BATRACHOCHYTRIUM DENDROBATIDIS PREVALENCE IN NORTHERN RED-LEGGED FROGS (*RANA AURORA*)—10 YEARS LATER

By

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ABSTRACT

BATRACHOCHYTRIUM DENDROBATIDIS PREVALENCE IN NORTHERN RED-LEGGED FROG (*RANA AURORA*)—10 YEARS LATER

Michael Cheng Sun

Batrachochytrium dendrobatidis (*Bd*), is an emerging pathogenic fungus of amphibians that has caused species decline and extinction worldwide. A decade ago, Nieto (2004) found that *Bd* existed in larval *Rana aurora* at a low (6.4%) overall prevalence throughout the coast of northern California. The primary goal of this research was to assess the current prevalence of *Bd* in the same populations of *R. aurora*. Both larvae and adults were sampled to better elucidate the dynamics of *Bd* infection in both life stages. All larval and adult *R. aurora* were non-lethally swabbed for qPCR analysis. Additionally, the same larval *R. aurora* were also processed for histological examination. Prevalence of *Bd* was consistently low, with no significant differences between studies or life stages. As in Nieto's (2004) study, oral anomalies remained a significant predictor of *Bd* infection in larval *R. aurora*. For *Bd* identification, molecular and microscopic diagnoses yielded nearly identical results. My results suggest that *Bd* may be endemic in these *R. aurora* populations.

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INTRODUCTION

Chytridiomycosis

Chytridiomycosis, an emerging infectious amphibian disease caused by a chytrid fungus *Batrachochytrium dendrobatidis*, is one of the main factors contributing to the rapid decline and extinction of amphibian species worldwide (Berger et al. 1998, Daszak and Cunningham 2003, Stuart et al. 2004, Skerrat et al. 2007). *Batrachochytrium dendrobatidis* (*Bd* hereafter) was first discovered in 1998 from records of massive amphibian die-offs in Australia and Central America (Berger et al. 1998). A year after the discovery of *Bd*, the pathogenic fungus was described and placed in a novel genus, *Batrachochytrium*, under Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales, and Family *incertae sedis* (Longcore et al. 1999). Since then, chytridiomycosis has been recorded from all continents except Antarctica, where amphibians are absent (Berger et al. 1998, Mutschmann et al. 2000, Hopkins and Channing. 2003, Ouellet et al. 2005, Goldberg et al. 2007, Lips et al. 2006, Fisher et al. 2009, Bai et al. 2010, Swei et al. 2011).

Chytridiomycetes (chytrid) belongs to a basal phylum (Chytridiomycota) in the Kingdom Fungi (Taylor et al. 1992). Some chytrids can be traced back to the 400-million-year-old Lower Devonian Rhynie chert (Taylor et al. 1992). Chytrids are cosmopolitan from the Arctic to the tropics (Sparrow 1960) in wet soils, freshwater bodies, and saline estuaries (Taylor et al. 1992). Chytrids are also found in aerial parts of

plants (Voos and Olive 1968). Chytridiomycota consists of about 127 genera and 1,000 species. Although most chytrids are saprotrophic (degrading cellulose, chitin, and keratin), some are parasitic on plants, protists, invertebrates, and other fungi (Karling 1981). Chytrids have a plethora of life histories and morphologies, yet all consistently produce motile asexual reproductive spores (zoospores), often with a single posterior flagellum (Sparrow 1960). *Batrachochytrium dendrobatidis* in particular belongs to a monotypic genus and is the only chytrid known to infect vertebrates (specifically amphibians).

Batrachochytrium dendrobatidis has two major life stages (Berger et al. 2005a). The first is a water-borne motile zoospore that tracks down, attaches to, and feeds on keratinized tissues of amphibians (e.g., mouthparts of a tadpole, skin of a frog) (Berger et al. 1998, Pessier et al. 1999). Chytrid zoospores can chemotactically recognize and attack amphibian hosts via chemical cues released by the host (Bruning 1991). Following asexual reproduction, the zoospore ultimately develops into the second major life stage: a sessile zoosporangium (Figure 1) that often forms a network and grows in the superficial epidermis (stratum granulosum and stratum corneum) of the adult amphibian host, or in the mouthparts of tadpoles, where keratinized cells are found (Berger et al. 2005a). A mature zoosporangium bears a discharge tube that releases zoospores towards the surface of the skin, which re-infect the host and/or enter the environment (Longcore et al. 1999, Berger et al. 2005a). The life cycle of *Bd in vitro* lasts 4 – 5 days at 22°C (Berger et al. 2005a). Unlike other chytrid fungi, there are no known drought-resistant resting and saprotrophic stages in *Bd* (Longore et al. 1999). *Batrachochytrium dendrobatidis*

