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EVALUATION OF TADPOLE MOUTHPART DEPIGMENTATION AS A DIAGNOSTIC TEST FOR INFECTION BY *BATRACHOCHYTRIUM DENDROBATIDIS* FOR FOUR CALIFORNIA ANURANS

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ABSTRACT: The objective of this study was to evaluate the utility of gross morphologic examination of larval mouthpart defects as a diagnostic screening test to detect *Batrachochytrium dendrobatidis* infection in four California, USA, anuran species. We examined mouthparts of 2,034 tadpoles of *Bufo boreas*, *Pseudacris regilla*, and *Rana catesbeiana* collected in 2003 and 2004 and *Bufo canorus* collected in 2004. Data were recorded for three morphologic features: upper toothrows, lower toothrows, and combined jaw sheaths. Mouthpart defects were observed in all four species ($n=757$), but only two species were infected with *B. dendrobatidis* ($n=84$). Sensitivity and specificity of the mouthparts test were 76% and 58%, respectively. Forty-two percent of *B. dendrobatidis*–negative animals would have been designated positive based on mouthpart defects. Observed prevalence was 43%, and true prevalence was 3.0%. Tests of the null hypothesis using logistic regression analysis showed that anuran larval mouthpart defects were not associated with *B. dendrobatidis* infection whether mouthparts scores were tested by individual morphologic feature or in combination ($P=0.37$). We conclude that *B. dendrobatidis* infection and anuran larval mouthpart defects are two separate processes that may occur concurrently and that evaluation of tadpole oral morphology is neither an accurate nor a reliable diagnostic test for *B. dendrobatidis* infection for the four species tested.

Key words: Amphibian chytridiomycosis, *Bufo boreas*, *Bufo canorus*, diagnostic, morphology, mouthparts, *Pseudacris regilla*, *Rana catesbeiana*.

INTRODUCTION

Since the early 1980s, ultraviolet B (UV-B) radiation, introduced exotic species, pesticides, and habitat loss have been postulated as causative agents of global and local amphibian declines (Hayes and Jennings, 1986; Davidson et al., 2002; Davidson 2004). Disease was not considered in the literature until 1996 when Laurance et al. (1996) offered evidence that an unidentified disease was the likeliest cause of local extinctions in the rainforests of eastern Australia. Their work catalyzed a scientific debate (Alford and Richards, 1997; Laurance et al., 1997) and numerous subsequent investigations (Berger et al., 1998, 1999; Nichols et al., 1998; Daszak et al., 1999; Lips, 1999; Pessier et al., 1999).

This previously unknown disease, amphibian chytridiomycosis, was further described in 1998 in Australian wild populations (Berger et al., 1998) and in 1999 in captive amphibians in North America (Pessier et al., 1999). The causative agent, *Batrachochytrium dendrobatidis*, a chytridiomycete fungus, was isolated, identified, and described in 1999 (Longcore et al., 1999). The associated disease has since been implicated in the global decline of amphibians, particularly in Australia, New Zealand, Central America, and parts of North America (Berger et al., 1998; Lips, 1999; Waldman et al. 2001; Bradley et al., 2002; Muths et el., 2003).

Batrachochytrium dendrobatidis is a fungal pathogen that infects only keratinized tissues of amphibians (Berger et al., 1998; Longcore et al., 1999; Pessier et al., 1999). Infections in epidermal keratinizing tissues of postmetamorphic anurans can be fatal (Berger et al., 1998, 1999; Daszak et al., 1999; Nichols et al., 2001; Bradley et al., 2002; Blaustein et al., 2005), but the effects of *B. dendrobatidis* vary

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among the few species that have been tested (Nichols et al., 1998; Davidson et al., 2003; Rachowicz and Vredenburg., 2004; Blaustein et al., 2005). *Batrachochytrium dendrobatidis* in metamorphic (Gosner stage 41-46; Gosner, 1960) and postmetamorphic anurans can be diagnosed by microscopic examination of skin scrapings, histologic sections, or DNA tests of skin samples (e.g., Boyle et al., 2003, 2004; Obendorf 2005).

