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REPLY COMMENT

On the biological relevance of a single *Batrachochytrium dendrobatidis* zoospore: a reply to Smith (2007)

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Smith (2007, this issue) raises important issues regarding the interpretation of the results of chytrid diagnostic assays, and highlights the blurred distinctions between the presence of *Batrachochytrium dendrobatidis* on a sample, infection by *B. dendrobatidis*, and the disease chytridiomycosis. In this reply, we (1) clarify the distinction between a chytrid infection and the disease chytridiomycosis; (2) suggest that the detection of chytridiomycosis (as opposed to the detection of *B. dendrobatidis*) has only been possible for dead or dying frogs, and is therefore of limited use to researchers; (3) demonstrate the biological relevance of a single *B. dendrobatidis* zoospore, and thus the importance of maintaining a conservative approach to the interpretation of diagnostic results; (4) confirm the usefulness of the quantifications produced by the quantitative (real-time) polymerase chain reaction (qPCR) assay; and (5) re-iterate our view that qPCR outperforms histology in virtually all respects, that the benefits of histology are far outweighed by its shortcomings, and thus qPCR should indeed be adopted as the primary means of detecting *B. dendrobatidis* on live amphibians.

Infection as opposed to disease. While an infection is merely the 'the establishment of a pathogen in its host after invasion', disease is 'a condition of the living animal or plant body or of one of its parts that impairs normal functioning and is typically manifested by distinguishing signs and symptoms' (http://merriam-webster.com). We will rely on these definitions for the remainder of this reply.

When and how can we detect chytridiomycosis? It has long been clear that the visual assessment of disease status in infected frogs is unreliable. While frogs often do not die until approximately 1 mo after being exposed to *Batrachochytrium dendrobatidis* (Longcore et al. 1999, Nichols et al. 2001, Daszak et al. 2004), distinguishing signs and symptoms of chytridiomycosis (i.e. lethargy, anorexia, excessive sloughing of skin) are often restricted to infected animals in the final stages of disease progression and are thus not evident until a few days prior to death (Berger et al. 1999, 2004, Nichols et al. 2001). Sometimes they go completely undetected: in a die-off of 28 captive poison dart frogs (*Dendrobates azureus* and *D. auratus*), only 2 frogs showed premonitory clinical signs (anorexia and lethargy), and these not until 1 d prior to death (Pessier et al. 1999). Thus the need to develop more sensitive diagnostic techniques, such as histology and PCR, to detect the potential for disease in individuals and populations before disease outbreaks actually occur.

Smith (2007) states that since a positive histological result is dependent on the presence of clusters of *Batrachochytrium dendrobatidis* zoospores, histology provides a robust confirmation of chytridiomycosis. We disagree. Using histology, Hanselmann et al. (2004) found high prevalence (96%, n = 48) of chytrid infection in bullfrogs *Rana catesbeiana* in Venezuela. Many frogs had *B. dendrobatidis* infecting up to 40% of their epithelial cells, yet no mortality or clinical signs of disease were observed. Further, the bullfrog population appeared to be rapidly increasing in numbers, rather

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than declining, making it unlikely that the chytrid fungus was impairing normal function in these frogs. Similarly, Daszak et al. (2004) exposed bullfrogs to 300 million *B. dendrobatidis* zoospores. While histology detected the chytrid fungus infecting many of the frogs, none died of chytridiomycosis or exhibited negative effects. In contrast to Smith's suggestion, in these cases histology clearly detected infection, yet failed to provide a robust confirmation of the disease chytridiomycosis.

Can histology ever detect chytridiomycosis? Yes. Whereas no studies have reported finding hyperkeratosis or hyperplasia in apparently healthy, chytrid-infected frogs (Lamirande & Nichols 2002, Daszak et al. 2004, Hanselmann et al. 2004), virtually all dead or dying chytrid-infected frogs that have been examined reveal these symptoms (Berger et al. 1998, Pessier et al. 1999, Bosch et al. 2001, Green & Kagarise Sherman 2001, Lips et al. 2003, Muths et al. 2003, Daszak et al. 2004). Thus the presence of major epidermal changes alongside chytrid zoosporangia appears to be a robust confirmation of chytridiomycosis. Histology, therefore, is indeed useful for performing necropsies. However, it is of limited use for the detection of chytridiomycosis on live frogs. While no published data exist regarding the temporal progression of epidermal changes in diseased frogs, it is likely that hyperkeratosis and hyperplasia do not appear until a few days prior to death, as is the case with the clinical symptoms anorexia and lethargy. As such, the likelihood of histology detecting anything more than chytrid zoosporangia (infection) on a live frog is minimal. On captive frogs displaying obvious clinical symptoms, it is likely that death would be witnessed before the histological result could be obtained, further limiting the usefulness of the histological technique.

