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ABSTRACT: Chytridiomycosis is a devastating infectious disease of amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). A growing number of studies have examined the role of amphibian adaptive immunity in response to this pathogen, with varying degrees of immune activation reported. Here we present immunologic data for the mountain yellow-legged frog, *Rana muscosa*, and the Sierra Nevada yellow-legged frog, *Rana sierrae*, which are two endangered and ecologically important species experiencing Bd-inflicted declines. Previous studies on these species that examined transcriptional response during Bd infection, and the effective of immunization, provided little evidence of immune activation to Bd. However, the studies did not directly assay immune effectors in the frog hosts. We performed experiments to examine antibody production, which is a hallmark of systemic adaptive immune activation. We used controlled laboratory experiments and enzyme-linked immunosorbent assays to examine the antibody response to Bd immunization and live Bd exposure. *Rana muscosa* and *R. sierrae* individuals did not produce detectable antibodies with the capacity to bind to denatured Bd antigens under our experimental conditions. While we cannot rule out antibody response to Bd in these species, our results suggest weak, poor, or inefficient production of antibodies to denatured Bd antigens. Our findings are consistent with susceptibility to chytridiomycosis in these species and suggest additional work is needed to characterize the potential for adaptive immunity.

Key words: Antibodies, *Batrachochytrium dendrobatidis*, chytridiomycosis, ELISA, *Rana muscosa*, *Rana sierra*.

Batrachochytrium dendrobatidis (Bd), the causative agent of chytridiomycosis, is responsible for amphibian population declines around the world (Skerratt et al. 2007). Although many factors interact to determine the outcome to Bd exposure, the host immune system likely plays a critical role in disease progression. Few investigators have attempted to directly measure immune system effector responses to Bd (but see Ramsey et al. 2010). Here we examine the antibody-mediated adaptive immune response to Bd in two sister species: *Rana muscosa* (the mountain yellow-legged frog) and *Rana sierrae* (the Sierra Nevada yellow-legged Frog). *Rana muscosa* and *R. sierrae* are highly susceptible species and have suffered widespread population declines due to Bd (Vredenburg et al. 2010). Although *R. muscosa* may have some innate defenses against Bd (i.e., antimicrobial peptides that inhibit Bd growth in vitro [Rollins-Smith et al. 2006]), previous work suggests that *R. muscosa* does not mount a robust adaptive immune response to Bd. Prior Bd immunization with formalin-killed Bd and adjuvant (administered via subcutaneous injection) did not diminish the progression of infection or the mortality upon subsequent Bd exposure (Stice and Briggs 2010). In addition, a gene expression experiment, which included *R. muscosa* and *R. sierrae*, suggested that critical immune pathways do not activate during infection (Rosenblum et al. 2012). However, prior experiments with *R. muscosa* and *R. sierrae* did not directly measure immune effectors (e.g., antibodies); thus, we currently have an incomplete understanding of the potential for adaptive immunity in these susceptible species.

We performed two controlled, laboratory, live-animal experiments where we immunized frogs with killed Bd and assayed the production of antibodies. In the first experiment, we immunized subadult *R. muscosa*

with killed Bd cells and inoculated the frogs with live Bd. The epidemiologic results of this experiment were described by Stice and Briggs (2010). Briefly, juvenile *R. muscosa* frogs (6 mo postmetamorphosis) were immunized via subcutaneous injection into the dorsal lymph sac of 100,000 formalin-killed Bd cells enriched for zoospores. The frogs were divided into three groups for immunization: Bd+adjuvant, adjuvant only, and saline control. The frogs received two immunizations, separated by 1 mo, which included Freund's complete adjuvant and Freund's incomplete adjuvant, respectively. One month after the second immunization, the frogs were inoculated with 100,000 live Bd zoospores. Blood samples were collected via cardiac puncture 110 d after the live Bd inoculation, and serum was isolated by centrifugation. We used enzyme-linked immunosorbent assay (ELISA) to detect circulating Bd-specific antibodies. For the ELISA (described in the upcoming text), we tested nine Bd+adjuvant frogs and seven saline-injected frogs.