Batrachochytrium dendrobatidis infection in premetamorphic (Gosner stages 26-40; Gosner, 1960) tadpoles appears to be restricted to the mouthparts (Berger et al., 1998; Pessier et al., 1999; Marantelli et al., 2004). Keratin is present throughout much of the larval period, primarily in the formative tissues of the larval teeth and jaw sheaths (Altig and McDiarmid, 1999). Larval responses to *B. dendrobatidis* infection have included decreased body mass at metamorphosis and increased larval periods (Parris, 2004), but tadpoles often appear to be otherwise healthy and generally do not seem to be negatively impacted by infection (Berger et al., 1998). Thus, it has been hypothesized that tadpoles may be reservoirs for the pathogen (Berger et al., 1998, 1999; Daszak et al., 1999; Daszak and Cunningham, 2003). *Batrachochytrium dendrobatidis* infection in anuran larvae is most reliably diagnosed through either histologic serial sectioning of mouthparts or DNA testing of mouthpart tissues or mouthpart swabs (Obendorf 2005).

The theory that observed anuran larval mouthpart defects might be related to *B. dendrobatidis* infection began with one of the first publications on *B. dendrobatidis* in Panama (Lips, 1999), wherein it was anecdotally reported that tadpole mouthpart defects had been observed at sites where amphibian populations were experiencing an amphibian chytridiomycosis epizootic event. Despite the observation, no testing was done to determine if tadpoles were infected (Lips, 1999) or to identify a relationship between tadpole mouthpart defects and *B. dendrobatidis* infection. Two articles published in 2001 on *Rana muscosa* in California, USA (Fellers et al., 2001; Vredenburg and Summers, 2001) also noted tadpole mouthpart defects in sites where *B. dendrobatidis* had been detected and the authors recommended examining gross morphology of tadpole mouthparts as an indicator of *B. dendrobatidis* infection. Requiring only a hand lens as equipment, the visual evaluation of larval mouthparts as suggested by Fellers et al. (2001) and Vredenburg and Summers (2001) appeared to be a useful field technique for detecting populations with *B. dendrobatidis* infections.

Subsequent publications on mouthpart defects in *R. muscosa* have shown an association between upper jaw sheath defects and *B. dendrobatidis* infection for *R. muscosa* tadpoles between Gosner stages 30 and 40 during temperate months 2–3 wk after snowmelt (Rachowitz, 2002; Knapp and Morgan, 2006). Obendorf (2005) found that three of five anuran species in Tasmania, Australia, displayed mouthpart abnormalities that were concurrent with *B. dendrobatidis* infection, whereas the other two of the five species showed no mouthpart defects despite the presence of the pathogen. Although the detection of the fungus, population die-offs, and larval mouthpart defects have been noted together (Lips, 1999; Fellers et al., 2001; Vredenburg and Summers, 2001) and may be related for some species (Rachowitz, 2002; Rachowitz and Vredenburg, 2004; Obendorf 2005; Knapp and Morgan, 2006), the potential relationships among these occurrences remain unclear for most other species.

Prompt detection of diseases can prevent a localized occurrence from becoming an epizootic event (Cooper, 2002), and wildlife veterinarians rely on immunology, histology, and DNA testing to diagnose diseases. Although valuable, these kinds of tests are not usually feasible in field situations, and furthermore, most herpe-

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tologists are not veterinary pathologists trained in these specialized techniques. An easily observed signal, like a morphologic anomaly, that indicates the presence of an infective agent would be the most useful tool for field biologists, particularly when in remote locations. The objective of our study was to provide an accurate assessment of the use of tadpole mouthpart defects as an indicator of *B. dendrobatidis* infection for four anuran species occurring in California, USA, and to provide further information on the potential application of the technique to other amphibian species.