Does qPCR provide a more reliable indication of disease status? While few data exist with which to judge the effect a given quantity of chytrid zoospores would have on an infected amphibian, one would intuitively assume, as Smith (2007) states, that the presence of a large number of zoospores would be functionally equivalent to chytridiomycosis. Indeed, inoculations of only 100 zoospores were required to kill great barred frogs *Mixophyes fasciolatus* (Berger et al. 1999). However, Kriger & Hero (2006) detected 4421 *Batrachochytrium dendrobatidis* zoospores on a wild stony creek frog *Litoria wilcoxii,* and this frog not only survived for several months after its positive diagnosis, but it cleared its chytrid infection (at least to the point where the assay could no longer detect it). We suggest then that this frog was infected, yet not diseased, in which case qPCR should also be considered an unreliable indicator of disease status.

Neither histology or qPCR are capable of detecting the sub-lethal effects *Batrachochytrium dendrobatidis* can have on infected frogs (e.g. Parris & Cornelius 2004). As sub-lethal effects constitute an impairment of normal function, and thus (according to our definition above) confer disease status upon the infected amphibian, this represents another failure of the techniques in the detection of chytridiomycosis. So if none of our current diagnostic techniques successfully detect chytridiomycosis in live frogs, where to from here? We suggest that in most instances, the search for chytridiomycosis will prove unnecessary: knowledge of an amphibian's disease status is of limited assistance to a researcher if it cannot be detected until immediately prior to death. Instead, it is the detection and quantification of *B. dendrobatidis* that will be of primary interest to researchers, as this information can be obtained from dying, dead, or apparently healthy frogs, and as early as 7 d post-infection (Hyatt et al. 2007, this issue). The remainder of this reply thus focuses on the detection and quantification of *B. dendrobatidis*, and the interpretation of the diagnostic results.

The biological relevance of a single *Batrachochytrium dendrobatidis* zoospore. Smith (2007) states the lack of a consensus regarding how many chytrid zoospores must be detected on a sample before we can classify the amphibian as infected. According to our definition above, only a single zoospore would be necessary, if indeed the zoospore had encysted in the amphibian's skin. However, Smith correctly notes that some zoospores may have merely come into contact with the amphibian, and that furthermore these zoospores could be inviable. As such, he suggests that 'when used at its highest level of sensitivity for the detection of a small number of zoospores, qPCR loses its biological relevance and becomes a molecular party trick', and he then asks if the qPCR assay is *too* sensitive.

We feel that an assay cannot be too sensitive. Instead it is the responsibility of the researcher to use sound judgment in the interpretation of results. With regard to amphibian disease research, we feel it is important to apply the precautionary principle and take a conservative approach to the interpretation, for the presence of a single *Batrachochytrium dendrobatidis* zoospore on a sample implies the amphibian was exposed to and therefore potentially susceptible to both chytrid infection and chytridiomycosis.

Furthermore, it is important to recognize that the number of *Batrachochytrium dendrobatidis* zoospores detected on a swab is likely a significant underestimation of the amphibian's true infection load. For instance, Hyatt et al. (2007) swabbed the mouthparts of infected tadpoles, then excised those mouthparts and used qPCR to quantify the number of chytrid zoospores

present on both the swabs and the excised mouthparts (the latter representing the number of zoospores truly present on the individual). While an average of only 75 chytrid zoospores were detected on swabs (n = 27), the excised mouthparts had average infection loads of 17 367 zoospores, a 232-fold difference. One tadpole's swab held only 2 zoospores, yet the excised mouthparts of the same individual yielded 18 104 zoospores. Clearly, it would be imprudent to classify a *B. dendrobatidis*-positive individual as uninfected simply because we failed to detect a large number of zoospores on its swab. The detection of a single zoospore on a swab is indeed biologically relevant.

