including jaw sheaths and toothrows, were dissected out of each specimen and soaked in 1X TE buffer (10 mM Tris.Cl pH 7.6, 1 mM EDTA pH 8.0) for two days prior to DNA extraction. For extractions performed in 2003 (76 specimens, 20 collected in 2002 and 56 collected in 2003), the right half of each mouthpart was extracted using a DNeasy Tissue Kit (Qiagen), and we screened this extraction for *B. dendrobatidis* using standard PCR. For samples that produced no amplification of *B. dendrobatidis* DNA, we extracted the left half of the mouthpart using Prepman Ultra (Applied Biosystems), pooled left and right mouthpart extractions, and used real-time PCR to provide a quantitative measure of the *B. dendrobatidis* load in the entire mouthpart. For extractions conducted in 2004 (from 40 specimens collected in 2004), we extracted entire mouthparts and screened all specimens using only real-time PCR. Both extraction methods effectively recover *B. dendrobatidis* DNA for standard and real-time PCR amplification (Boyle et al., 2004).

Standard and real-time PCR assays both targeted the ribosomal DNA (rDNA) internal transcribed spacers (ITS). For standard PCR, a 235 bp fragment containing the 5.8S rRNA gene was amplified using *B. dendrobatidis*-specific primers, forward CHYITS1F3 5′-ACAAAATTTATTTATTTTTTCGAC-3′ (located in ITS1) and reverse CHYITS2R2 5′-CATGGTTCATATCTGTCCAG-3′ (located in ITS2). Although *B. dendrobatidis* zoospores display within-individual variability in ITS regions, these primers were designed in portions of sequence that are conserved across isolates originating from the U.S., Australia, Panama, and Ecuador (J. Morgan, unpubl. data). Each PCR reaction contained 0.5 µM of each primer, 10–100 ng of extracted DNA, 1X Taq buffer, 0.8 mM dNTP, 2.5 mM magnesium, and 0.05 units/µl of Taq polymerase (Roche Molecular Systems). This mix was thermocycled for 30 cycles. Cycle 1 was 95 C for 60 sec, 50 C for 45 sec, and 72 C for 90 sec. This was followed by 29 shorter cycles of 95 C for 30 sec, 50 C for 30 sec, and 72 C for 90 sec. The mix was held at 72 C for 7 min to complete extension and then held at 4 C. All PCR products were viewed on an ethidium bromide-stained 1.5% agarose and TAE gel.

The primers used for real-time PCR assays were also anchored in conserved regions and amplify a 95-base fragment between forward ITS-1Chytr3 5′-CCTTGATATAATACAGTGTGCCATATGTC-3′ (located in ITS1) and 5.8Schytr 5′-TCGGTTCTCTAGGCAACAGTTT-3′ (located in 5.8S; Boyle et al., 2004). Real-time Taqman (Roche Molecular Systems) PCR assays were performed using the probe ChytrMGB2 5′-CGAGTCGAACAAAAT-3′. Reaction conditions and quantitation standards follow Boyle et al. (2004) with the following modifications. A 20 µl final reaction volume was used, and assays were conducted in triplicate on a DNA Engine Opticon 2 System (MJ Research). The threshold value was manually set at 0.05 for all assays, the baseline was averaged over the cycle range and subtracted, and blanks were not subtracted. The standards gave reproducible results with $R^2 > 0.99$ for all assays. Negative extraction and PCR controls were run alongside all samples to allow detection of sample contamination with *B. dendrobatidis* DNA.

Samples were determined to be positive for *B. dendrobatidis* if standard PCR yielded a 235-base band or if the number of genomic equivalents detected in the real-time PCR assay was >1 (= 80 genomic equivalents in the total extracted tissue). This lower limit for the real-time PCR assay was a conservative measure taken to minimize the chance of calling an uninfected individual infected due to low-level contamination.

