COMMENTS

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A Conceptual Framework for Detecting Oral Chytridiomycosis in Tadpoles

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We comment on the results of a previous study which evaluate the accuracy of mouthpart depigmentation as an indicator of *Batrachochytrium dendrobatidis* (*Bd*) infection in anuran larvae. Macroscopic mouthpart inspection is a useful technique for *Bd* identification since it is practical and nonlethal; however, this method is also expected to result in increased rates of false negatives and false positives when compared to other methods. We therefore suggest that there are scenarios in which the use of mouthpart depigmentation is not appropriate, and we present a conceptual framework for deciding when mouthpart depigmentation is an appropriate indicator of oral chytridiomycosis in tadpoles. We conclude that more sensitive techniques are preferable when false positives or false negatives could result in the incorrect classification of populations as infected or uninfected, namely when *Bd* is absent or present at low prevalence. In contrast, mouthpart depigmentation is an appropriate indicator of infection when *Bd* prevalence is high and especially when conservation or practical concerns preclude lethal methods such as histological preparation.

ACCUMULATING evidence suggests that the fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, can cause mass mortality of infected individuals potentially leading to amphibian population declines and extinctions (Berger et al., 1998; Lips et al., 2006). The accurate identification of *B. dendrobatidis*-infected individuals and populations is important to the understanding of the role played by this pathogen in amphibian declines and is consequently relevant to amphibian conservation (Knapp and Morgan, 2006).

In their recent paper, Knapp and Morgan (2006) present and analyze an alternative to microscopic inspection of tadpole mouthparts, which typically includes destructive sampling and histological preparation of oral tissues and is the traditional method for detecting oral chytridiomycosis in tadpoles. This process necessarily requires collecting and euthanizing individual tadpoles, which for ethical reasons limits the practicality of detecting oral chytridiomycosis in the larvae of rare species. This limitation, combined with the fact that histological preparation is expensive in terms of equipment, time, and expertise, has led to the search for inexpensive, rapid, noninvasive techniques for identifying *Bd* infection in field-collected tadpoles. Following observations of tadpole mouthpart abnormalities (e.g., depigmentation or malformation) in *Bd*-infected individuals (Fellers et al., 2001; Lips et al., 2004; Rachowicz and Vredenburg, 2004), Knapp and Morgan (2006) evaluate the efficacy of using mouthpart depigmentation to detect oral chytridiomycosis in the tadpoles of one species, the Mountain Yellow-Legged Frog *Rana muscosa*. The authors conclude that this convenient, noninvasive technique results in a high proportion of correctly-classified individuals (86% of individuals and 92% of populations correctly classified) and urge that similar studies be conducted on other amphibian species.

The findings of Knapp and Morgan (2006) are compelling and are likely to be useful to other researchers. However, we suggest that there are scenarios in which the use of mouthpart depigmentation, though convenient, is inappropriate. In this comment, we will address this issue by discussing two important topics related to the findings of Knapp and Morgan (2006). First, we will discuss the general advantages and disadvantages of using mouthpart depigmentation to identify oral chytridiomycosis. Second, we will present a conceptual framework which shows how knowledge of these advantages and disadvantages can be used to help guide the choice of detection method based on factors such as study scope and *Bd* prevalence.

INDIRECT METHODS: ADVANTAGES AND DISADVANTAGES

For the purposes of this paper, we use the term ''indirect'' to describe methods of identification that rely not on identification of the *B. dendrobatidis* organism itself, but instead on symptoms

TABLE 1. SENSITIVITY AND SPECIFICITY OF MOUTHPART DEPIGMENTATION AS AN INDIRECT METHOD FOR THE IDENTIFICATION OF CHYTRID INFECTION IN *Rana muscosa* TADPOLES. Values are calculated from Knapp and Morgan (2006) Table 3.

	Definition	Individual tadpoles	Population average
Sensitivity	(# of diseased individuals testing positive)/ (# of diseased individuals)	90.0%	100%
Specificity	(# of well individuals testing negative)/ (# of well individuals)	82.1%	84.4%

caused by *B. dendrobatidis*. Because tadpole mouthpart depigmentation and malformation are symptoms of oral chytridiomycosis, they are indirect methods of identifying underlying *Bd* infection in tadpoles. In this paper we follow the orginal terminology of Knapp and Morgan (2006) in the classification of oral deformities in *Rana mucosa* as 'mouthpart depigmentation.' In contrast, ''direct'' methods of *Bd* identification rely on identification of the *B. dendrobatidis* organism itself, either by microscopic inspection (e.g., histology) or detection of *B. dendrobatidis* genetic material, for example via PCR (Boyle et al., 2004).