MATERIALS AND METHODS

Study species

The western toad, *Bufo boreas*, is distributed throughout much of the northern and central portions of California, USA, with a range extending south to Baja California, Mexico. The species is now uncommon and appears to be in decline in the Greater Yellowstone Ecosystem and other parts of the western United States (Leonard et al., 1993), although it is still documented in most of its historic range (Wright and Wright, 1949; Stebbins, 1985). *Bufo boreas* has a wide elevational distribution occurring from sea level up to 3,600 m (Wright and Wright, 1949; Stebbins, 1985). The species is found in ponds, lakes, reservoirs, rivers, and streams in a diversity of habitat types, such as grasslands, woodlands, mountain meadows, and desert riparian zones.

The Yosemite toad, *Bufo canorus*, is an endemic species with a relatively small distribution that is restricted to open montane meadows in the boreal zones (1950–3450 m) of the Sierra Nevada Mountains of California, USA. Once abundant where it occurred (Mulally, 1953), *B. canorus* has disappeared from >50% of sites where it has historically been documented (Sherman and Morton, 1993; Jennings and Hayes, 1994).

The Pacific treefrog, *Pseudacris regilla*, is the most abundant and widely distributed anuran in California, USA, and is found in a variety of habitat types throughout the state from sea level up to the tree line at ~3,540 m (Wright and Wright, 1949; Stebbins, 1985). This ubiquitous species shows no sign of decline and can be found any place where there is constant moisture within grasslands, chaparral, oak woodlands, forest, desert oases, and farmland.

The nonnative bullfrog, *Rana catesbeiana*, was first introduced into California, USA, in 1896 (Hayes and Jennings, 1986). The species is now widespread throughout the state; although it has not been documented at elevations above 2,000 m. *R. catesbeiana* occurs in all types of perennial waters across a wide variety of habitats.

Anuran larval sampling

Nineteen ponds were sampled for *B. boreas, P. regilla*, and *R. catesbeiana* larvae in August 2003, and 59 ponds were sampled in May 2004 at Joseph D. Grant County Park, Santa Clara County, California, USA (37°20′07″N; 121°42′06″W), Blue Oak Ranch Preserve, Santa Clara County, California, USA (37°22′34″N, 121°44′34″W), and East Bay Municipal Utilities District, Contra Costa County, California, USA (37°57′38″N, 122°13′23″W). A maximum of 30 larvae of all three species were collected from each pond when numbers and species occurrence allowed. *Bufo canorus* tadpoles originally collected as eggs from Edith Lake (37°33′18″N, 118°53′31″W) and Glacier Bench (37°55′12″N, 119°14′14″W), Inyo National Forest, California, USA, were sent to one of us (G.E.P-F.) as preserved tadpoles for *B. dendrobatidis* testing and were also included in this study because 1) the tadpoles were part of a larger mixed-age group of animals collected in the field, some of which were positive for *B. dendrobatidis*, 2) the screening of mouthparts for *B. dendrobatidis* has been used in laboratory-reared animals, and 3) the possibility that *B. dendrobatidis* zoospores may have been present in the water surrounding the collected egg mass and could have infected the larvae after hatching. All anuran larvae included in the study were preserved in 70% ethanol.

Tadpole identification

Labial toothrow formulae and jaw sheath characteristics are typically used in tadpole species identification and to differentiate among larval ecomorphologic guilds (e.g., Altig and Johnston, 1989). Keratinized jaws first appear at Gosner stage 24 (Kaung 1975), and amphibian larval mouthparts are fully formed and keratinized by Gosner stages 25-26 (Altig and Johnston, 1989). Larval mouthparts begin to atrophy and are shed during Gosner stages 41-46 when the hind limbs become fully formed and the forelimbs are appearing. Keratinized labial teeth and jaw sheaths develop from mitotic tissues at their bases

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and are constantly replaced. Labial toothrow formulae were used to identify each tadpole to species in this study (Stebbins 1985).

