

Short Communication

The Value of Well-Designed Experiments in Studying Diseases with Special Reference to Amphibians

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Abstract: Relatively few studies of amphibian diseases have employed standard ecological experimental designs. We discuss what constitutes a well-designed ecological experiment and encourage their use in disease studies. We illustrate how well-designed experiments can be used to determine the effects of pathogens on amphibians and we illustrate how ancillary information, including that collected using molecular tools, can be used to enhance the value of such experiments.

Keywords: ecological experiments, amphibian pathogens, *Batrachochytrium*

Ecologists and conservation biologists are increasingly recognizing the importance of disease in the dynamics of animal populations and how it may affect biodiversity and management of threatened species (McCallum and Dobson, 1995; Daszak et al., 2000; Dobson and Foufopoulos, 2001; Altizer et al., 2003). As part of an overall “Biodiversity Crisis,” numerous populations of amphibians are declining, amphibian ranges are diminishing, and extinctions of whole amphibian species are occurring (Stuart et al., 2004). One recent assessment suggests that amphibians are more threatened and are declining more rapidly than mammals and birds (Stuart et al., 2004). Pathogens are among several factors that contribute to amphibian population declines. One particular fungal pathogen, *Batrachochytrium dendrobatidis* (BD), is associated with many amphibian population declines and extinctions (Daszak et al., 2003; Mendelson et al., 2006). This has prompted a worldwide response stimulating numerous investigators

from a variety of subdisciplines to investigate the dynamics of BD. Techniques and paradigms from immunologists, physiologists, epidemiologists, and ecologists along with perspectives from herpetologists and mycologists have greatly advanced our knowledge of the effects of BD on amphibian hosts.

This strong interdisciplinary approach has led to a burgeoning literature (<http://www.jcu.edu.au/school/phtm/PHTM/frogs/batrachochytrium.htm>). It has become clear that the effects of BD on amphibians are strongly modulated by amphibian behavior, ecology, and species interactions (Woodhams et al., 2003; Parris and Beaudoin, 2004; Parris and Cornelius, 2004; Woodhams and Alford, 2005; Rowley and Alford, 2007; Han et al., 2008). Ecologists have long recognized that many questions about such relationships can be clearly resolved using well-designed experiments (Ramsey and Shafer, 1997; Resetarits and Bernardo, 1998; Jenkins, 2004). However, relatively few studies of BD have employed standard ecological experimental designs. Our goals were to 1) explain what constitutes a

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well-designed ecological experiment and encourage their use in disease studies, 2) illustrate how well-designed experiments can be used to determine the effects of BD on amphibians, and 3) illustrate how ancillary information, including that collected using molecular tools, can be used to enhance the value of such experiments.

Determining the dynamic relationships between host and pathogen is difficult. However, well-designed experiments are useful tools that can provide unambiguous answers to specific questions about host-pathogen interactions. For example, in a simple experiment, one could set up a regime where hosts are exposed to pathogen X in one treatment (experimental) and simultaneously an equal number of hosts are not exposed to pathogen X (controls). All treatments are applied the same way under the same conditions (e.g., same temperature, same lighting conditions) in the same laboratory, and experimental and control enclosures are spatially interspersed. If hosts are fed, in all treatments they are fed at the same time, equal amounts of the same food. If the addition of the pathogen requires a technique, such as adding the pathogen to a substrate with a pipette or a syringe, a sham pipette or syringe containing pathogen medium is used in the control treatments. In other words, the *only* difference between the experimental treatment and the control treatment is that hosts are exposed to a pathogen in the experimental treatment and not exposed in the control treatment.

