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CITIZEN SCIENTISTS MONITOR A DEADLY FUNGUS THREATENING AMPHIBIAN COMMUNITIES IN NORTHERN COASTAL CALIFORNIA, USA

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ABSTRACT: Ecoclub youth and supervising family members conducted citizen science to assess regional prevalence and distribution of *Batrachochytrium dendrobatidis* (*Bd*) among amphibians at Humboldt Bay National Wildlife Refuge (Refuge) and Redwood National and State Parks (Parks), Humboldt County, California, US, May 2013 through December 2014. Using quantitative real-time PCR, 26 (17%) of 155 samples were positive for *Bd*. Positive samples occurred in four frog and toad species: foothill yellow-legged frog (*Rana boylii*), northern red-legged frog (*Rana aurora*), Pacific chorus frog (*Pseudacris regilla*), and western toad (*Anaxyrus* [*Bufo*] *boreas*); no salamanders or anuran larvae were positive. Except for *R. aurora*, all infected anurans were first-time species reports for coastal northern California. At the Refuge, significantly fewer (6/71) postmetamorphic amphibians were positive compared to the Parks (20/69; P=0.0018). We assessed the association of being PCR-positive for *Bd*, season of sampling, and age of sampler (child, teen, or adult). The full model with season, species, and sampler age had the greatest support. Frogs tested in winter or spring were more likely to be positive than those tested in summer or fall; foothill yellow-legged frogs, northern red-legged frogs, and western toads were more likely to be positive than were Pacific chorus frogs; and the probability of being positive nearly doubled when a child (≤12 yr old) collected the sample compared to a teen or adult. Our results support other chytrid studies that found amphibians are more susceptible to *Bd* when temperatures are cool and that species differ in their susceptibility. The Ecoclub's findings provide new information important to conservation of northern California's coastal amphibians and demonstrate the value of involving children in citizen science.

Key words: Amphibian disease, amphibian monitoring, *Batrachochytrium dendrobatidis*, *Bd*, chytrid fungus, Northern California, youth citizen science.

INTRODUCTION

Spread of the deadly amphibian disease, chytridiomycosis, has become a prominent threat to amphibian biodiversity worldwide (Skerratt et al. 2007; Olson et al. 2013; Botzler and Brown 2014). Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*; class Chytridiomycetes), commonly referred to as the chytrid fungus, has caused decline or extinction of >200 amphibian species (Skerratt et al. 2007). It affects a broad range of amphibian hosts and has been reported in 516 (42%) of 1,240 amphibian species evaluated (Olson et al. 2013). The fungus infects keratinized tissue of amphibians including the skin of postmetamorphic animals and mouthparts of larvae (Berger et al. 1998, 2005). In postmetamorphic animals, *Bd* compromises osmotic regulation and can lead to mortality from cardiac arrest (Voyles et al. 2009).

Citizen science projects increasingly are making important contributions to conservation science (Citizen Science Association 2016). Although many projects focus on participation by adults, the inclusion of children in citizen science can be beneficial because children are enthusiastic and curious observers. For example, Minnesota school

children discovered a high proportion of malformed frogs in a local pond, leading to a wide-scale investigation into the scope and cause of amphibian malformations (Vandenlangenberg et al. 2003). Incorporating children in scientific research also enhances their teamwork skills to accomplish common goals and further develops awareness of conservation issues, providing science education through direct experience.

Youth of the Bilingual McKinleyville Ecoclub in Humboldt County, California, US, searched the literature about chytridiomycosis and learned that severe declines were occurring in frog populations in California's mountains (Rachowicz et al. 2006; Fellers et al. 2008; Piovia-Scott et al. 2014). California, and the north coast in particular, has a rich diversity of amphibians with 27 species of frogs and toads (order Anura) and at least 43 salamanders (order Caudata; Stebbins and McGinnis 2012), yet information about the status of many of the species found in coastal northern California is lacking. Nieto et al. (2007) reported a *Bd* prevalence of 6.4% among 6,830 northern red-legged frog (*Rana aurora*) tadpoles in 13 ponds associated with Redwood National Park, Humboldt County. In a follow-up study of the same sites, Sun (2012) found a 5% prevalence among 81 red-legged frog tadpoles and a 14% prevalence among 42 metamorphosed red-legged frogs. Adams et al. (2010) reported the presence of a northern red-legged frog infected with *Bd* on a map in the vicinity of Humboldt Bay National Wildlife Refuge, Humboldt County; no methods or other pertinent information is given for this report. These are the only known reports of *Bd* in coastal northern California.