Statistical analysis.—To describe the general association between the extent of mouthpart pigmentation and *B. dendrobatidis* presence/absence for individual tadpoles, we used both univariate and multivariate analyses. In univariate analyses, we compared the extent of upper and lower jaw sheath pigmentation for infected versus uninfected tadpoles using Wilcoxon rank-sum tests. The frequency of toothrow pigmentation categories for infected versus uninfected tadpoles was compared using a χ^2 test. For multivariate analysis, we used logistic regression. Because we were interested not only in determining the relative strength of the relationships between the predictor variables and the probability of *B. dendrobatidis* presence but also in describing the actual shape of the significant relationships, we utilized a generalized additive model. Generalized additive models (GAMs) are similar to generalized linear models, but relax the assumption that the relationships between the response variable (when transformed to a logit scale) and predictor variables are linear. Relaxation of this assumption is accomplished by estimating a nonparametric smooth function (using loess) to describe the relationships between the response and predictor variables (Cleveland and Devlin, 1988; Hastie and Tibshir-

ani, 1991). The response variable was *B. dendrobatidis* presence/absence, and predictor variables included upper jaw sheath pigmentation (%), lower jaw sheath pigmentation (%), toothrow pigmentation (3 categories), and tadpole Gosner stage. Regression methods were identical to those described in Knapp et al. (2003) and Knapp (2005). The relationship between the probability of *B. dendrobatidis* presence and the one statistically significant predictor variable (upper jaw sheath pigmentation; see Results) is shown graphically as a response curve that describes the log odds of *B. dendrobatidis* presence as a function of upper jaw sheath pigmentation (throughout this paper, ''log'' refers to $\log_e$).

Having demonstrated that mouthpart depigmentation was associated with the presence of *B. dendrobatidis* and that this association was strongest for upper jaw sheaths (see Results), we assessed the predictive accuracy of using the extent of upper jaw sheath pigmentation to classify tadpoles as infected or uninfected. Tadpoles were categorized based on having upper jaw sheaths that were 100%, 90–99%, and <90% pigmented. The strength of the association between upper jaw sheath pigmentation category and the presence/absence of *B. dendrobatidis* was analyzed using χ^2 tests.

If tadpole mouthparts are occasionally depigmented by factors other than the presence of *B. dendrobatidis*, then using the extent of mouthpart pigmentation from a single tadpole per site may result in an unacceptably high level of false-positives (i.e., depigmented tadpole mouthparts suggest chytridiomycosis, but PCR assays fail to detect *B. dendrobatidis*). In this case, the rate of false-positives could be reduced by averaging the extent of mouthpart pigmentation across numerous tadpoles from the same site. To do this, we used only those 82 sites where we were able to inspect at least five tadpoles (range = 5–24; mean = 12), and for each of these sites, we averaged upper jaw sheath pigmentation across all tadpoles to create a pigmentation ''score.'' Scores were rounded to create integer values, and sites were grouped into three categories based on having scores of 100%, 90–99%, or <90%. A χ^2 test was used to analyze the strength of the association between average pigmentation score and *B. dendrobatidis* presence/absence. Although inspecting an even larger number of tadpoles per site might have increased the accuracy of our pigmentation scores, it was our intention to determine whether pigmentation scores provided an accurate indication of the presence/absence of *B. dendrobatidis* despite being based on a relatively small number of tadpoles.

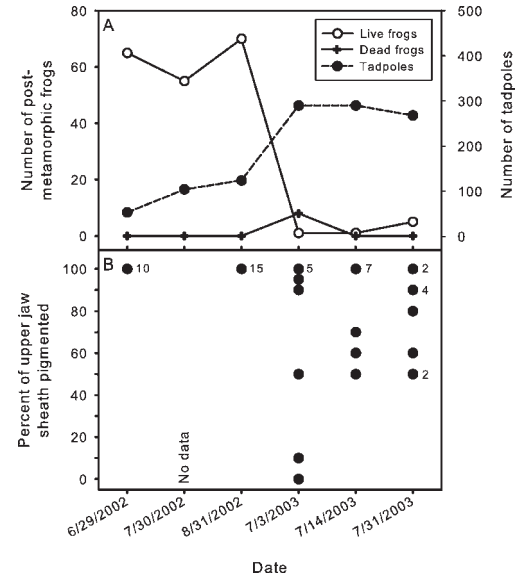

Fig. 1. Counts of post-metamorphic *Rana muscosa* (live and dead) and tadpoles at the intensive survey site before (2002) and after (2003) the appearance of *Batrachochytrium dendrobatidis* (A), and jaw sheath pigmentation scores for tadpoles at the same site (B). Numbers next to dots in (B) indicate the number of tadpoles represented by a single dot. The higher tadpole counts in 2003 than in 2002 are likely attributable to the fact that in 2002 all tadpoles were age-1, an age at which they are difficult to see due to their small size. In 2003, all tadpoles were age-2, were considerably larger, and as a result were much more visible. This *R. muscosa* population was extinct by 2004.