In the second experiment, we immunized adult *R. sierrae* with killed Bd and, again, assayed Bd-specific antibodies. We obtained wild-caught frogs from the Ebbett's Pass in Alpine County, California, US (38°31′42′′N, 119°46′32′′W). The average snout-vent length of the frogs was 49.1 ± 5.9 mm (mean $\pm$ SD). Frogs were housed in individual tanks, maintained at the preferred temperature of 17 C, and were fed 15–20 calcium-dusted crickets once weekly. We immunized seven adult frogs via intraperitoneal injection with 100,000 killed Bd cells prepared according to Stice and Briggs (2010). Four control frogs were injected with amphibian-grade phosphate-buffered saline (PBS). We collected blood samples via cardiac puncture at 14 and 21 d after immunization. We isolated the serum by centrifugation and used ELISA to test the serum samples for Bd-specific antibodies. For the control group, only samples from the 21 d time-point were analyzed by ELISA.

We developed the primary antibody, rabbit anti-*R. muscosa* immunoglobulin Y heavy chain, for the ELISA experiments. We purified the heavy chain of immunoglobulin Y (i.e., antibody) from *R. muscosa* serum as described by Chen et al. (2005) and performed a western blot in parallel to determine the molecular weight of the immunoglobulin heavy chain Y (IgY) using polyclonal rabbit anti-*Xenopus* IgY as the primary antibody. We also performed a western blot with rabbit anti-*Xenopus* IgX as the primary antibody in order to differentially identify isotype bands. The IgY heavy chain protein band was isolated by cutting and destaining the gel band. Once purified, we submitted the IgY heavy chain antigen to a custom antibody production company (Pacific Immunology, Ramona, California, USA) to develop the primary antibody reagent for the ELISA: rabbit anti-*R. muscosa* IgY (H). We tested the primary antibody reagent by western blot to confirm reactivity to the heavy chain. Briefly, a sodium dodecyl sulfate polyacrylamide gel electrophoresis was run to electrophoretically separate proteins in 1:100 diluted *R. muscosa* serum and *Rana catesbeiana* (American bullfrog) serum. We included the *R. catesbeiana* serum lane to test the feasibility of using this reagent with related species. The separated proteins were transferred to a nitrocellulose blot overnight at 4 C and then blocked with 3% nonfat dry milk in PBS with 0.05% Tween®20. We incubated the western blot with 1:1,000 rabbit antiserum for 1 h at room temperature with gentle agitation. Following five blocking buffer washes, the secondary antibody (1:5,000 anti-rabbit conjugated to horseradish peroxidase [Southern Biotech, Birmingham, Alabama, USA]) was added and incubated for 1 h at room temperature. Finally, we used the chemiluminescent detection method to develop the blot with the Immobilon Western Chemiluminescence Substrate kit (Millipore, Bedford, Massachusetts, USA). We used a protein standard ladder to estimate the molecular weight of the protein bands on the western blot.
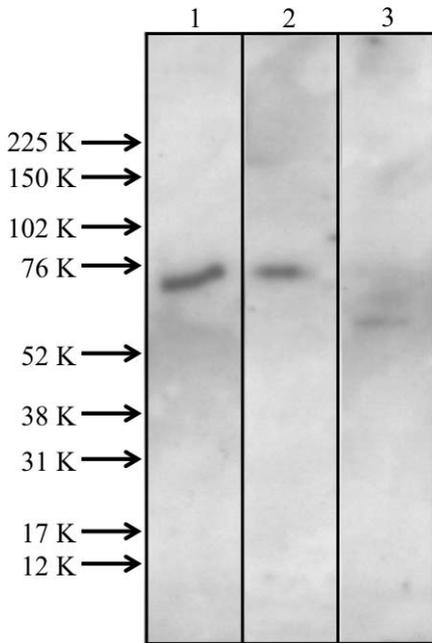
FIGURE 1. Western blot of sera from the mountain yellow-legged frog (*Rana muscosa*) and American bullfrog (*Rana catesbeiana*) confirming the binding activity of the primary antibody: rabbit anti-*Rana muscosa* immunoglobulin heavy chain. Frog sera and primary antibody were diluted 100 fold. Lanes 1 and 2 show strong binding to the *R. muscosa* immunoglobulin Y heavy chain at 1:100 and 1:200 dilutions, respectively. Lane 3 shows weaker binding to *R. catesbeiana* immunoglobulin heavy chain at ~65 kDa and two additional faint bands, which likely signifies weak binding to two other IgY (H) isoforms, at ~70 and ~75 kDa. The arrows indicate the locations of molecular weight standards.