With volunteer help from local scientists, the Bilingual McKinleyville Ecoclub members designed a monitoring program to assess the status and distribution of *Bd* in amphibians in protected habitats of Humboldt County. The club is part of an international organization, Ecoclubs International, promoting youth leadership development with a special focus on healthy and sustainable natural environments and human communities (Pan American Health Organization 2013). We had two objectives. Firstly, we used citizen science to understand the status and distribution of *Bd* within local amphibian communities. Secondly, we evaluated whether citizen science with children could provide viable research on a significant wildlife disease problem. In addition to the conservation importance of the project, studying the prevalence and distribution of *Bd* in local amphibian populations seemed an appropriate project for Ecoclub youth because 1) frogs and salamanders are relatively easy and safe to catch; 2) testing for *Bd* involves a simple skin swab that youth can be trained to do correctly; and 3) *Bd* causes no harm to humans.

MATERIALS AND METHODS

Study areas

We surveyed sites at the Humboldt Bay National Wildlife Refuge (Refuge) (40°42′59″N, 124°13′04″W) and Redwood National and State Parks (Parks) (41°04′–41°49′N, 123°53′–124°10′W). Both locations are in coastal Humboldt County and support a diversity of protected freshwater habitats where red-legged frogs have been found infected with *Bd* (Nieto et al. 2007; Sun 2012). The Refuge includes 1,619 ha in the southern part of the County and is used during the year by >260 species of birds, 50 species of mammals, 95 species of fish, 15 species of reptiles, and eight species of amphibians (US Fish and Wildlife Service 2009). The Parks are a World Heritage Site about 85 km north of the Refuge along the coast and are jointly managed by the National Park Service and the California Department of Parks and Recreation (US National Park Service 2001). The Parks include 53,420 ha of temperate rainforest coast redwood (*Sequoia sempervirens*) set among several perennial streams and coastal freshwater ponds. Both the Refuge and Parks support a high diversity of amphibians. Species sampled by the Ecoclub Amphibian Group included foothill yellow-legged frog (*Rana boylii*), northern red-legged frog (*R. aurora*), Pacific chorus frog (*Pseudacris regilla*), western toad (*Anaxyrus* [*Bufo*] *boreas*), coastal tailed frog (*Ascaphus truei*), Ensatina (salamander; *Ensatina eschscholtzii*), California slender salamander (*Batrachoseps attenuatus*), coastal giant salamander (*Dicamptodon tenebrosus*), and rough-skinned newts (*Taricha granulosa*). Amphibians known to occur in similar aquatic habitats in the Refuge and Parks, but not found

by the Ecoclub, include northwestern salamander (*Ambystoma gracile*) and southern torrent salamander (*Rhyacotriton variegatus*).

We sampled three sites at the Refuge: Cattail Marsh (40°40′46″N, 124°12′06″W) comprises a riparian forest at its eastern edge, freshwater, and then a brackish water slough. Frog City (40°41′7″N, 124°12′30″W) and Wildwing Dock (40°41′9″N, 124°12′33″W) are freshwater habitats but become saltier from historic salt marsh soils as freshwater declines during the dry season.

Four sites were evaluated at the Parks. One was on Redwood Creek (41°17′49″N, 124°1′56″W), the major waterway of the Parks. This site was difficult to access and was not a visitor-destination site. The other study areas were frequent visitor destinations and included Tall Trees Trail (41°17′N, 124°1′W) parallel to Redwood Creek, Fern Canyon (41°24′5″N, 124°3′55″W), and Freshwater Lagoon (41°15′58″N, 124°5′50″W); the Lagoon is a brackish body of water adjacent to the Pacific Ocean.