In this discussion we will also focus on two types of error that may occur in *Bd* screening: false negatives (or omission error) and false positives (or commission error). These relate, respectively, to the concepts of sensitivity and specificity of a diagnostic test, in which a test with a high sensitivity results in few false negatives while a test with high specificity results in few false positives (for a brief but helpful introduction to these concepts see Loong, 2003).

Using these definitions, an important disadvantage of the use of indirect detection methods is the enhanced likelihood of false negatives. This is because indirect methods assume the presence of a high correlation between *Bd* infection and the expression of a symptom, in this case mouthpart depigmentation. For two reasons we expect that this assumption is often violated. First, it is unlikely that symptoms become evident immediately following infection by *B. dendrobatidis*, resulting in latent infections that are undetected. Second, not all individuals or species will necessarily respond to *B. dendrobatidis* infection with similarly detectable symptoms. This latter point is an important one, as it may preclude the identification of asymptomatic reservoirs of *B. dendrobatidis*. Identifying reservoirs of *B. dendrobatidis* is critical to understanding if and how *B. dendrobatidis* causes declines, extinctions, or extirpations (McCallum, 2005), so this is an important limitation of indirect detection methods. Although false negatives are associated with most detection techniques to some degree (e.g., skin histology; Olsen et al., 2004), the rate of false negatives can reasonably be expected to be higher in indirect as compared to direct methods of *Bd* detection. This is because a new potential source of error exists in indirect methods: misclassification of asymptomatic infections. In other words, the sensitivity of inspection for mouthpart depigmentation is expected to be lower than the sensitivity of microscopic inspection.

Another disadvantage of indirect methods of *Bd* infection is that they are also likely to result in false positives. This is because mouthpart depigmentation is not exclusively associated with oral chytridiomycosis, but can also result from abiotic factors such as pollution, temperature, and season as well as from other unknown causes (Rowe et al., 1998; Rachowicz, 2002; Knapp and Morgan, 2006). In contrast, false positives are not known to result from direct microscopic inspection done by experienced observers, owing to the distinct morphological characteristics of *B. dendrobatidis* (Berger et al., 1998, 2005; Fellers et al., 2001; Rachowicz and Vredenburg, 2004). In other words, whereas the specificity of direct detection of oral chytridiomycosis approaches 100%, the specificity of relying on mouthpart depigmentation is $<100\%$ (Table 1). Thus, the use of indirect methods results in potentially higher false negative rates and, importantly, the introduction of a new and significant category of possible errors, false positives.

The advantages of using indirect methods of detection are primarily logistical, in that mouthpart depigmentation can be determined quickly, with little expertise, and with very little expense. Perhaps most importantly, mouthpart pigmentation can be assessed nonlethally, obviating the permanent removal of tadpoles from wild populations.

In sum, the adoption of mouthpart depigmentation as an indirect method of *Bd* detection represents a tradeoff between test accuracy on the one hand and ethical and practical concerns on the other. Because classification errors were relatively infrequent, Knapp and Morgan (2006) provide convincing evidence that the errors introduced by the use of mouthpart depigmentation are not of such magnitude as to contrain-

dicate the use of mouthpart depigmentation in detecting *B. dendrobatidis* in *R. muscosa* (Table 1). Importantly, because *R. muscosa* has experienced severe declines over the past century, shifting to indirect methods of *Bd* detection is a favorable tradeoff and the authors suggest evaluating this technique for other species. However, we suggest that the favorability of the accuracy–ethics tradeoff will vary not only with amphibian species, but also with factors such as the desired scale of inference and prevalence of *Bd* infection in the population(s) of interest.

Conceptual Framework for Indirect Methods of Detection

Here we present a simple conceptual framework to aid researchers in the selection of method for detecting *B. dendrobatidis* in field studies of tadpoles. We suggest that this decision should be made with information on the study design, especially the desired scale of inference, and with information on the expected prevalence of *Bd* within populations. We will also discuss the situation in which information on *Bd* prevalence is not available.

Batrachochytrium dendrobatidis prevalence should influence the choice of detection method because the relative importance of sensitivity and specificity can change with disease prevalence. For example, if a disease is absent from a monitored population, then false positives may result in spuriously categorizing the population as infected. In contrast, false negatives cannot occur in the absence of disease, so false negatives will have no effect (Table 2). In this scenario, methods with high specificity should be favored over those which can produce false positives.

In contrast, false negatives are more important when they could result in misclassification of the population as uninfected, specifically when *Bd* is present at low prevalence. In this case false positives will result in the overestimation of the prevalence of *Bd*, arguably a less significant error than misclassification of the population as uninfected.