We prepared the plates and performed the ELISA following Ramsey et al. (2010) using pelleted Bd cells (mixture of sporangia and zoospores) from an actively growing culture to coat the wells. To perform the ELISA, we first added 50 μL of 1:100 diluted frog serum in blocking buffer to plate wells. We ran each serum sample in triplicate. Positive control wells received polyclonal rabbit anti-Bd serum diluted to 1:100 in blocking buffer. In this context, the positive control served to confirm that the ELISA plate wells contain Bd antigens that were receptive to antibody binding interactions, albeit with rabbit anti-Bd antibodies. Negative control wells received blocking buffer. We used a 1:100 dilution to ensure that we did not dilute antibodies

beyond the point of detection. Ramsey et al. (2010), working with the South African clawed frog (*Xenopus laevis*), showed that immunized frogs produced an antibody titer of 1:32,000 at 14 d postimmunization. We prepared the primary antibody (rabbit anti-*Rana muscosa* immunoglobulin heavy chain) by dilution to 1:500 in blocking buffer. Then we added 100 μL of ABTS substrate (Southern Biotech, Birmingham, Alabama, USA) to each well and measured the optical density (OD) in each well at 405 nm using a Bio-Rad 680 Microplate Reader (Bio-Rad, Hercules, California, USA). We used Student's *t*-tests to compare mean antibody concentrations (OD) at different sampling points. Each posttreatment sampling point (immunization or exposure) was compared to the respective preliminary sampling points and to the negative control measurements.

We tested the efficacy of the primary antibody (rabbit anti-*Rana muscosa* IgY [H]) by western blot to confirm its reactivity to the heavy chain of *R. muscosa* antibodies. Figure 1 shows the specific binding of the primary antibody to the *R. muscosa* IgY (H; at 76 kDa) by the presence of a single band at the appropriate molecular weight.

In the first experiment, juvenile *R. muscosa* frogs subcutaneously immunized with killed Bd and subsequently inoculated with live Bd did not produce detectable antibodies against Bd antigens (Fig. 2). The ELISA for *R. muscosa* antibodies with binding specificity against Bd antigens was negative for all experimentally inoculated frogs. The frogs in the procedural control group (saline-injected and live Bd-inoculated) also did not produce detectable Bd-specific antibodies following Bd inoculation. There was no detectable difference in Bd-specific antibody concentration between the immunized frogs and the ELISA-negative control (blocking buffer only; $P>0.100$ for all comparisons). The positive control measurements confirmed that the assay effectively detects the presence of antibodies bound to Bd antigens ($P<0.01$ positive versus negative controls).
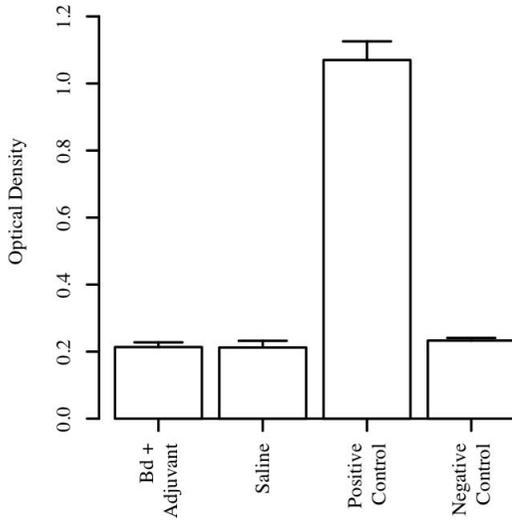
FIGURE 2.   Juvenile mountain yellow-legged frogs *Rana muscosa* did not produce antibodies specific to *Batrachochytrium dendrobatidis* (Bd) following subcutaneous immunization and subsequent exposure to Bd. Mean optical density is shown for the enzyme linked immunosorbent assay (ELISA) results. Error bars represent standard deviation. Optical density is a quantitative measure of relative concentration of Bd-specific antibodies in the ELISA. The first bar shows the treatment group ($n=9$): *R. muscosa* immunized with Bd and adjuvant. The second bar shows the procedural control group ($n=7$): *R. muscosa* injected with saline. The positive and negative controls refer to ELISA assay controls: rabbit anti-Bd reagent and amphibian phosphate-buffered saline, respectively.
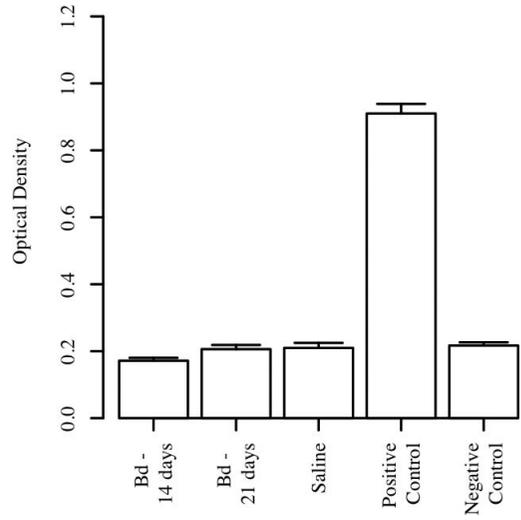