Sampling and analysis

All participants, including the youth (4–16 yr) and supervising adult family members, attended an educational workshop that included a review of the scientific method and training in the specific techniques required for the collection and sampling of amphibians. During this study, 28 youth and 26 supervising adults completed a workshop and participated in the field sampling of amphibians. The age distribution of participating youth on 1 January 2014, the approximate mid-point of this study was: 5 yr olds (n=4), 6 yr (n=5), 7 yr (n=4), 8 yr (n=1), 9 yr (n=5), 10 yr (n=2), 11 yr (n=2), 12 yr (n=2), 13 yr (n=2), 14 yr (n=0), 15 yr (n=1), and 16 yr (n=0). As the ages changed during the 2 yr of the study, individual youth made collections in more than one age class.

Field collections generally were conducted 2–3 h monthly, alternating each month between the Refuge and the Parks. The corresponding author (R.G.B.) arranged and monitored all field collections. He attended the swabbing of every amphibian to ensure consistency and uniformity in techniques. Other biologists (K.L.P., D.T.A.) participated as their schedules allowed. Boots were rinsed in didecyl dimethyl ammonium chloride (HDQ Neutral®), at the start and end of a visit to any site, according to the manufacturer's instructions (Spartan Chemical Company, Maumee, Ohio, USA). All used field equipment was disinfected in HDQ Neutral or stored at –18 C for ≥24 h (US Forest Service 2014).

We visually searched appropriate habitats and captured amphibians by hand or dip-net. In order to minimize the risk of transmission from an infected to uninfected amphibian within a site, hands were rinsed in the ponds or streams between captures in an effort to physically remove chytrids from the hands so the next captured frog would have no more exposure to chytrids than already was present in the natural waters. Most samples were collected by Ecoclub youth (≤16 yr); adults primarily supervised youth but sometimes also helped catch and swab amphibians. Captured postmetamorphic animals were weighed, measured by snout–urostyle length, and swabbed with rayon swabs following Boyle et al. (2004). Sex was determined when possible. Each postmetamorphic animal was swabbed five times in seven body areas: the ventral surface of the abdomen, the left and right sides between the front and hind legs, the left and right inner thighs, and the webbing of each hind foot. For larval frogs and toads, a swab was gently inserted into the mouth and rotated six times. All swabs were placed in individual sterile vials and stored at –18 C upon return from the field until DNA was extracted from the samples. Because animals were not marked, recapture was possible.

The DNA was extracted from swab samples (by G.M.W.) using PrepMan™ (Applied Biosystems, Foster City, California, USA) following the protocol of Retallick et al. (2006); Buffer solution (0.5 mL of 1% tris-ethylenediaminetetraacetic acid) was added to each swab and the preparation was shaken overnight at room temperature. Swabs were removed and tubes centrifuged at 15.7 × G for 10 min. The DNA extraction was performed using 40 μL of PrepMan Ultra according to the manufacturer's specifications.

Extracted DNA was diluted 1:20 with molecular grade water to minimize PCR inhibitors and stored at –20 C. For quantitative PCR (qPCR) analyses to test for DNA of *Bd*, samples were run using a StepOnePlus™ Real-time PCR machine (Applied Biosystems) as reported by Boyle et al. (2004). Fungal genomic equivalents were estimated by multiplying the DNA quantity found by qPCR in each sample by 160 to account for earlier dilution of the DNA. The DNA analysis was done in the laboratory of J.E.F. at the School of Veterinary Medicine, University of California Davis, California.

We first conducted summary comparisons to assess differences among species and between the Parks and Refuge. We then used generalized linear mixed models and multimodel inference based on information-theoretic approaches (Anderson 2008) to assess the effects of three predictor variables (species, survey timing, and age of sampler [child: ≤12 yr, vs. teen and adult: ≥13 yr]) on the probability of animals being PCR positive for *Bd*. These variables were selected based on results from published research and on

TABLE 1. Prevalences (number infected/number sampled) of *Batrachochytrium dendrobatidis* among amphibians collected at Redwood National and State Parks (Parks) and Humboldt Bay National Wildlife Refuge (Refuge), Humboldt County, California, USA, May 2013 through December 2014.