Treatments also should be replicated. Moreover, the physical layout of the experiment must be randomized and could be blocked in a randomized block design (Ramsey and Shafer, 1997). In a randomized block experiment, treatment combinations are assigned randomly to subjects within a block and separate randomizations occur within each block. In a complete randomized block design, each block contains all possible combinations. In a randomized block design, each treatment may be replicated several times within each block. When there is more than one replicate, within group variation can be measured. Thus, the researcher can explore block by treatment interactions. Randomizations must be done formally, using random number tables or generators, because no other technique can guarantee truly random layouts. These techniques have been used for years in ecological studies (Ramsey and Shafer, 1997; see also Discussion in Jenkins, 2004).

The experimental designs discussed above can provide an unambiguous rejection of the null hypothesis that pathogen X is not harmful to its host. If significantly more animals die in the experimental treatments than in control

treatments, the hypothesis that exposure to pathogen X is harmful to host Y is supported unequivocally. It is not necessary to conduct detailed postmortem examinations or diagnostic assays to quantify pathogen load, or even to determine whether the hosts were infected. In a properly designed and conducted experiment, *if the only difference between the experimental and control regimes was the addition of the pathogen, the only possible reason for a treatment effect is that exposure to the pathogen caused the death of the hosts in the experimental treatments.*

To illustrate these points further, we provide a specific example of experimental testing of the effects of BD on amphibians. Blaustein et al. (2005) tested the hypothesis that BD was lethal to the larvae of frogs. In one series of experiments, 50 plastic cups filled with dechlorinated water were placed on a laboratory bench. Each cup was randomly assigned to contain Petri dishes, agar and BD ($N = 25$), or control Petri dishes ($N = 25$; agar but no BD). One larva of the western toad (*Bufo boreas*), matched for size and developmental stage, was placed in each cup. The experiment ran for 48 h, after which there were significantly more dead larvae in cups where larvae were exposed to BD compared with those in control cups. Thus, the null hypothesis of no effect was unequivocally rejected at $\alpha = 0.05$; Blaustein et al. concluded that exposure to BD killed larvae. In this study the mechanism of mortality was not determined. The authors suggested that chytridiomycosis, the disease caused by BD, was probably not the mechanism that caused mortality, because in the experiment, BD did not have enough time to go through one generation. The authors suggested the possibility that a toxin produced by BD caused mortality. This possibility has been suggested previously (Berger et al., 1998; Daszak et al., 1999). However, the experiment is entirely sufficient to indicate that *exposure* to BD can cause mortality in larvae of some amphibian species and provides an empirical foundation for the suggestion that mortality from this source may affect whole amphibian populations. Obviously, much more information could have been obtained from this simple experiment. For example, to determine whether there were sublethal effects, such as anomalies of the mouth, one characteristic of BD infection, examination of mouthparts would have been useful. However, it was not necessary to conduct detailed anatomical examinations or autopsies to test the hypothesis that BD can be lethal to amphibian larvae; the statistical result from a properly designed experiment was sufficient.

As a further illustration of the fact that demonstration of an effect is sufficient evidence to establish that it exists, even if the mechanism of its cause is unknown, consider the analogous complementary situation in which a randomized replicated drug trial with the proper controls is conducted. If the statistical analysis reveals that taking a drug helps to reduce mortality or morbidity, then we can conclude that the drug acted in some way to limit the negative effects of the disease. It is not necessary to show how the drug did this or even that the drug was absorbed into the bloodstream. The mode of action of many effective drugs is not known, but humanity has greatly benefited from their discovery and use.

We do not argue that ecological experiments should not be supported by more mechanistic explorations. On the contrary, we employ molecular tools regularly to more thoroughly investigate ecological problems (Blaustein et al., 1994; Harris et al., 2009; Lauer et al., 2008). Other procedures can help address pertinent questions concerning pathogens and their effects. When examining amphibian hosts for chytridiomycosis, the polymerase chain reaction (PCR) is a technique commonly used and considered by many to be an important tool for detecting and answering certain questions concerning infection of BD on its hosts (Boyle et al., 2004; Retallick et al., 2006).

PCR can confirm the presence of pathogen DNA. Quantitative PCR (qPCR) can confirm the presence and the amount of pathogen DNA. However, neither of these techniques provides answers regarding how an animal becomes infected or the consequences of infection by the pathogen. These are tools that can be used to supplement information derived from experiments.