The desired scale of inference should also influence researchers' choice of detection method because certain types of error will matter more at small spatial scales (e.g., single populations) than at relatively large spatial scales (across populations or regionally). This is because some types of error will average out over larger spatial scales, with a concomitant reduction in the magnitude of the error (Table 1). For example, when *Bd* is present in low prevalence, false negatives can easily result in the misclassification of a single population. Across many populations, however, it is unlikely that false negatives will occur in every case, reducing the likelihood of misclassification of an entire region or group of populations.

After considering these and several other possible scenarios of scale of inference and *Bd* prevalence, we conclude that direct microscopic examination of tadpole mouthparts for *B. dendrobatidis* is more appropriate than mouthpart depigmentation in a plurality of cases (Table 2). These scenarios are those in which the higher rates of false positives and false negatives inherent in indirect inspection result in the generation of spurious results in the form of misclassification errors (e.g., labeling infected populations as uninfected or vice versa). In this framework, indirect detection methods are only preferable when false positives and false negatives are unlikely to influence conclusions, as when *Bd* prevalence is high across a number of populations. In two scenarios when misclassification errors are unlikely to occur, direct and indirect methods are expected to perform similarly. In these scenarios, practical and conservation considerations should have primacy, generally favoring the nonlethal indirect method of mouthpart depigmentation.

There are several important limitations to this conceptual framework. First, the expected prevalence of *Bd* within populations will initially be unknown for many studies. In these cases, if mouthpart depigmentation is known to occur in the focal species we recommend that researchers first conduct a pilot study using indirect methods of mouthpart inspection to estimate *Bd* prevalence. These data can then be applied to the conceptual framework to guide the decision of whether additional surveys should use direct or indirect methods of *Bd* detection.

A second limitation is that not all species will exhibit mouthpart abnormalities when infected with *B. dendrobatidis*. Although many species in the Americas do exhibit *Bd*-associated mouthpart abnormalities (Kappp and Morgan's Table 4), the strength of the relationship between abnormalities and *Bd* infection has been studied in very few species. We join Knapp and Morgan (2006) in encouraging additional studies on this topic. In our own experience in South Africa, we have found mouthpart depigmentation to be an unreliable predictor of oral chytridiomycosis in several species. Indeed, we have found that oral chytridiomycosis in one species, *Heleophryne natalensis*, is associated not with depigmentation, but with hyperpigmentation (Smith et al., 2007).

Finally, our conceptual framework does not explicitly account for conservation status of the focal species. In some situations, as when the

TABLE 2. A TABULAR CONCEPTUAL MODEL FOR THE SELECTION OF DIRECT VS. INDIRECT TECHNIQUE FOR THE DETECTION OF *Batrachochytrium dendrobatidis* IN TADPOLES. Items marked with an asterisk (*) are considered to be major errors.

Scale of inference	Within-population prevalence of *B. dendrobatidis*	Effect of false negatives	Effect of false positives	Preferred detection technique	Reason for preference
Small (e.g., single population)	Not present	No effect	Misclassification of population as infected*	Direct	Few/no false positives
	Low	Misclassification of population as disease-free*	Overestimation of prevalence	Direct	Fewer false negatives
	High	Underestimation of prevalence	Overestimation of prevalence	Equivocal	Decision should be based on practical or conservation concerns
Large (e.g., several populations or regional)	Not present	No effect	Misclassification of region as infected*	Direct	Few/no false positives
	Low	Occasional underestimation of proportion of populations infected	Occasional overestimation of proportion of populations infected	Equivocal	Decision should be based on practical or conservation concerns
	High	No effect	No effect	Indirect	Practical, nonlethal

focal amphibian is rare, this should be the primary concern, in which case invasive or lethal detection may be ruled out. In these cases our framework can still be used as a general guide to understand the limitations and errors that are associated with the use of indirect methods of *Bd* detection.

We agree with Knapp and Morgan (2006) that the methods used to detect oral *Bd* infection in amphibian larvae should be practical in terms of the investment of resources and in minimizing impact to the populations being studied. Ideally, highly accurate, convenient, nonlethal methods of *B. dendrobatidis* detection would be readily available to all researchers. The increased use of Real-time PCR using swabbed (i.e., nonlethal) samples may soon make indirect and direct lethal methods obsolete, and the recent establishment of a rigorous sampling protocol (Hyatt et al., 2007) makes a shift to more widespread use of Real-time PCR even more likely. However, nonlethal swab-sampling of tadpole mouthparts for PCR analysis is less sensitive than destructive sampling methods both for PCR (Hyatt et al., 2007) and histology (Retallick et al., 2006). Equally important is the fact that PCR expertise and technology are not available to all researchers, so other methods must suffice in some cases. Microscopic inspection and assessment of mouthpart depigmentation are two good methods, and we hope that the conceptual model presented here will help make the decision to use one technique in lieu of another an informed one.

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