Species	Parks	Refuge	Total
Foothill yellow-legged frog (*Rana boylii*)	16/22	0/0	16/22
Northern red-legged frog (*Rana aurora aurora*)	1/9	2/20	3/29
Pacific chorus frog (*Pseudacris regilla*)	0/3	4/51	4/54
Western toad (*Anaxyrus* [*Bufo*] *boreas*)	3/22	0/0	3/22
Others[a]	0/13	0/0	0/13
Tadpoles[b]	0/14	0/1	0/15
Totals	20/83	6/72	26/155

[a] Includes 3 rough-skinned newts (*Taricha granulosa*), 2 California slender salamanders (*Batrachoseps attenuatus*), 5 coastal giant salamanders (*Dicamptodon tenebrosus*), and 3 Ensatina (*Ensatina eschscholtzii*).

[b] Includes 5 coastal tailed frogs (*Ascaphus truei*), 5 northern red-legged frogs, 4 western toads, and one unidentified species.

our objective of understanding if using youth citizen scientists was effective for detecting *Bd* in sampled amphibians. We used only postmetamorphic frogs and toads for the analysis because none of the samples from the salamanders or larvae were positive (see Results). For Season, we combined winter and spring (December–May) surveys as the cold season (the city of Eureka mid-range daily temperature generally <11 C) and summer and fall (June–November) surveys as the warm season (Eureka mid-range daily temperature generally >11 C; 2015 US Climate Data). We used 127 samples from seven sites (three in the Refuge and four in the Parks) in the models and included the site sampled as a random factor to account for the expected similarity of samples collected from the same site. We ran a null model (intercept and site only) as a reference for assessing model importance (Anderson 2008).

We calculated corrected Akaike information criterion (AIC_c)-based model probabilities, or "Akaike weights," for every model in the candidate set (Anderson 2008). Model-averaged parameter estimates were obtained from the weighted average of parameter estimates from each of the candidate models, with a value of zero assigned for models in which the parameter being estimated does not appear (Anderson 2008; Lukacs et al. 2010). Approximate 95% confidence intervals for each parameter were calculated as the model-averaged mean±two times the model-averaged standard errors (Anderson 2008; Lukacs et al. 2010); for ease of interpretation *P*-values also were estimated for each parameter in the final model candidate set. Analyses were conducted by K.L.P. using R (R Development Core Team 2012). Mixed models were fit using the 'glmer' function in the 'lme4' package (Bates and Maechler 2010).

RESULTS

Twenty-six of 155 (17%) skin swabs and buccal swabs were PCR positive for *Bd* (Table 1). Four species of frogs and toads had positive samples; none of 13 salamanders or 15 late-stage anuran larvae was infected. Infection prevalences varied among anuran species with 16/22 foothill yellow-legged frogs, 3/29 northern red-legged frogs, 4/54 Pacific chorus frogs, and 3/22 western toads found positive for *Bd* (Table 1).

Prevalence of *Bd* among postmetamorphic amphibians differed between the Refuge and Parks with 6 of 71 (8%) amphibians from the Refuge positive for *Bd* compared to 20 of 69 (29%) from the Parks (χ^2=9.76, df=1, *P*=0.002). However, the mean chytrid zoospore density ('genome equivalent') per positive sample was more than twice as high at the Refuge (mean=1342.1, SE=335.9) compared to the Parks (mean=546.8, SE=60.4; *t*-test on log-transformed data: *t*=1.9, *P*=0.09).