FIGURE 3.   Adult Sierra Nevada yellow-legged frogs *Rana sierrae* did not produce antibodies specific to *Batrachochytrium dendrobatidis* (Bd) following intraperitoneal immunization with Bd. Mean optical density is shown for the enzyme linked immunosorbent assay (ELISA) results. Error bars represent standard deviation. Optical density is a quantitative measure of relative concentration of Bd-specific antibodies in the ELISA. The first and second bars show the two sampling points of the treatment group ($n=7$): *R. sierrae* immunized with Bd. The third bar shows the procedural control group ($n=4$): *R. sierrae* injected with saline. The positive and negative controls refer to ELISA assay controls: rabbit anti-Bd reagent and amphibian phosphate-buffered saline, respectively.

In the second experiment, adult *R. sierrae* frogs immunized with killed Bd did not produce detectable antibodies against Bd antigens (Fig. 3). There was no detectable difference in Bd-specific antibody concentration between the immunized frogs and the assay negative control at both time-points: 14 and 21 d postimmunization ($P>0.100$ for all comparisons). As mentioned earlier, the positive control measurements confirmed that the assay effectively detects antibodies bound to Bd antigens ($P<0.01$ positive versus negative controls).

The negative results of our antibody assays suggest a lack of antibody generation in our experimental conditions. One interpretation is that the *R. muscosa* and *R. sierrae* immune system responds weakly to Bd antigens due to ill-equipped immunoreceptor repertoires

(i.e., major histocompatibility complex, antibodies, T-cell receptor). The ineffective immunization seen by Stice and Briggs (2010) may be accounted for by the lack of immune activation, as measured by antibody production. Although we had negative results for both species in our experimental conditions, there may be differences in host response between species or age classes. Subadults that have recently undergone metamorphosis frequently have less well-developed immune defenses than do adults (Robert and Ohta 2009), and previous studies have shown that wild *R. muscosa* and *R. sierrae* subadults are very susceptible during Bd outbreaks and suffer high infection intensities and mortality rates (Vredenburg et al. 2010).

While we did not detect antibody responses, we do not rule out the possibility

of an antibody response under different conditions. For example, experimental factors including inoculum preparation (number of cells and fixation method), number of injections, environmental conditions (e.g., temperature), and choice of sampling time-point could affect the elicitation and detection of an antibody response. For example, Ramsey et al. (2010) observed antibody production under different conditions in *X. laevis*: higher inoculum (2×10^7 mixed sporangia and zoospore) with a higher laboratory-controlled temperature (20–24 C). While 17 C is the standard temperature for *R. muscosa* laboratory husbandry, higher temperatures may increase rates of physiologic processes, including immune response, in amphibians. Also given the dynamic nature of antibody production over time, our sampling point may have affected our ability to detect antibodies in our experiments. Future work should include an experimental positive control protocol (e.g., bacteria or model antigen for immunization) to show that an antibody response can be elicited in a laboratory experiment. Once this is confirmed, researchers can test the effect of ELISA variations (e.g., fixation method) on detectability of antibodies. The immunization method (e.g., with or without adjuvant) may also affect immune activation in these species. McMahon et al. (2014) suggest that such cutaneous exposure to live Bd (with clearance by antifungal drugs) might elicit the strongest immune response.

Examining the host response to chytridiomycosis is critical for understanding disease-caused declines and for designing effective mitigation strategies. More work is needed to examine how mechanisms of adaptive immunity potentially operate in amphibian species. Future work that incorporates population and species variation in susceptibility will offer a powerful way to investigate mechanisms of host response.

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