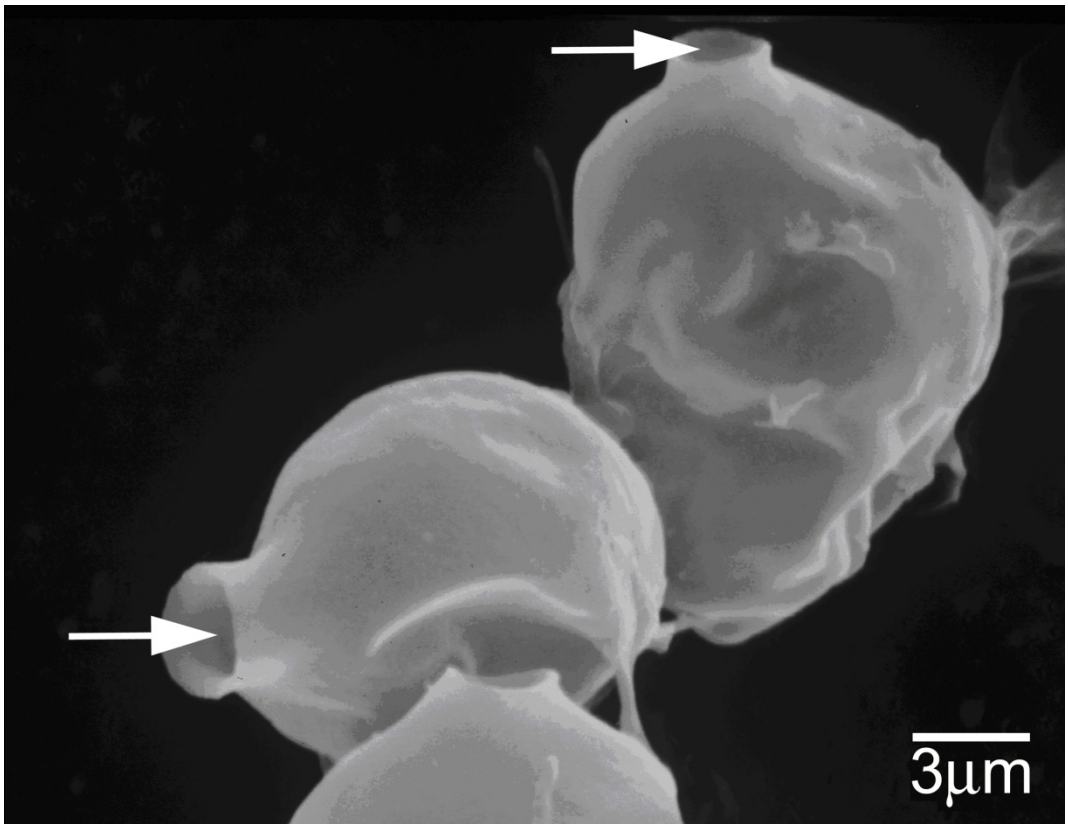

Figure 1. Scanning electron micrograph (SEM) of a network of zoosporangia. The opening (arrows) of the zoosporangium is where a discharge tube releases zoospores towards the epidermal tissue to re-infect the host and/or release into the environment.

zoospores have the ability to remain viable for 3 – 4 weeks in tap water and deionized water (Johnson and Speare 2003), but they are unable to survive desiccation (< 3 hours at room temperature), freezing, or temperatures above 29°C (Johnson et al. 2003, Piotrowski et al. 2004).

Batrachochytrium dendrobatidis only infects the superficial layers of the epidermis and causes no pathological changes to the internal organs (Berger et al. 2005a). The pathological mechanisms have been controversial, but it is generally agreed that chytridiomycosis impedes the physiological functions of the skin, such as respiration, water exchange, and electrolyte balance (Pessier et al. 1999, Berger et al. 2005a, Blaustein et al. 2005, Voyles et al. 2007, Voyles et al. 2012). Specifically, chytridiomycosis can cause hyperkeratosis, a thickening of the skin that prohibits permeability, which leads to amphibian death resulting from cardiac arrest and dehydration (Voyles et al. 2007, Voyles et al. 2012). Clinical signs of chytridiomycosis include lethargy, hyperemia, irregular epidermal sloughing, loss of appetite, and hyperextension of hindlimbs (Kriger et al. 2007, Voyles et al. 2007, Forzán et al. 2008).

Despite being highly pathogenic to many post-metamorphic frogs and other amphibians, *Bd* infection is not necessarily directly pathogenic to larval anurans, but rather impacts them indirectly through other means such as altered foraging behavior with reduced efficiency (Venesky et al. 2009). As a result, *Bd* can increase the length of larval period and reduce body mass at metamorphosis (Parris and Cornelius 2004, Venesky et al. 2010). *Batrachochytrium dendrobatidis* infects the jaw sheaths (upper and lower) and tooth rows of anuran larvae (Berger et al. 1998). Several studies have

suggested that *Bd* infection can cause pigmentation loss or lateral asymmetry of the mouthparts in tadpoles (Berger et al. 1999, Fellers et al. 2001, Knapp and Morgan 2006). Nieto et al. (2007) concluded that mouthpart anomalies (e.g. complete or partial absence of pigmentation in oral disc and/or asymmetry) in larval *Rana aurora* were good indicators of *Bd* infection. However, reports from other anuran species have found that mouthpart depigmentation is not a reliable indication of *Bd* infection (Rachowicz 2002, Padgett-Flohr and Goble 2007). The disagreement is presumably caused by interspecific variation in the manifestation of oral depigmentations due to *Bd* infection (Rachowicz 2002, Blaustein et al. 2005, Padgett-Flohr and Goble 2007).

Batrachochytrium dendrobatidis infection can be transmitted among tadpoles, and from tadpoles to post-metamorphic animals, which implies that the congregation of amphibian tadpoles serves as a disease reservoir, not only for the larvae themselves but also for post-metamorphic frogs (Rachowicz and Vrendenburg 2004). Since no previous data exist comparing the fungal prevalence in adult and larval stages of *Rana aurora*, the dynamics of *Bd* infection transmission between these two life stages remained unknown in this species. Additionally, information on *Bd* infection in post-metamorphic *R. aurora* was exceptionally scarce in California. Only one study has recorded *Bd* infection in a single post-metamorphic individual of *R. aurora* collected from northern California (Adams et al. 2010).

Even though *Bd* infects more than 350 species of amphibians worldwide (Fisher et al. 2009), not all of the infected species develop chytridiomycosis. Certain frog species, such as North American bullfrogs (*Lithobates catesbeianus*) and African clawed

frogs (*Xenopus laevis*), are highly resistant to *Bd* (Weldon et al. 2004, Garner et al. 2006, Kielgast et al. 2010) apparently due to their effective antimicrobial skin peptides (Rollins-Smith et al. 2002, Ramsey et al. 2010). Consequently, *L. catesbeianus* and *Xenopus* spp. may act as vectors of *Bd* (Daszak et al. 2004, Weldon et al. 2004, Garner et al. 2006). Other non-amphibian vectors include waterfowl, snakes, lizards, scientists, and recreationists, who may carry and spread *Bd* by traveling between water bodies (Daszak et al. 2000, Johnson and Speare 2005, Kilburn et al. 2011, Garmyn et al. 2012).

Origin and Spread of *Bd*

Evidence and early records of *Bd* have accumulated worldwide over the last decade, yet the origin and spread of *Bd* remains enigmatic and fascinating. In general, there are two competing hypotheses for the apparent spread of *Bd*. The first is the Novel Pathogen Hypothesis (NPH), which suggests that an emerging disease has recently dispersed to a new geographic area or increased in transmissibility (Berger et al. 1999, Daszak et al. 1999, Alford 2001, Skerratt et al. 2007). The second is the Endemic Pathogen Hypothesis (EPH), which suggests that an emerging disease has always existed in the environment but has recently increased in prevalence or host range because of environmental changes, or had simply escaped human notice previously (Rachowicz et al. 2005, Pounds et al. 2006). Molecular evidence suggests that that NPH is the more probable explanation for the dispersal of *Bd*. However, the two hypotheses are not mutually exclusive. *Bd* can be endemic in one region while novel to another.