RESULTS

At the intensive survey site, repeated counts of *R. muscosa* conducted in 2002 prior to the appearance of *B. dendrobatidis* indicated a relatively stable number of post-metamorphic frogs (Fig. 1A). Inspections of tadpole mouthparts were conducted during two of the three surveys in 2002 and indicated that all inspected individuals had fully pigmented upper jaw sheaths (Fig. 1B). During the first survey in 2003, very few live but several dead post-metamorphic *R. muscosa* were observed (Fig. 1A). Two recently-dead frogs were collected and based on PCR assays were found to harbor *B. dendrobatidis*. Tadpole mouthpart inspections on this same date and subsequent dates in 2003 indicated that 30–80% of tadpoles had upper jaw sheaths that were <100% pigmented (Fig. 1B). The temporal association between the appearance of *B. dendrobatidis* in this *R. muscosa* population and the observation of depigmented tadpole mouthparts suggests that *B. dendrobatidis* was the cause of the observed mouthpart depigmentation.

TABLE 1. INFECTION STATUS OF *Rana muscosa* TADPOLES (*Batrachochytrium dendrobatidis* PRESENT/ABSENT) AS A FUNCTION OF TOOTHROW PIGMENTATION CATEGORY.

Toothrow pigmentation category	*B. dendrobatidis*[a]	
	Present	Absent
Fully pigmented	17 (35%)	32 (65%)
Partially depigmented	32 (60%)	21 (40%)
Completely depigmented	11 (79%)	3 (21%)

[a] Presence/absence of *B. dendrobatidis* determined using standard or real-time PCR assays.

Over the three years of field surveys, 116 tadpoles (collected from 105 sites) were subjected to both mouthpart inspections and PCR assays for the presence of *B. dendrobatidis*. Of these, PCR assays indicated that 52% were *B. dendrobatidis*-positive. We refer to these tadpoles as ''infected'' and *B. dendrobatidis*-negative tadpoles (based on PCR assays) as ''uninfected.'' The average extent of pigmentation for upper and lower jaw sheaths was significantly lower in infected than uninfected tadpoles, but this difference was much more pronounced for upper jaw sheaths (upper jaw sheath: $\overline{X}_{\text{infected}} = 46\%$, $\overline{X}_{\text{uninfected}} = 94\%$, two-sided Wilcoxon rank-sum test: $Z = 7.66$, $P < 0.0001$; lower jaw sheath: $\overline{X}_{\text{infected}} = 82\%$, $\overline{X}_{\text{uninfected}} = 88\%$, $Z = 2.02$, $P = 0.04$). The proportion of tadpoles with fully pigmented, partially depigmented, and completely depigmented toothrows also differed significantly between infected and uninfected animals (Table 1; $\chi^2 = 11.3$, df = 2, $P = 0.004$), although the distribution of infected and uninfected tadpoles amongst these three toothrow pigmentation categories overlapped broadly.

The results from the GAM indicated a highly significant relationship between *B. dendrobatidis* presence and upper jaw sheath pigmentation, but no significant relationships with lower jaw sheath pigmentation, toothrow pigmentation, or tadpole stage (Table 2). Therefore, after the relationship between upper jaw sheath pigmentation and *B. dendrobatidis* presence was accounted for, no significant relationship remained between *B. dendrobatidis* presence and the other predictor variables. Based on the response curve for upper jaw sheath pigmentation (Fig. 2), the odds of *B. dendrobatidis* presence were high and relatively constant in tadpoles with upper jaw sheaths that were 0–50% pigmented, and decreased sharply as the extent of upper jaw sheath pigmentation increased from 50% to 100%. Because the pigmentation status of upper jaw sheaths was of overwhelming importance in predicting *B. dendrobatidis* presence relative to the other predictor variables (Table 2), we present results for upper jaw sheaths only in subsequent analyses.

TABLE 2. ANALYSIS OF DEVIANCE TABLE SHOWING THE STATISTICAL SIGNIFICANCE (*P*-VALUE) OF THE PREDICTOR VARIABLES IN THE GAM ANALYSIS.