We compared eight models to determine the relative importance of the predictor variables Species, survey Season, and adult or child Sampler (Table 2). The full model including Season+Species+Sampler was the most supported model tested although the model with Season+Species received a comparable ranking within 2 AIC_c units of the full model (Table 2). Post hoc comparisons of the full model revealed Season to be more influential than Species and Sampler (Season

TABLE 2. Linear mixed-effects models with model deviances, number of parameters in the models (K), and difference in corrected Akaike information criterion (AIC_c) scores from the best model and AIC_c weights. Models are ordered by AIC_c. All models include site as a random variable. Season is either winter/spring or summer/fall. Species include foothill yellow-legged frog (*Rana boylii*), northern red-legged frog (*Rana aurora*), Pacific chorus frog (*Pseudacris regilla*), and western toad (*Anaxyrus* [*Bufo*] *boreas*). Samplers were either children (4–12 yr) or teens and adults (>12 yr old).

Model	Model deviances	K	AIC_c	Delta AIC_c	AIC_c weight
Season+Species+Sampler	63.60	7	72.07	0	0.59
Season+Species	66.75	6	72.83	0.77	0.41
Season	81.58	3	82.87	10.80	0
Season+Sampler	81.48	4	84.89	12.82	0
Species	86.19	5	92.64	20.57	0
Species+Sampler	85.31	6	94.03	21.96	0
Null	106.64	2	104.89	32.82	0
Sampler	106.62	3	106.94	34.88	0

χ^2=7.6, df=1, P=0.006; Species χ^2=7.4, df=3, P=0.06; Sampler χ^2=2.6, df=1, P=0.10). Frogs or toads sampled in winter or spring were more likely to be positive for *Bd* than those tested in summer or fall. By species, the probability of being *Bd*-positive was greater for foothill yellow-legged frogs, northern red-legged frogs, and western toads in winter/spring and lower for Pacific chorus frogs irrespective of season (Fig. 1). The probability of being *Bd*-positive was higher when a child collected the sample than when an adult collected a sample (child probability=0.20, SE=0.25, and adult probability=0.03, SE=0.05; P=0.10), while controlling for season and species. Positive *Bd* samples were taken by samplers ranging from 4–73 yr old.

DISCUSSION

We used citizen science to assess the status and distribution of *Bd* within amphibian communities. The Bilingual McKinleyville Ecoclub partnered with local scientists to collect and process samples over 20 mo (the project still is ongoing) and obtained informative results that support findings of other studies on chytridiomycosis around the world. All anuran species sampled had *Bd*-positive individuals. Our overall prevalence of 17% is comparable to similar surveys conducted in the Pacific Northwest (Pearl et al. 2007; Piovia-Scott et al. 2011). We conclude that citizen science programs can be useful for understanding and monitoring the threat of chytridiomycosis to local amphibian populations and that children with trained adult supervision are highly effective at collecting the necessary data.

The prevalence of *Bd* was highly correlated with survey timing. Prevalences of *Bd* were higher in the colder winter and spring than summer and fall. Based on recent laboratory and field research, *Bd* growth and frog immune responses are sensitive to temperature. Optimal growth in culture occurs between 17 and 25 C (Piotrowski et al. 2004), but on an amphibian host growth is faster at 15 C than at 25 C, likely due to enhanced host resistance to infection at the higher temperature (Raffel et al. 2013). This pattern is consistent with field observations, with most amphibian infections and die-offs occurring in cool climes and seasons (Berger et al. 1998; Kriger and Hero 2006). Recently, more-nuanced associations with climate and weather have been hypothesized. Raffel et al. (2013) used laboratory experiments and field data to show that unpredictable temperature variation decreases frog resistance to *Bd*, supporting the hypothesis that *Bd* acclimatizes to temperature shifts quicker than frog hosts.

We also found different prevalences among *Bd*-positive anurans. Ranid frogs (northern red-legged frog and foothill yellow-legged