Supporters of the NPH argue that *Bd* is not distributed globally (Fisher et al. 2009), but rather in patches or "hotspots" where devastating amphibian declines have taken place due to chytridiomycosis, namely in Australia, and Central and South America (Berger et al. 1998, Lips et al. 2006, Lips et al. 2008). Many amphibian hosts/vectors that have been identified both in the pet trade (Fisher and Garner 2007) and in the wild (Garner et al. 2006, Walker et al. 2008) could have recently introduced this pathogen. Molecular work has also shown few genetic differences in *Bd* strains using genetic markers (Morehouse et al. 2003, James et al. 2009), suggesting that *Bd* is a novel pathogen. In addition, the genomes of 20 *Bd* isolates have been compared from around the world (Australia, South Africa, Europe, Central and North America), and this work identified a hypervirulent recombinant lineage, possibly created by the global amphibian trade, which further increases the support for the NPH (Farrer et al. 2011).

Much evidence points to an origin in Africa. *Batrachochytrium dendrobatidis* is widely distributed in Africa and is common among many *Bd*-resistant African frogs (e.g., *Xenopus* spp.) (Weldon et al. 2004). The earliest known instance of *Bd* infection in Africa dates back to 1933, from a specimen of *X. fraseri* collected in Cameroon (Soto-Azat et al. 2010), while another early *Bd*-infected specimen is a *X. laevis* collected from the Western Cape coastal lowland in 1938 (Weldon et al. 2004). Since *X. laevis* can carry *Bd* asymptomatically, the international trade of wild-caught *X. laevis* may have prompted a global spread of *Bd* in the 1930s (Weldon 2002, Weldon et al. 2004). In importing countries, escaped *X. laevis* have since established feral populations in the United Kingdom, Chile, and the United States (Tinsley and McCoid 1996). Conversely,

in Australia, where serious native anuran declines have been recorded (Berger et al. 1998), imported *X. laevis* were confined to laboratories (Rachowicz et al. 2005). In California, feral populations of *X. laevis* are restricted to southern California (Tinsley and McCoid 1996), but major outbreaks and many recorded instances have occurred much further north (Davidson et al. 2001, Fellers et al. 2001, Rachowicz et al. 2006, Nieto et al. 2007, Pearl et al. 2007). Therefore, other vectors (e.g., *L. catesbeianus*) are likely involved in spreading this fungal pathogen from *X. laevis* (Rachowicz et al. 2005, Garner et al. 2006, Fisher and Garner et al. 2007). As noted above, thirty-five strains of *Bd* obtained from Africa, Australia, and North America were sequenced for genetic diversity (Morehouse et al. 2003). A difference between African strains and the others was almost non-existent (Morehouse et al. 2003). This contradicts the hypothesis of an African origin, because African strains exhibited the same founder effect (low genetic diversity) as other strains found around the world. The global genetic uniformity of *Bd* makes determination of its origin especially problematic.

Batrachochytrium dendrobatidis is widespread in North America, and many cases of *Bd* infection have been recorded from Canada, the United States, and Mexico (Ouellet et al. 2005, Rothermel et al. 2008, Pearl et al. 2009, Cheng et al. 2011). The earliest known North American *Bd*-infected specimens are two *L. catesbeianus* from central California collected in 1961 (Padgett-Flohr and Hopkins 2009). The importation of exotic *X. laevis* may have introduced *Bd* to northern California (Santa Clara County) between the late 1950's and early 1960's (Padgett-Flohr and Hopkins 2009), with subsequent dispersal by *L. catesbeianus* and *Pseudacris regilla* (Padgett-Flohr and

Hopkins 2009). Evidence on when and how *Bd* arrived on the North Coast is lacking, but given that the NPH is correct, it is likely that the spread was facilitated both by anthropogenic means and *Bd*-resistant animal vectors.

In contrast, proponents of the EPH have noted that in some places *Bd* existed for decades before any of the disease outbreaks occurred, namely Japan (Goka et al. 2009), South Africa (Weldon et al. 2004), and Canada (Ouellet et al. 2005), while *Bd* also appeared in pristine and well-protected areas (Berger et al. 1998, Bosch et al. 2001). Those observations support the notion that *Bd* was a widespread fungus present in many trans-continental regions prior to the onset of an epizootic disease. Under this hypothesis, environmental factors such as global warming could have driven the onset of chytridiomycosis (Pounds et al. 2006, Bosch et al. 2007). In the case of California's North Coast, it is possible that *Bd* has always been here, but had never previously been recognized.

Northern Red-legged Frog

Rana aurora (Northern Red-legged Frog) is a coastal species ranging from Mendocino County, California, to southwestern British Columbia, Canada. This medium sized, semi-aquatic frog plays a vital role in its ecosystem. It transfers aquatically-derived resources to the terrestrial environment by serving as an important food source for many birds of prey, snakes, and medium to small carnivorous mammals. In this sexually dimorphic species female adults can reach over 100 mm in snout-vent length

(SVL), while mature male frogs are often less than 70 mm SVL (Nussbaum et al. 1983). Even though males can reach the age of sexual maturity during the first breeding season following metamorphosis, most males first breed at age two (Licht 1971). Females can reach the age of sexual maturity two years after metamorphosis, but the majority of females breed after reaching age three (Licht 1971). *Rana aurora* breeds in wetlands that are less than 1,200 m in elevation (Stebbins 2003), utilizing a variety of breeding waters. For instance, the study sites of this research included ephemeral ponds, permanent freshwater lagoons, and small ponds formed by overflows from large lakes (>0.5 hectares).

The breeding season for *R. aurora* typically occurs between December and March. Males arrive at breeding sites soon after the first rains, as early as November, followed by females (Storm 1960). Egg masses are usually attached to vegetation in shallow and slow-moving water (Storm 1960, Licht 1971), but oviposition can occur in pools that are up to 5 m deep (Calef 1973). Breeding begins when water temperature exceeds 6°C (Storm 1960). Average clutch size is from 500 – 800 eggs per mass and ranges from 200 – 1,100 (Storm 1960, Licht 1971). Eggs hatch after 10 – 30 days and metamorphosis occurs in about 11 – 14 weeks, typically between June – July (Storm 1960, Licht 1971).