Tadpole staging

Anuran tadpoles have a distinct sequence of development that begins with the egg stage and climaxes with metamorphosis (Altig and McDiarmid, 1999). Each stage of development has distinguishable morphologic characteristics that are common to most anuran taxa and thus allow comparisons of disparate species that exhibit variations in size and developmental periods. Gosner (1960) described 46 stages of anuran larval development and provided illustrations of the key morphologic changes associated with each stage. The Gosner (1960) system of staging tadpoles was used to identify the developmental stage of each tadpole used in this study.

Anuran larval mouthparts evaluations

All tadpoles were visually examined for mouthpart defects in the laboratory using a dissecting microscope. Mouthpart data recorded were divided into upper toothrows, lower toothrows, and jaw sheaths (upper and lower combined). For this study, defects were characterized by estimating the percentage of pigment absent where structures are normally fully pigmented (Stebbins 1985). For each individual, these structures were categorized as 1) fully pigmented, 2) 1–50% pigment absent, 3) 51–90% pigment absent, or 4) no pigment. Individual mouthpart scores were recorded for each structure and then summed giving a total mouthparts score for each individual ranging from three to 12.

After evaluations, we removed the entire oral disc intact, including the underlying soft tissue, and then divided the oral disc into left and right halves. All instruments were flame-sterilized between each procedure. Left halves were preserved in 70% ethanol in individually numbered vials and cross-referenced to the right halves, which were batched by species for each of the 78 ponds. Batched samples ($n \leq 30$) preserved in 70% ethanol were tested for *B. dendrobatidis* infections by DNA analysis. Batching samples is routinely done to decrease the cost of testing large numbers of samples. Batched samples are homogenized and centrifuged to coagulate DNA, and the pelleted DNA is the subject of the polymerase chain reaction (PCR) assay. Large numbers of samples can decrease the sensitivity of the PCR assay; however, published information is lacking on the maximum total tissue volume that can comprise a batch. Positive DNA results on batched samples were followed up by DNA testing every individual comprising the batched sample.

DNA analyses were carried out at Pisces Molecular Laboratory (Boulder, Colorado, USA). DNA analysis followed the procedure outlined in Annis et al. (2004) with the following modifications: 35-cycle was increased to 45-cycle, annealing temperature was increased from 60 C to 65 C and $[Mg^{2+}]$ was also increased from 1.5 mM to 3.5 mM (J. Wood, K. Rogers, L. Livo, and J. Epp, unpubl. obs.). Each PCR run included controls of positive DNA, negative DNA, and contamination detection. The contamination detection control consists of a sterile water sample to test for any possible contamination of the master mix used in the PCR assay. The *B. dendrobatidis* PCR assay test is highly specific for the *B. dendrobatidis* ribosomal RNA Internal Transcribed Spacer, and the test is very sensitive as it will detect the presence of <10 *B. dendrobatidis* zoospores in a 2 µl sample (Annis et al., 2004). The modified protocol has not been published yet, but has been tested on $>13{,}000$ samples.

Mean scores were calculated for each mouthpart variable (upper toothrow, lower toothrow, and jaw sheath) for all tadpoles of each species. Logistic regression analysis tested for independence between the frequencies of *B. dendrobatidis* infection and mouthpart abnormalities for all tadpoles. The Hosmer-Lemeshow goodness-of-fit test was used to verify the data were adequately described by the logistic regression analyses. Mouthpart scores were tested for each of the three variables separately and as a combined total mouthparts score (values ranged from three to 12). For graphic comparative presentation in Fig. 1, mean combined scores were divided by three to obtain a total mean mouthpart score from 1–4. A separate logistic regression restricted to tadpoles between Gosner stages 30 and 40 (Knapp and Morgan 2006) was also conducted.