Although useful, molecular tools such as PCR are expensive and must be done in a laboratory with special equipment and trained personnel. Through productive collaborations, these methods have become more common in current research in disease ecology. However, disease ecologists often do not have the equipment or the expertise to conduct the assays. Samples often must be sent to specialists who can perform this relatively expensive, time-consuming work even though the original question of interest may be answered by a simple, well-designed experiment. In the case of BD, we feel that well-designed experiments, carefully interpreted, are more than sufficient to test whether BD can be harmful to its hosts. For species affected by an emerging infectious disease, time is of the essence in terms of understanding the disease and how to manage it. Therefore, laboratories without access to PCR

should be encouraged to conduct well-designed and focused experiments as a means of further understanding chytridiomycosis and other diseases.

Despite the commonness of pathogens and the harmful effects that they have on their hosts, data from manipulative experimental studies, especially in amphibians, are rare. However, they are necessary for a true understanding of host-pathogen interactions in nature. Even very simple ecologically focused experiments can reveal important facts that would not be apparent if all investigations of disease were conducted using molecular techniques under standard laboratory conditions. For example, Woodhams et al. (2003) demonstrated that one reason for the puzzling patterns of variation among species in susceptibility to BD is likely to be basking behavior; short exposures to body temperatures of 37°C cured infected animals. No amount of investigation at the molecular level or under standard laboratory conditions would have uncovered this fact, and because they performed a properly designed experiment, no molecular analysis was needed to confirm their result. More complex experiments can illustrate how diseases interact with other influences on species populations. In some cases, these effects may be in directions opposite to those that would be inferred based on less integrative studies; for example, Kiesecker and Blaustein (1999) showed that embryonic mortality caused by the water mold *Saprolegnia ferax* can reach relatively high levels, but may actually benefit both the host and species it interacts with at the population level by reducing competition among larvae. The effects of disease also may be exacerbated in more complex situations, as illustrated in the study by Parris and Beaudoin (2004), which showed that the negative effects of BD on larval *Hyla chrysoscelis* increased in the presence of predators. Experimental studies are clearly essential for identifying differences among species in susceptibility to disease and determining their causes, and for examining the effects of pathogens at the individual, population, and community levels. There is a large body of literature where investigators have used experiments to examine the effects of pathogens on nonamphibian hosts (Hudson et al., 1998; Tompkins and Begon, 1999). Experimentally based results have been used to show that certain human diseases are caused by microparasites (Marshall et al., 1985; Thagard, 1999). These experimental studies generally yield unequivocal, noncontroversial results without using PCR or similar techniques.

If a conclusion is based on a properly designed and executed experiment, then it is unreasonable and can im-

pede progress in understanding chytridiomycosis, or any disease, to reject its outcome. Any reader who has had a reviewer or colleague reject the outcome of a properly designed experiment can share our frustration. We agree that it is always useful to extract the maximum information possible from the outcome of any experiment, but by definition, a properly designed and conducted experiment, such as the above-mentioned larval example, eliminates the effects of all other variables, however subtle, thus providing an unambiguous answer to a scientific question.

We do not argue that it is not useful to collect data on host pathogen interactions using a wide range of approaches. Using molecular and other diagnostic techniques can provide answers to specific questions about BD-host dynamics, such as those addressed earlier, but these techniques are not a necessary follow-up to well-designed ecological experiments. Understanding and ultimately mitigating the serious problems imposed by diseases, such as chytridiomycosis, requires increased understanding at all levels, from molecules through population dynamics and epidemiology to system-level effects. Attempting to address all of these levels simultaneously often is impossible, and ignoring the outcomes of well-designed experiments because they are conducted at a phenomenological rather than a mechanistic level can be a serious impediment to understanding, as can placing undue weight on poorly designed data collected at the mechanistic level.

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