Rana aurora lives in a year-round cool climate, which coincides with conditions that are somewhat favorable to *Bd*. The embryos of *R. aurora* can tolerate temperatures between 4°C and 21°C (Licht 1971). The embryo temperature tolerance span overlaps with the range of growing temperature of *Bd*, which grows optimally at temperatures

between 17°C to 25°C (Piotrowski et al. 2004). Nevertheless, *Bd* can still grow at a slower rate between 4°C to 17°C (Piotrowski et al. 2004). Although *R. aurora* is a potential victim of this virulent amphibian disease, there is currently no evidence for the occurrence of chytridiomycosis in this species. According to the IUCN (International Union for Conservation of Nature Red List), the populations of these frogs are assessed to be of "least concern" in the most recent 2008 update (http://www.iucnredlist.org/apps/redlist/details/58553/0). However, *R. aurora* is listed as a Species of Special Concern by the California Department of Fish and Game (www.dfg.ca.gov).

Research Goals

Many studies on chytridiomycosis have focused their efforts in the neotropics and Australia, where major amphibian declines have happened (Berger et al. 1998, Lips 1999, Lips et al. 2006), or on species threatened by the impact of chytridiomycosis (Murray et al. 2009), if not both (Ryan et al. 2008). While a few studies have explored the prevalence and dynamics of *Bd* in anurans occupying the Pacific Northwest (Pearl et al. 2007, Pearl et al. 2009), none have recorded the dynamics of *Bd* infection in an apparently healthy population. Three years after the first documentation of *Bd*, Nathan Nieto, then a Humboldt State University graduate student, found *Bd* present in larval *R. aurora* in and around Redwood National Park, northern California (Nieto 2004, Nieto et al. 2007). Notably, Nieto's research stemmed from the initial discovery of Dr. Gary

Fellers, who found a *Bd*-infected *R. aurora* tadpole in Humboldt County as early as 1999 (Nieto 2004). Nieto detected low overall *Bd* prevalence (6.4%) in larval samples collected in 2001 and 2002. Since the original documentation of *Bd* infection in larval *R. aurora* (Nieto 2004), this species has not shown any apparent signs of population decline in northern California. The time that had passed since Nieto's work thus provide a unique opportunity to examine the time course of *Bd* infection in an apparently healthy population. Therefore, the main goal of this study was to detect any changes in *Bd* prevalence from the same *R. aurora* populations 10 years later.

Besides surveying for *Bd* prevalence, the disease dynamic of this pathogenic fungus within *R. aurora* populations in this particular region of California also remained unknown. A secondary goal of this study was to elucidate *Bd* dynamics between larval and adult life stages of *R. aurora* by sampling both non-lethally for *Bd* infection. Histology was the most efficient and accurate method of *Bd* diagnosis when Nieto conducted his research, but it is time consuming and occurred at the expense of the life of the specimen. Since then, a non-lethal quantitative polymerase chain reaction (qPCR) assay has been developed, and PCR has become the most common diagnostic method for *Bd* (Hyatt et al. 2007). In the current study the combination of histology with non-lethal qPCR makes this research comparable with other studies using similar techniques. To prevent any discrepancies due to varying sensitivity of the different detection techniques (histology vs. qPCR), it was valuable to have histology-based data to compare data between studies. This also provided the opportunity to examine the relative sensitivity of the two diagnostic techniques.

Finally as noted above, Nieto (2004) found mouthpart abnormalities to be a good predictor of *Bd* infection in *R. aurora* larvae, whereas in other species this has not been shown to be the case. Re-sampling these same populations provided the opportunity to confirm this association.

The four main objectives of this study were thus to:

1. Assess the current prevalence of *Bd* infection in larval *R. aurora* from the same study sites in Northern California, and compare this with Nieto's data from 2001 – 2002.
2. Determine if *Bd* prevalence differs between larval and adult *R. aurora*.
3. Assess relative sensitivity of the techniques (histology vs. qPCR).
4. Verify the association of *Bd* infection with mouthpart abnormalities in *R. aurora* tadpoles.

MATERIALS AND METHODS

Study Area

To be as comparable as possible, the same 13 ponds as in Nieto's study (Figure 2; Table 1) were sampled during the summer months of 2011. All study sites were located in and around Redwood National Park, in Humboldt and Del Norte Counties, California. Redwood National Park is located on the coast of northern California, and is under 670 m in elevation (Noss 2000). Annual precipitation is 630 – 3100 mm, most falling from October to April, and experiences a mild climate, with temperature ranging from 4 – 15°C (www.weather.com/weather/wxclimatology/monthly/USCA0928) and averaging 10.2°C (www.noaa.gov) annually.

Common plants that occur at study sites include coastal redwood (*Sequoia sempervirens*), red alder (*Alnus rubra*), willow (*Salix sp.*), several species of native ferns (e.g., *Pteridium aquilinum*, *Polystichum munitum*, etc.), redwood sorrel (*Oxalis oregana*), rush (*Juncus* sp.), sedge (*Scirpus* sp.), cattail (*Typha latifolia*), hydrocotyle (*Hydrocotyle* sp.), and duckweed (Lemnoideae). Besides *R. aurora*, the study sites were also used by many other species of breeding amphibians (e.g., *P. regilla*, *L. catesbeianus*, *Taricha granulosa*, *Ambystoma gracile*, *Anaxyrus boreas*), fishes (e.g., *Gasterostelus aculeatus*, *Rhinichthys osculus*, etc.), and a variety of aquatic invertebrates.