RESULTS

Sampling in 2003 occurred in August, and therefore, no *B. boreas* and few *P. regilla* larvae were present, but large numbers of *R. catesbeiana* were collected. In May and June 2004, the most frequently occurring species was *P. regilla*, but large samples of *B. boreas* and moderate numbers of *R. catesbeiana* were collected (Table 1). A total of 373 tadpoles in 2003

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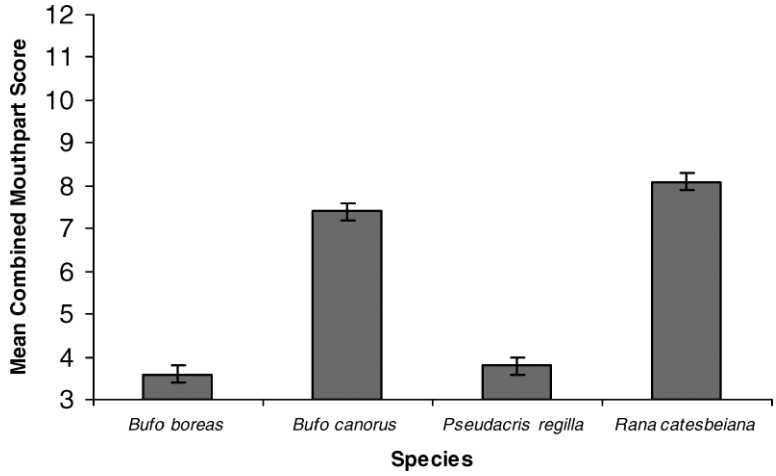


FIGURE 1. Mean combined mouthpart scores by species sampled in 2003–2004. Upper toothrow, lower toothrow, and jaw sheaths for *Bufo boreas*, *Bufo canorus*, *Pseudacris regilla*, and *Rana catesbeiana* larvae (n=2,034) were examined and categorized as 1) fully pigmented, 2) 1–50% pigment absent, 3) 51–90% pigment absent, or 4) no pigment. Individual mouthpart scores were recorded for each of the three variables and then summed giving a total mouthparts score for each individual ranging from three to 12.

and 1,661 tadpoles in 2004 were evaluated for mouthpart defects and tested for *B. dendrobatidis* infection. Gosner stages for all tadpoles ranged from 26 to 41, and mean stages for each species were as follows: *B. boreas*=33, *B. canorus*=34, *P. regilla*=35, and *R. catesbeiana*=29.

B. dendrobatidis infections were confirmed in *R. catesbeiana* and *P. regilla* tadpoles (Table 1), whereas no infections were detected in *B. boreas* or *B. canorus*. Mean total mouthpart scores varied between 3.6 (*B. boreas*) and 8.1 (*R. catesbeiana*) (Fig. 1). Mean individual mouthpart scores were highest for *R. catesbeiana* and *B. canorus* and lowest for *B. boreas* and *P. regilla* (Fig. 2). Logistic regression analyses tested for independence of disease status and mouthpart scores for *H. regilla* and *R. catesbeiana*. There was no significant relationship between *B. dendrobatidis* and total mouthpart scores for either species (χ^2=0.80, df=1, P=0.37; χ^2=1.35, df=1, P=0.25, respectively), and results of the Hosmer-Lemeshow goodness-of-fit test showed that each data set was adequately described by the logistic regression (χ^2=1.81, df=2, P=0.40;

TABLE 1. Number of tadpoles with mouthpart defects and incidence of *Batrachochytrium dendrobatitis* (Bd) infection for four anuran species sampled in 2003–2004. Tadpoles with mouthpart defects were defined as those tadpoles having a score >3 when all three variable (upper toothrow, lower toothrow, and jaw sheath) scores were combined. Each variable was categorized as 1) fully pigmented, 2) 1–50% pigment absent, 3) 51–90% pigment absent, or 4) no pigment.

	2003			2004		
Species	Number sampled	Number with mouthpart defects	Number infected with Bd	Number sampled	Number with mouthpart defects	Number infected with Bd
Bufo boreas	0	—	—	305	82	0
Bufo canorus	0	—	—	53	50	0
Pseudacris regilla	7	6	1	1,235	339	19
Rana catesbeiana	366	212	1	68	68	63
Total	373	218	2	1,661	539	81