Model	Model		Test		
	Deviance	df	Deviance[a]	df[b]	*P*-value
Null model	161	115			
Full model	77	106			
Full model less:					
Upper beak pigmentation	134	108	57	2	$<10^{-10}$
Tadpole stage	83	108	6	2	0.08
Toothrow pigmentation	81	108	4	2	0.13
Lower beak pigmentation	78	108	1	2	0.59

[a] Test deviance = (deviance of full model less one covariate) − (deviance of full model)
[b] Test df = (df of full model less one covariate) − (df of full model)

The infection status of individual tadpoles was quite predictable based on the percent pigmentation of upper jaw sheaths (Table 3A). Tadpoles with 100% pigmented upper jaw sheaths were much less likely to be infected than were those with <90% pigmented upper jaw sheaths (Table 3A; $\chi^2 = 62.2$, df = 1, $P < 0.0001$). Tadpoles in

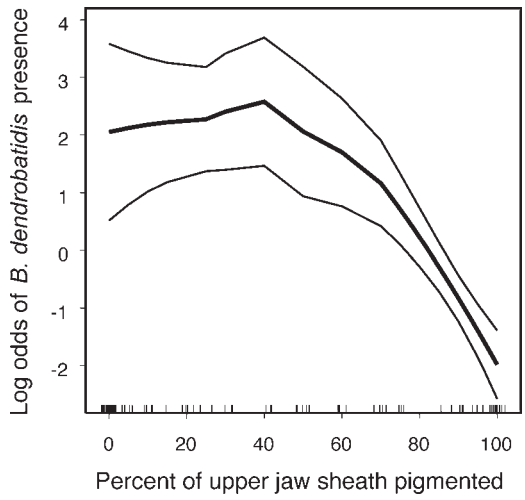

Fig. 2. Estimated relationship between the log odds of *Batrachochytrium dendrobatidis* presence and the degree of upper jaw sheath pigmentation (including approximate 95% confidence interval), as determined from the generalized additive model. Log odds on the y-axis were standardized to have an average value of zero. Hatch marks above the x-axis indicate observed values, with some marks slightly offset to allow display of identical values.

TABLE 3. SUCCESS OF UPPER JAW SHEATH PIGMENTATION SCORES IN PREDICTING *Batrachochytrium dendrobatidis* PRESENCE OR ABSENCE IN *Rana muscosa* (A) TADPOLES OR (B) POPULATIONS.

A. Individual Tadpoles					
Percentage of jaw sheath pigmented (observed)	*B. dendrobatidis*[a]		Predicted number	Classification rate[b]	
	present	absent	infected/uninfected[b]	% correct	% incorrect
100	6	46	0/52	88	12
90–99	4	4	8/0	50	50
<90	50	6	56/0	89	11
B. Population Average					
Percentage of jaw sheath pigmented (observed)	*B. dendrobatidis*[a]		Predicted number	Classification rate[b]	
	present	absent	infected/uninfected[b]	% correct	% incorrect
100	0	38	0/38	100	0
90–99	11	7	18/0	61	39
<90	26	0	26/0	100	0

[a] Presence/absence of *B. dendrobatidis* determined using standard or real-time PCR assays. In (B), infection status of each population is based on PCR assays conducted on a single tadpole.
[b] Based on rule that upper jaw sheath pigmentation scores of 100% indicate non-infection and scores <100% indicate infection.

the intermediate pigmentation category (90–99%) were evenly split between uninfected and infected animals (Table 3A).

Results based on average pigmentation scores were similar to those based on individual tadpoles. Tadpoles collected from sites with average jaw sheath pigmentation scores of 100% were again much less likely to be infected than were those from sites with pigmentation scores of <90% (Table 3B; $\chi^2 = 59.9$, df = 1, $P < 0.0001$). The intermediate pigmentation score category contained similar numbers of both infected and uninfected tadpoles. Overall misclassification rates based on average pigmentation scores were slightly lower than those for individual tadpoles (8% [7/82] vs. 14% [16/116], respectively; Table 3).