To establish statistical independence, and to avoid cross infection by the same individual frog, all study sites were >2 km apart. This is greater than the maximum

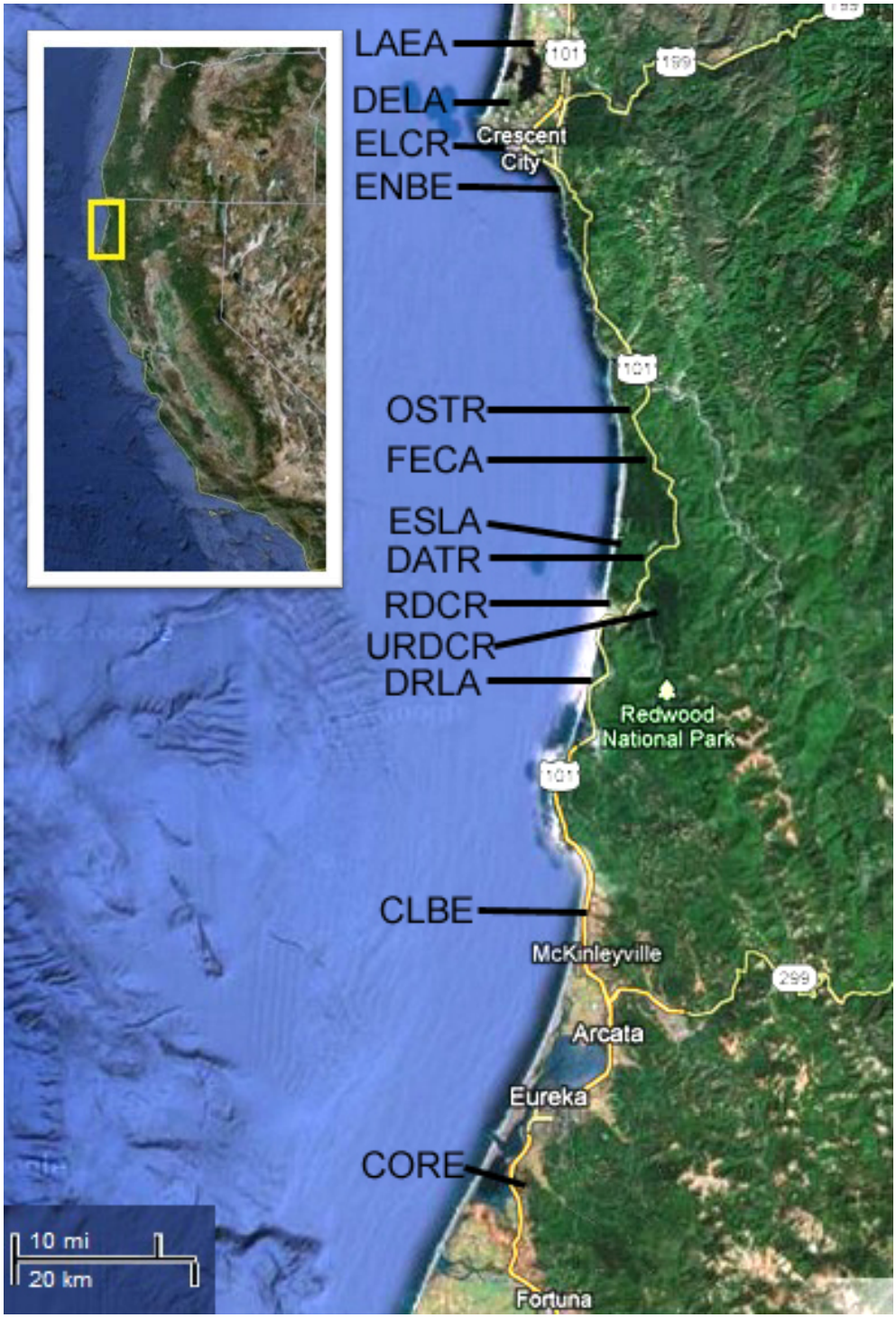

Figure 2. Study sites located on the coast of northern California (https://maps.google.com).

Table 1. Study sites within and around Redwood National and State Parks, California.

Site Number	Identity Code	Site Name	County	Latitude / Longitude
1	LAEA	Lake Earl	Del Norte	41° 48' 17" N / 124° 12' 17" W
2	DELA	Dead Lake	Del Norte	41° 47' 18" N / 124° 13' 46" W
3	ELCR	Elk Creek	Del Norte	41° 45' 21" N / 124° 11' 18" W
4	ENBE	Endert's Beach	Del Norte	41° 43' 33" N / 124° 09' 03" W
5	OSTR	Ossagon Trail	Del Norte	41° 26' 26" N / 124° 03' 43" W
6	FECA	Fern Canyon	Humboldt	41° 24' 13" N / 124° 03' 55" W
7	ESLA	Espa Lagoon	Humboldt	41° 21' 29" N / 124° 04' 12" W
8	DATR	Darlington Trail	Humboldt	41° 19' 12" N / 124° 02' 29" W
9	RDCR	Redwood Creek	Humboldt	41° 17' 19" N / 124° 05' 26" W
10	URDCR	Upper Redwood Creek	Humboldt	41° 16' 50" N / 124° 01' 51" W
11	DRLA	Dry Lagoon	Humboldt	41° 13' 26" N / 124° 06' 13" W
12	CLBE	Clam Beach	Humboldt	40° 59' 37" N / 124° 06' 41" W
13	CORE	College of the Redwoods	Humboldt	40° 41' 46" N / 124° 14' 42" W

distance an adult *R. aurora* can travel based on radio telemetry data (Fellers and Freel 1995, Haggard 2000).

The three northernmost study sites were located in Crescent City, California. The Lake Earl site (LAEA) is a marshy pond located near the southern border of the lake, dominated by cattails, and possibly formed from rain overflow from the lake. The Dead Lake site (DELA) is an ephemeral pond, formed from overflow of Dead Lake, situated immediately adjacent to the lake and close to the dirt road. Both Lake Earl and Dead Lake are large lakes (> 0.5 hectare). The Elk Creek (ELCR) site is a large permanent pond in the Elk Creek Wildlife Area into which the slow-flowing Elk Creek flows.

Eight sites were located within Redwood National and State Parks. The northernmost site within Redwood National Park was Endert's Beach (ENBE). This lotic pond is located right next to a beach highly used by local residents. The west end of the pond is dammed by large drift wood with pond water draining off to the Pacific Ocean.