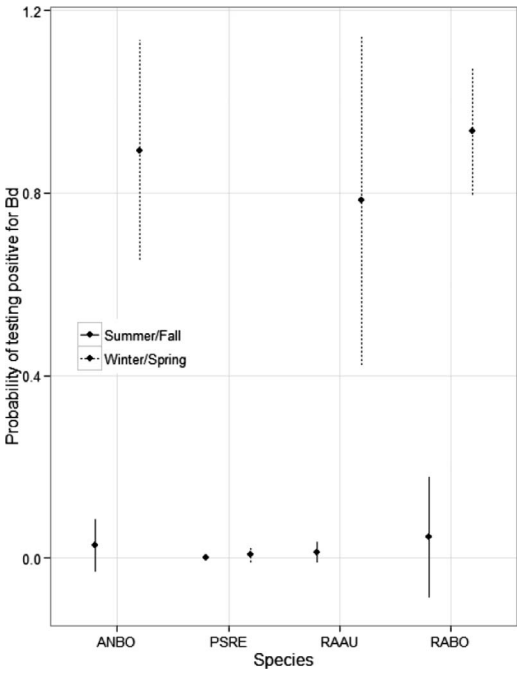

FIGURE 1. Model estimates for the probability of an amphibian being PCR positive for *Batrachochytrium dendrobatidis* (Bd) by species and season. Species include western toad (*Anaxyrus boreas*, ANBO), Pacific chorus frog (*Pseudacris regilla*, PSRE), northern red-legged frog (*Rana aurora*, RAAU), and foothill yellow-legged frog (*Rana boylii*, RABO). Dots represent predicted probability and lines represent ±1 SE. Summer and fall were joined as one season and winter and spring were joined.

frog) had higher prevalences than did the hylid Pacific chorus frog. Pacific chorus frogs were the most-common species captured overall and were very common at the Refuge. Pacific chorus frogs are less susceptible to *Bd* than are other, more-aquatic frogs and can persist at high populations at sites in which other species undergo severe declines; they also tolerate loads of *Bd* well above those lethal to other amphibian species at the same site (Reeder et al. 2012). The two species we found to have the highest prevalence of *Bd* are sister taxa (in the same genus) to the two species in California with documented declines due to *Bd*, Sierra Nevada yellow-legged frogs (*Rana sierrae/R. mucosa*) and Cascades frog (*Rana cascadae*; Vredenburg et al. 2010; Pope et al. 2014). Therefore it seems important to determine if *Bd* is affecting the population dynamics of northern red-legged frogs and foothill yellow-legged frogs in the cool north coast of California. The mean density of *Bd* zoospores per positive sample were significantly lower than the 10,000 zoospore density hypothesized as a threshold for mortality (Vredenburg et al. 2010); however, four frogs carried zoospore densities >5,000.

Skin samples collected by children (≤12 yr) were positive for *Bd* more frequently than were skin samples collected by teens and adults. The children may have taken greater care than older participants and the children were more closely supervised during swabbing by accompanying biologists than were adults. We do not believe that the swabs collected by the children included more false positives than adults because, in other studies, both in the laboratory and field, we have only documented false negatives and have yet to discover a false positive (K.L.P. unpubl. data). Based on these data, the children's techniques were proper and complete, and there is no basis for concern that children might not be able to provide the same quality work as teens and adults.

A notable aspect of this research is that virtually all field work was conducted by young citizen scientists and supervised by their family members. This was a multigenerational (4–73 yr) program that integrated traditionally underserved portions of our community: about 40% of the school district families qualify as being under 130% of US federal poverty guidelines, making them eligible for assistance via school lunch programs. Also, 10 of the 25 (40%) participating students were part of our Latino, Native American, or African-American communities; this is similar to the general figures of 34% of the McKinleyville Union School District families identifying as other than Caucasian. Thus the participants reflected the local community structure and were not a select group of academic or other professional families.

This study supports the perspective that the care, rigor, and quality of design and sampling

techniques used by Ecoclub youth and families were comparable to those of professional scientists. These findings further support the potential value of increased involvement of multigenerational and ethnically diverse youth and families in citizen science projects. Such members of the public can provide an important source of interested and capable workers to conduct field studies with researchers (Citizen Science Association 2016). In addition, many members of the public, including traditionally underserved socioeconomic or cultural groups, sometimes have distrust about science in general—often based on a lack of understanding of science or on misrepresentations of science they might hear or read. Good citizen science programs can address such misunderstandings through direct, positive experiences among members of the general public. Having better information about the natural world can improve understandings among the public about environmental changes and enhance public stewardship. Active citizen science programs have an important potential to contribute to these goals.

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