DISCUSSION

Our interpretation of analysis results are based on two explicit assumptions. First, we assumed that results from PCR assays were 100% accurate in assessing *B. dendrobatidis* presence/absence on an individual tadpole. The validity of this assumption is supported by the fact that both standard and Real-time PCR assays for *B. dendrobatidis* are extremely sensitive, capable of detecting an amount of *B. dendrobatidis* DNA equivalent to that found in 1–10 zoospores (Annis et al., 2004; Boyle et al., 2004). Second, in the analysis of the association between presence/absence of *B. dendrobatidis* at a site and mouthpart pigmentation averaged across all inspected tadpoles at that site, we assumed that the results of PCR assays conducted on a single specimen accurately reflected the infection status for the entire population. This assumption could be violated if the incidence of *B. dendrobatidis* amongst tadpoles at a site was <100%. In this case, PCR assays on a single specimen (that by chance was *B. dendrobatidis* negative) could classify some infected sites as being uninfected. The results of assays conducted on multiple tadpoles (average = 11, range = 5–21) collected from each of 20 *B. dendrobatidis*-positive sites indicated that the site-specific incidence of *B. dendrobatidis* was close to 100% (average = 83%, range = 40–100%). In addition, violation of this assumption would only affect those results based on average tadpole mouthpart pigmentation scores because data in all other analyses were based on the association between the extent of mouthpart depigmentation for an individual tadpole and the infection status of that tadpole. Finally, errors resulting from the violation of either assumption should be conservative, because misclassification of the *B. dendrobatidis* status of sites would likely reduce the strength of the relationship between the degree of mouthpart depigmentation and chytridiomycosis.

Our results indicate that in *R. muscosa*, depigmentation of tadpole jaw sheaths and toothrows is associated with chytridiomycosis. This association was much stronger for upper jaw sheaths than either lower jaw sheaths or toothrows. The extent of upper jaw sheath pigmentation, based on measurements from individual tadpoles or averaged across multiple tadpoles per site, was a reasonably accurate indicator of *B. dendrobatidis* presence/absence. When infection status of tadpoles was characterized based on upper jaw sheaths being 100% pigmented (un-

TABLE 4. DOCUMENTED MOUTHPART PIGMENTATION STATUS OF TADPOLES FROM 30 ANURAN SPECIES EXPOSED TO *Batrachochytrium dendrobatidis*.

Family	Species	Mouthpart depigmentation?	References
Bufonidae	*Bufo boreas*	No	Muths et al., 2003; Blaustein et al., 2005
	Bufo canorus	No	Green and Kagarise Sherman, 2001
Centrolenidae	Unknown	Yes	Lips, 1998; Lips, 1999
Hylidae	*Duellmanohyla ignicolor*	Yes	Lips et al., 2004
	Hyla calypsa	Yes	Lips, 1998
	H. colymba	Yes	Lips, 1999
	H. cyclada	Yes	Lips et al., 2004
	H. debilis	Yes	Lips, 1999
	H. lancasteri	Yes	Lips, 1999
	H. melanomma	Yes	Lips et al., 2004
	H. nephila	Yes	Lips et al., 2004
	H. pentheter	No	Lips et al., 2004
	H. sabrina	Yes	Lips et al., 2004
	H. uranochroa	Yes	Lips, 1999
	H. sumichrasti	No	Lips et al., 2004
	Hyla sp.	Yes	Lips et al., 2004
	Pseudacris regilla	No	Blaustein et al., 2005; R. Knapp, unpubl. data
	Ptychohyla erythromma	Yes	Lips et al., 2004
	P. hypomykter	Yes	Mendelson et al., 2004
	P. leonhardschultzi	Yes	Lips et al., 2004
	P. zophodes	Yes	Lips et al., 2004
	Smilisca baudini	No	Lips et al., 2004
Ranidae	*Rana aurora*	Yes	Nieto and Reiss, 2002
	R. berlandieri	No	Lips et al., 2004
	R. cascadae	Yes	Blaustein et al., 2005
	R. catesbeiana	Yes	L. Rachowicz and J. Bettasso, pers. comm.
	R. forreri	No	Lips et al., 2004
	R. muscosa	Yes	Fellers et al., 2001; Rachowicz and Vredenburg, 2004; this study
	R. sierramadrensis	Yes	Lips et al., 2004
	R. zweifeli	No	Lips et al., 2004

infected) or <100% pigmented (infected), misclassification error rates ranged from 8–14%. Assuming that *B. dendrobatidis* presence/absence for an *R. muscosa* population can be accurately assessed by PCR assays on a single tadpole, using average jaw sheath pigmentation scores based on inspections of five or more tadpoles provided the most accurate method of classifying populations as infected or not infected. The misclassification error rate of this method could be further reduced by subjecting one or more tadpoles from sites with average jaw sheath pigmentation scores of 90–99% to PCR assays, as the infection status of sites in this category was the most poorly classified (39% misclassified).