The Ossagon Trail (OSTR) pond is situated on the beach. It is formed by the outflow of Ossagon Creek, and is surrounded by sedge and red alder. The Fern Canyon (FECA) site is located two miles south of the Ossagon Creek. This pond, dominated by rushes, receives water from Fern Creek and flows directly into the ocean. Espa Lagoon (ESLA) is a deep and small permanent lagoon. The littoral zone of this freshwater lagoon is encircled by cattails. Two other sites were located on Redwood Creek, 1) Upper Redwood Creek (URDCR) pond sits on the upstream end of the creek and is adjacent to the Tall Trees Grove Trail, and 2) Redwood Creek (RDCR) pond is located just upstream from the mouth of the Redwood Creek. The water's edge of this site is

dominated by cattails. The southernmost site within Redwood National and State Parks boundaries was Dry Lagoon (DRLA). This shallow but extensive freshwater lagoon is quite close to a road and parking lot. Vegetation consists mostly of rushes, sedges, and cattails in and around the lagoon.

Only two study sites were located south of Redwood National Park. The Clam Beach (CLBE) pond is located right next to the east side of Highway 101. This pond drains directly to the ocean under the highway. Cattails, sedges, and red alders surround this turbid pond. The southernmost site in this study is located behind the police academy of College of the Redwoods Eureka campus (CORE). This is an artificial pond surrounded by cattails and willows. Hydrocotyle and duckweed often floated on top of the shallow water.

Field Methods

Animal handling was approved by the Humboldt State University's Institutional Animal Care and Use Committee (IACUC Protocol 10/11.B./68-A), animals were collected under the California Department of Fish and Game Collecting Permit (Permit # SC-11461), California Department of Parks and Recreation Collecting Permit (Permit # 11-635-027), and a Redwood National and State Parks Scientific Research and Collecting Permit (Permit # REDW-2011-SCI-0017).

The same general sampling design and protocol were followed as described in Nieto (2004). At each pond, at least 300 *R. aurora* tadpoles were collected. Visual

encounter surveys were used along the littoral zone and tadpoles were removed with a dip net. The tadpoles were anesthetized using a 0.5% solution of tricaine methanesulfonate (MS-222) and examined under a dissecting microscope for mouthpart abnormalities. Only tadpoles between Gosner stages 33 and 42 (Gosner 1960) were examined because tadpole mouthparts become fully keratinized at stage 33, but tadpoles start to lose their larval mouthparts due to metamorphosis past stage 42 (Nussbaum et al. 1983). Abnormality was defined by loss of pigments in the upper or lower jaw sheath, tooth rows, or any sign of asymmetry (Fellers et al. 2001).

At each site, five randomly chosen normal-appearing tadpoles plus all of the abnormal tadpoles were collected and euthanized by an overdose of 0.5% MS-222 for laboratory analyses, while the uncollected individuals were returned to the pond. A total of 16 abnormal tadpoles plus 65 random normal-appearing tadpoles (five tadpoles from each of the 13 sites) were collected for further laboratory examination. The oral disc of each collected tadpole was swabbed using a sterile synthetic-tipped swab (Fisherbrand, Hampton, NH) for molecular analysis. Each tadpole was held ventral side up firmly in one hand, while the other spun the swab tip on the oral disc for 25 complete rotations. Each swab was processed by the same protocol as for frogs (see below). A total of 3900 tadpoles from the 13 sites were examined during the summer months (late-May to late-June) of 2011.

To compare differences in *Bd* prevalence between adult and larval *R. aurora*, up to 10 frogs per study site were sampled for *Bd* infection. At each study site, the first frogs that were visually encountered were captured, swabbed, and released. To swab,

each frog was restrained in one hand, while the other firmly ran a swab five times over each of the ventral surface, inner thigh area, and plantar and palmar foot webbing (Kriger et al. 2006). The tip of each swab was then air dried for three to five minutes, broken off, and placed in a 1.5 mL Eppendorf tube with the tip side facing down. All swabs were kept near 0°C in the field and stored at - 20°C in the lab, before transported for qPCR analysis.

To prevent disease transportation between sites, all field equipments were sterilized using concentrated bleach (≥ 10%) (Speare et al. 2004). To avoid sample contamination, disposable gloves were changed and dip nets were decontaminated between captures. All used dip nets were thoroughly soaked and stirred in a bleach solution for at least 5 minutes and allowed to air dry before next use. Due to concerns regarding the contamination of New Zealand mud snails, coolection adhered to the Aquatic Invasive Species Decontamination Policy issued by the State of California, which included placing the field gear in a large plastic bag and freezing at <0°C for a minimum of six hours (Aquatic Invasive Species Decontamination Policy, 2009). Surface water temperature of each pond was measured using a mercury thermometer rounding to the nearest degree Celsius, and pH was measured by pH indicator strips (Fisher Scientific, Hampton, NH).

Laboratory Methods

Quantitative PCR Assay

Nucleic acids of *Bd* were extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), with the following modifications to the manufacturer's standard protocol to increase DNA yield: 1) DNA samples (swabs) were incubated at 58°C (± 1°C) for 24 hours, 2) during the final elution, four volumes of 50 µl distilled nuclease-free water were used instead of 200 µl Buffer AE, 3) after each aliquot of 50 µl distilled nuclease-free water, each DNA sample was incubated at 20°C for 10 minutes. The extracted DNA samples were immediately stored at -20°C until qPCR analysis. A total of 123 DNA extractions averaged 3.2 ng/µl, ranging from 0.5 – 24.8 ng/µl, as quantified by using Thermo Scientific's NanoDrop 1000 (Waltham, MA).

To determine *Bd* prevalence, the qPCR detection assays outlined in Boyle et al. (2004) were generally followed. Hybridization probe based PCR assays were run on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The target region of amplification is an internal transcribed spacer (ITS) region of the ribosomal DNA, which is now widely used in systematics, phylogenetic analyses, and environmental sampling of fungi (O'Brien et al 2005). The ITS region includes well-conserved priming sites that flank highly variable regions, making it a desirable genetic marker (Peay et al. 2008). The forward primer 5'-CCTTGATATAATACAGTGTGCCATATGTC-3', reverse primer 5'-AGCCAAGAGATCCGTTGTCAAA-3', and the FAM labeled probe 5'-6FAM CGAGTCGAACAAAAT TAMRA-3' were used to amplify a fragment of the target

region that is 176 base pairs long. Each qPCR reaction contained 10 µl of 2X SsoFast Probe Supermix (Bio-Rad, Hercules, CA), 1.2 µl of each 5 µM primer, 1 µl of 5 µM probe, 3 µl of distilled water, and 3.6 µl of template DNA, totaling 20.0 µl of a reaction volume. The qPCR followed a two step amplification protocol: 2 min hold at 50°C, 10 min hold at 95°C, then followed by 15 sec denaturation at 95°C, 30 sec annealing at 60°C, and 30 sec extension at 60°C for 50 cycles. Three negative controls and at least one positive control were included in each run. The negative control reactions contained 3.6 µl of distilled nuclease-free water instead of the template DNA. Positive control reactions contained 3.6 µl of *Bd* DNA (8 ng/µl) extracted from a *Bd* culture (JEL 720) kindly supplied by Dr. Joyce Longcore (University of Maine).