Quantification of *Batrachochytrium dendrobatidis* zoospores using qPCR assay. Smith (2007) is hesitant to conclude that qPCR quantifies the severity of chytrid infections in a meaningful way, largely because he is unsure that a 'heavy chytrid load' (a large number of zoosporangia) always produces a representatively large number of zoospores. However, it is intuitive that the number of zoosporangia and the number of zoospores on an animal should be inextricably linked: from zoosporangia come zoospores, and vice versa. There is empirical evidence to support the existence of this relationship. Hyatt et al. (2007) examined the toe clips of experimentally infected *Mixophyes fasciolatus*, and quantified the number of zoospores present using the qPCR assay, and the number of zoosporangia using immunoperoxidase (IPX) histology. There was a significant positive relationship between the number of zoospores and zoosporangia detected (Fig. 1a; $p = 0.015$; $r^2 = 0.276$; n = 21 frogs deemed chytrid positive by both histology and qPCR on Day 35). Data from Boyle et al. (2004) show that this relationship holds for wild frogs as well (Fig. 1b).

As with anything in science that we attempt to quantify, we must (1) conduct our sampling in a standardized manner, (2) ensure the patterns we see are not due simply to chance, and (3) not extrapolate our conclusions beyond what our data tells us of the population we sampled. When abiding by these rules, the qPCR assay is indeed an excellent method for examining and understanding the causes and consequences of differing chytrid infection loads on amphibians.

Histology vs. qPCR. As mentioned earlier, histology is well suited for performing necropsies on amphibians. Amphibians that die of lethal chytridiomycosis are likely to show epidermal changes that are easily detected, and have large numbers of *Batrachochytrium dendrobatidis* zoosporangia infecting their epidermis, making robust confirmation of disease possible, and false negatives unlikely. Histology, however, is of little value for chytrid research on live frogs. Multiple studies have confirmed that histology of toe clips taken from live amphibians yields an inordinate number of false

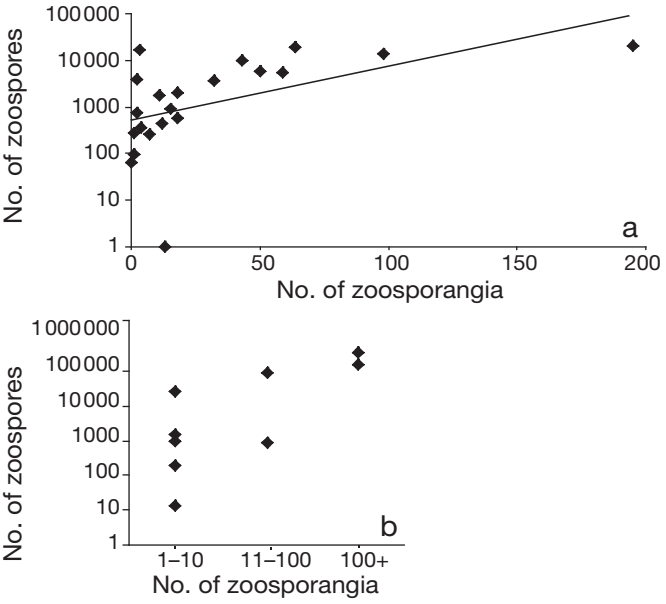

Fig. 1. *Batrachochytrium dendrobatidis.* Relationship between the number of zoosporangia and zoospores detected on toe clips from (a) experimentally infected frogs and (b) infected wild frogs. Zoosporangia were quantified using immunoperoxidase (IPX) histology, and zoospores using quantitative (real-time) polymerase chain reaction (qPCR). Data for Fig. 1a taken from Table 2 of Hyatt et al. (2007); data for Fig. 1b taken from Table 3 of Boyle et al. (2004)

negative results (Boyle et al. 2004, Speare et al. 2005, Kriger et al. 2006), and there is little doubt that the qPCR assay is significantly more likely to detect *B. dendrobatidis* than is histology. While the number of false negatives in histology could likely be reduced by increasing the number of toes (or amount of webbing) examined, this would increase the already significant ethical issues involved (McCarthy & Parris 2004), the harm incurred by the animal likely negating any positive aspects of the information obtained from the histological examinations. Further, qPCR quantifies the amount of *B. dendrobatidis* present on a sample in an unbiased, precise manner, and thus provides a valuable index of infection severity not possible with histology.

In summary, in the study of chytrid in live amphibians, the single benefit of histology (the ability to detect epidermal changes associated with chytridiomycosis) is certainly outweighed by (1) the likelihood of missed detection using the technique; (2) the harm done to the animal during sampling; (3) the technique's inability to accurately quantify infection severity; and (4) the additional time needed to obtain a result. As such, we confirm that qPCR outperforms histology in virtually all respects, that the benefits of histology are far outweighed by its shortcomings, and that there will be few instances when the removal of toes or webbing for histological diagnosis will be justified.

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