Misclassification of infection status was likely the result of at least two factors. First, false-negatives may be the result of a time lag between *B. dendrobatidis* infection and jaw sheath depigmentation. Of the six tadpoles with upper jaw sheaths that were 100% pigmented but that were *B. dendrobatidis*-positive (Table 3A), four showed some degree of jaw sheath abnormality, including small white spots in the black pigmentation and brown instead of black coloration of the entire jaw sheath. These abnormalities did not result in distinct gaps in jaw sheath pigmentation, and the percent of the upper jaw sheath that was pigmented was therefore recorded as 100%. Laboratory experiments with *R. muscosa* tadpoles indicate that the first evidence of jaw sheath depigmentation occurs 7–15 weeks following initial *B. dendrobatidis* infection (Rachowicz and Vredenburg, 2004) and often first appears as small white holes in the black upper jaw sheath, results that support the possibility that at least some of the false negatives in the current study were a consequence of a time lag

between *B. dendrobatidis* infection and jaw sheath depigmentation.

A second factor that may influence misclassification rates is jaw sheath deformities caused by factors other than chytridiomycosis. Of the six tadpoles with upper jaw sheaths that were <90% pigmented but were *B. dendrobatidis*-negative (Table 3A), four had jaw sheath and toothrow abnormalities that were not consistent with those typically seen in *R. muscosa* tadpoles infected with *B. dendrobatidis*. These included very misshapen jaw sheaths and completely depigmented upper jaw sheaths and toothrows but fully pigmented lower jaw sheaths and toothrows. The cause of these deformities is unknown.

Although the results of our study indicate that the extent of upper jaw sheath pigmentation is an accurate measure of the *B. dendrobatidis* infection status of *R. muscosa* tadpoles, similar studies are critically needed for other anuran species before this technique can be broadly applied. We have found accounts for 30 anuran species that specifically describe tadpole mouthpart pigmentation status at *B. dendrobatidis*-positive sites or following experimental infection with *B. dendrobatidis* (Table 4). Tadpole mouthpart depigmentation was reported for 21 species and a lack of mouthpart depigmentation was reported for nine species (Table 4). Therefore, although the available evidence suggests that tadpole mouthpart depigmentation is a common outcome of chytridiomycosis, this effect may not be universal.

We suspect that at least two factors could make *R. muscosa* tadpoles particularly susceptible to chytridiomycosis and resulting mouthpart depigmentation. First, *R. muscosa* tadpoles require at least two years to reach metamorphosis, providing ample time for mouthparts to be infected by *B. dendrobatidis* and for the resulting depigmentation to manifest itself. In *R. muscosa*, there is a 7–15 week time lag between initial infection and subsequent depigmentation (Rachowicz and Vredenburg, 2004). If other anuran species exhibit a similar time lag, this could explain why tadpoles of species with a short larval period (e.g., *Pseudacris regilla*, *Bufo canorus*, *B. boreas*) show no evidence of mouthpart depigmentation after being exposed to *B. dendrobatidis* (Table 4). Second, *R. muscosa* tadpoles are very large (up to 100 mm total length; Knapp, unpubl. data) with correspondingly large mouthparts that may provide an easier-to-find and more keratin-rich target for *B. dendrobatidis* zoospores than do the mouthparts of species with smaller tadpoles.

The extent of tadpole mouthpart depigmentation provides a reasonably accurate, quick, and inexpensive measure of *B. dendrobatidis* infection status in *R. muscosa*, which has allowed us to track the disease status of >500 *R. muscosa* populations on an annual basis during the past three years (Rachowicz et al., in press; R. Knapp, unpubl. data). This research would have been impossible if microscopic or molecular assays were necessary to determine infection status of populations. These data are providing key insights into the incidence of *B. dendrobatidis* and its spread between *R. muscosa* populations. Before tadpole mouthpart depigmentation can be used as an indicator of chytridiomycosis in anurans in general, however, further studies will be necessary to determine how broadly generalizable the results of the current study are to other anuran species.

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