Histological Examination

After swabbing, tadpoles were fixed for more than 24 hours in 10% neutral buffered formalin. The anterior head (including oral disc) of tadpoles was then dissected and prepared following standard histological techniques (Humason 1979, Berger et al. 2000). Tissues were sectioned at 8 µm in paraffin, stained with hematoxylin and eosin (H & E), and examined under 100X, 400X, and1000X magnification for *Bd* zoospores and/or sporangia.

Statistical Analysis

Logistic regression (R statistical software, version 2.15.1, Lucent Technologies, NJ) was used to: 1) test for differences in *Bd* prevalence in *R. aurora* tadpole between 2011 (present study) and 2001 and 2002 (Nieto 2004), 2) test for the association of *Bd* infection with mouthparts abnormalities in *R. aurora* tadpoles using histological data, 3) test for differences in sensitivity of the two detection methods (histology vs. qPCR) using tadpole data. Additionally, a 95% confidence interval around the detection probability was constructed for each detection method. Significance was assessed at the $p < 0.05$ level.

To compare prevalence difference between larval and adult *R. aurora* life stages, qPCR data were used. The estimated *Bd* prevalence in adult frogs was first calculated: $\hat{p}_{\text{Frogs}} = \frac{\text{Infected Frogs}}{\text{Total Frogs}}$ and a 95% confidence interval around $\hat{p}_{\text{Frogs}}$ was constructed. Then the estimated *Bd* prevalence of tadpoles was calculated using the following formula:

$$\hat{p}_{\text{Tadpoles}} = Pr\{Abnormal\}\,Pr\{Infected|Abnormal\} + Pr\{Normal\}\,Pr\,\{Infected|Normal\}.$$

Finally, $\hat{p}_{\text{Tadpoles}}$ was directly compared with the 95% confidence interval of $\hat{p}_{\text{Frogs}}$ for statistical significance.

RESULTS

Larval *R. aurora* occurred in ponds with a pH ranging from 6.2 – 7.2 (mean = 6.6), and surface water temperatures between 13 – 18°C (mean = 15.4°C) during data collection (Table 2). No morbidity or mortality of *R. aurora* was observed during the sampling events. Of the examined 3900 tadpoles, 16 (0.41%) individuals exhibited oral disc abnormality, mostly of mouthpart depigmentation to varying degrees.

Of the 81 larval *R. aurora* processed for histological diagnosis, nine (56.3%) of the 16 abnormal tadpoles and two (3.1%) of the 65 normal-appearing tadpoles were positive for *Bd* infection (Table 3.1). Forty-two frogs and the same 81 tadpoles were swabbed for qPCR analysis, eight (50%) of the 16 abnormal tadpoles, three (4.6%) of the 65 normal tadpoles (Table 3.2), and six (14.3%) of the 42 frogs tested positive for *Bd* infection (Table 3.3).

There was no difference in *Bd* prevalence in larval *R. aurora* between Nieto's (2004) and the present study (X_1^2= 0.78896, p = 0.374) (Figure 3). *Batrachochytrium dendrobatidis* occurred in six (46.2%) of the 13 sites in 2011, and similarly, 2001 and 2002 each had five (38.5%) of the 13 sites with *Bd* infection. However, *Bd* was repeatedly identified in only two sites (CORE, ESPA) between 2001 and 2002 (Nieto 2004). Overall, *Bd* occurred in 11 of the 13 sites over both studies (Table 4).

The sensitivity of the two detection techniques was identical (X_1^2= 0, p = 1). Both detection techniques identified 11 infected specimens out of 12, although with slightly different results. Nine (56.3%) of the 16 abnormal tadpoles were positively identified

Table 2. Habitat data from sampling events at 13 sites across Humboldt and Del Norte Counties, northern California.

Site	Habitat (Lentic/Lotic)	pH	Water Temperature (°C)
CORE	Lentic	6.4	16
CLBE	Lentic	6.0	14
DATR	Lotic	6.8	15
DELA	Lentic	7.0	18
DRLA	Lentic	6.6	18
ENBE	Lotic	6.4	14
ESLA	Lentic	6.6	13
FECA	Lotic	6.4	17
LAEA	Lentic	7.2	16
OSTR	Lotc	6.4	14
RDCR	Lotic	6.8	16
URDCR	Lentic	6.8	16
ELCR	Lotic	6.2	13
Mean		6.6	15.4
SD		0.33	1.7

Table 3.1 Prevalence of *Bd* based on histological examination of *R. aurora* tadpoles.

Site (Lentic)	Infected/Random Normal	Infected/Abnormal	Estimated Prevalence/Sample (%)
CORE	1/5	2/3	20.1
DELA	0/5	2/2	0.7
DRLA	0/5	0/4	0
ESLA	0/5	0	0
LAEA	0/5	2/2	0.7
URDCR	0/5	0	0
Mean Prevalence	1/30 (3.3%)	6/11 (54.5%)	3.60% SE = 3.31%
Site (Lotic)	**Infected/Random Normal**	**Infected/Abnormal**	**Estimated Prevalence/Sample (%)**
CLBE	0/5	0	0
DATR	1/5	0	20
ENBE	0/5	1/1	0.3
FECA	0/5	2/2	0.7
OSTR	0/5	0/1	0
RDCR	0/5	0	0
ELCR	0/5	0/1	0
Mean Prevalence	1/35 (2.9%)	3/5 (60%)	3.0% SE = 2.84%
Total Individuals	2/65 (3.1%)	9/16 (56.3%)	3.30%

Table 3.2 Prevalence of *Bd* based on qPCR analysis of *R. aurora* tadpoles.