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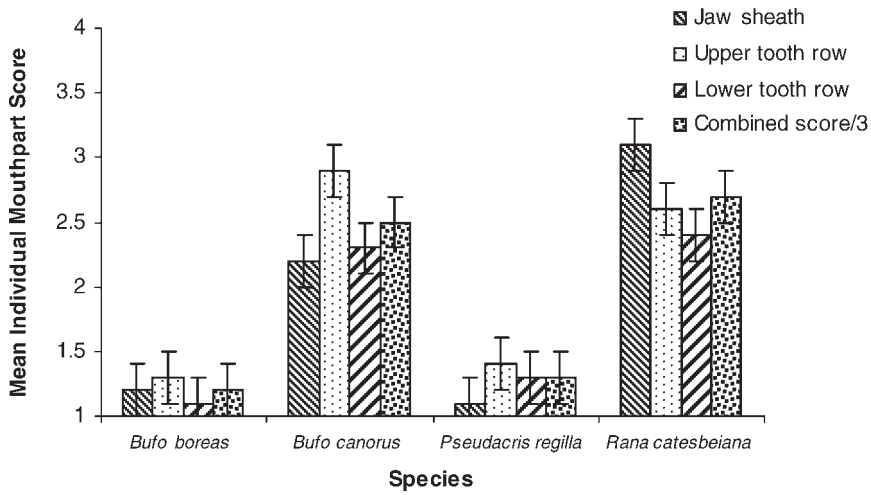


FIGURE 2. Mean mouthpart scores for each variable: upper toothrow, lower toothrow, and jaw sheaths for *Bufo boreas*, *Bufo canorus*, *Pseudacris regilla*, and *Rana catesbeiana* larvae (n=2,034) sampled in 2003–2004. Each variable was categorized as 1) fully pigmented, 2) 1–50% pigment absent, 3) 51–90% pigment absent, or 4) no pigment. For graphic comparison with the individual variable scores, the mean total combined score of all three variables was divided by three to obtain an average total score between one and four.

χ^2=1.65, df=4, P=0.80). Logistic regression results on means of individual mouthpart variables were similar to that obtained for the mean total mouthpart score. Subset analyses restricted to tadpoles at Gosner stages >30 and <41 were also similar to results obtained from analyses of the total sample. *B. boreas* and *B. canorus* were not tested because there were no *B. dendrobatidis*–infected samples.

Sensitivity and specificity of the mouthparts test were 76% and 58%, respectively. Forty-two percent of *B. dendrobatidis*–negative animals would have been designated positive based on mouthpart defects. Twenty-four percent of *B. dendrobatidis*–positive animals would have been designated negative based on mouthpart defects. Observed prevalence was 43%, and true prevalence was 3.0%.

DISCUSSION

Our study shows that different responses occur among species and also demonstrates a lack of correlation between *B. dendrobatidis* infections and mouthpart defects for *B. boreas*, *B. canorus*, *P. regilla*, and *R. catesbeiana*. These results agree with the laboratory study of Blaustein et al. (2005), who found no differences in the proportions of *B. boreas* (n=57) and *P. regilla* (n=44) tadpoles with mouthpart abnormalities when *B. dendrobatidis*–treated groups were compared with control groups. They found that all *R. catesbeiana* (n=30) in both regimes had abnormal mouthparts (Blaustein et al., 2005).

Our findings also indicate that for the four species tested, the occurrence of larval mouthpart abnormalities is neither an accurate nor a reliable diagnostic test for *B. dendrobatidis* infection. Similar to results obtained by Marantelli et al. (2004), we had animals with normal mouthparts that were positive for *B. dendrobatidis* and animals with defective mouthparts that were not infected. Two species (*R. catesbeiana, B. canorus*) showed a high prevalence of mouthpart abnormalities independent of *B. dendrobatidis* infection, and the actual cause of mouthpart defects remains unknown. Across the four taxa, we observed mouthpart defects in 58% of individuals from

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2003 and 32% from 2004. This is consistent with data (D. Drake, pers. comm.) on bufonid, hylid, and ranid tadpoles from Louisiana and Mississippi, which showed mouthpart defects in 13.5–98.0% of tadpoles ($n=300$) collected from 14 sites with no known history of chemical (e.g., pesticide) contamination and a grand mean of 1% ($n=3$) were infected with *B. dendrobatidis* (D. Drake, pers. comm.).

From our combined sample of 2,034 tadpoles, a grand mean of 37% had mouthpart defects, and 4% were infected with *B. dendrobatidis*. This infection prevalence is similar to Obendorf et al. (2005) and Ouellet et al. (2005), who found that *B. dendrobatidis* was widely distributed in North American amphibians based on museum specimens collected in years 1895–2001, and 7% of animals collected after 1960 were found to be infected with *B. dendrobatidis*.

Loss of pigmentation and other mouthpart defects can be caused by a number of factors. Rachowitz and Vredenburg (2004) found that mouthpart pigmentation in *R. muscosa* can be temperature dependent as pigmentation was lost at 6 C and regained at 14 C in laboratory studies. Burger and Snodgrass (2000) found that for some species, mouthpart defects increase with increasing hydroperiod. Rowe et al. (1996) found that oral deformities can be caused by contaminants (As, Ba, Cd, Cr, and Se) either singly or in combination. In 1998, they followed up their earlier study demonstrating a cause and effect relationship between site conditions of embryonic and larval development and oral deformities (Rowe et al., 1998). Pesticides such as DDT can also cause mouthpart damage (Hayes et al., 1997). Conversely, Drake (pers. comm.) found a high prevalence of defects at sites with no known history of chemical or heavy metal contamination.

The lack of an understanding of the total developmental biology of keratinized mouthparts is a deterrent to the generalized use of tadpole mouthparts as an indicator of *B. dendrobatidis*. The main problems include distinguishing between the normal range of mouthpart variation throughout tadpole ontogeny, identifying which factors cause which kinds of defects, and determining which defects are able to be repaired and how the repair is implemented. All of these facets of tadpole biology will likely differ widely between species. Studies are needed on the normal biology of tadpoles and the factors that modify their physiologic responses (e.g., cycles of mitotic activity in various keratinized mouthparts). Knowing the types, prevalences, and phenologies of oral defects that can occur by species throughout tadpole ontogeny at sites having no history of chemical or heavy metal contamination would provide baseline data on which further comparative studies could be based.

Given the range of factors already known to cause anuran larval mouthpart defects, it may not be possible to identify a single cause of defects in a given population. For example, *R. muscosa* occupies habitat that is within range of pesticide plumes originating from the Central Valley of California, USA (Davidson et al., 2002; Davidson 2004). Cory et al. (1970) documented relatively large amounts of DDT and its metabolites in *R. muscosa*, and thus, this species may be experiencing high levels of mouthpart defects due to pesticide contamination and not just because of *B. dendrobatidis* infection. Multivariate analyses may show that mouthpart defects can be related to more than one causative agent or, conversely, demonstrate that univariate analysis may lead to erroneous conclusions due to confounding, yet untested, variables.

In summary, it appears that there are actually two separate phenomena occurring: 1) the emergence of disease (i.e., amphibian chytridiomycosis) as a factor in amphibian declines, and 2) a high prevalence of anuran larval mouthpart defects across taxa. The fact that these two separate processes were noticed concur-

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rently is not an indication that the two processes are necessarily and exclusively related across all amphibian taxa. Widespread anuran larval mouthpart defects may have gone unnoticed because of the dearth of research on that aspect of anuran larval ontogeny.

Gross mouthpart morphology is not a reliable or accurate indicator of *B. dendrobatidis* for B. *boreas*, *B. canorus*, *P. regilla*, or *R. catesbeiana*. We conclude that there is no quick field technique to diagnose *B. dendrobatidis* infection across all taxa and that diagnosis based on gross mouthpart morphology can result in false positives and false negatives unless the methodology has been tested for the species in question. The most reliable methods still remain histologic examination or DNA analysis of tissue or culture swabs. Given the serious nature and widespread distribution of *B. dendrobatidis* infection in numerous species worldwide, we advocate a rigorous approach in testing diagnostic techniques for this disease for each individual species.

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