Site (Lentic)	Infected/Random Normal	Infected/Abnormal	Estimated Prevalence/Sample (%)
CORE	1/5	1/3	20.1
DELA	1/5	2/2	20.5
DRLA	0/5	0/4	0
ESLA	0/5	0	0
LAEA	0/5	2/2	0.7
URDCR	0/5	0/1	0
Mean Prevalence	2/30 (6.7%)	5/11 (45.5%)	6.90% SE = 4.24%

Site (Lotic)	Infected/Random Normal	Infected/Abnormal	Estimated Prevalence/Sample (%)
CLBE	0/5	0	0
DATR	1/5	0	20
ENBE	0/5	1/1	0.3
FECA	0/5	2/2	0.7
OSTR	0/5	0/1	0
RDCR	0/5	0	0
ELCR	0/5	0/1	0
Mean Prevalence	1/35 (2.9%)	3/5 (60%)	3.0% SE = 2.84%
Total Individuals	3/65 (4.6%)	8/16 (50%)	4.80%

Table 3.3 Prevalence of *Bd* based on qPCR analysis of adult frogs.

Site	Infected/Frog
CLBE	0
CORE	4/10
DATR	0/3
DELA	0/2
DRLA	0/1
ELCR	0
ENBE	0/3
ESLA	1/2
FECA	1/6
LAEA	0/2
OSTR	0/1
RDCR	0/10
URDCR	0/2
Mean Prevalence	6/42 (14.3%) SE = 5.5%

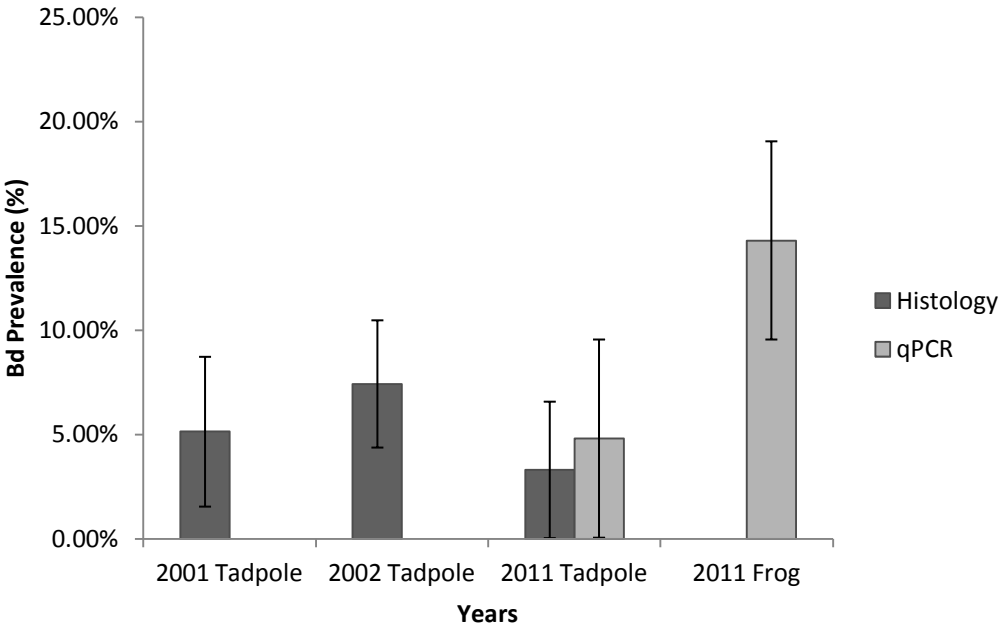

Figure 3. *Bd* prevalence across years, between life stages, and by different detection methods. *Bd* prevalence has been at a low level persistence across years. There is no statistically significant difference in *Bd* prevalence among larvae across years, between the two *R. aurora* life stages in 2011, or by different detection methods. Error bars represent the standard errors.

Table 4. Prevalence of *Bd* across sampling years at 13 sites across Humboldt and Del Norte Counties, northern California. Despite low prevalence, *Bd* was found in 11 out of the 13 sites, indicating a ubiquity of *Bd* on the coast of northern California. Presence/Absence (+/-) of *Bd* is based on histological data in *R. aurora* tadpoles.

Site	2001 & 2002	2011	2001, 2002, 2011
ENBR	-	+	+
OSTR	-	-	-
FECA	-	+	+
LAEA	-	+	+
ELCR	-	-	-
DELA	+	+	+
DRLA	+	-	+
DATR	+	+	+
RDCR	+	-	+
CLBE	+	-	+
CORE	+	+	+
ESLA	+	-	+
URDCR	+	-	+
Total Sites	8	6	11

with *Bd* via histological examination, while qPCR data detected 8 (50%) of the same 16 abnormal tadpoles (Table 5). Of the 65 normal-appearing tadpoles, two (3.1%) were identified with *Bd* through histological examination while qPCR detected three (4.6%) *Bd*-positive individuals. Both methods have an estimated detection probability of 0.92 (11/12) (95% C.I. = 0.58 – 0.99). For both histological and qPCR analyses oral abnormality is a significant predictor of *Bd* infection (X_1^2=23.670, p = 1.144x10^{-6}).

Data from qPCR were used to determine if infection prevalence differed between larval and adult *R. aurora*. The estimated *Bd* prevalence in adult frogs was 0.14, with a standard error of $SE_{\hat{p}} = 0.06$. For binomial distributions a normal approximation holds if the number of successes and number of failures are both greater than or equal to five, which was true for my data. Hence, the 95% confidence interval for adult prevalence = $\hat{p} \pm 1.96\ SE_{\hat{p}} = [0.037, 0.25]$. The estimated *Bd* prevalence in tadpoles was 0.048, within the 95% confidence interval of adult frog prevalence. Thus there is no evidence that *Bd* prevalence differed between life stages.

The relative *Bd* load (Figure 4) was indicated by a direct comparison of quantification cycles (C_q) in each infected sample from the qPCR assay. A C_q value is the cycle number at which fluorescence from the probe has increased above the threshold. The smaller the C_q value, the sooner the reaction amplified, and therefore heavier the infection. Adult frogs had a higher disease load than tadpoles. Within tadpoles, abnormal tadpoles tend to have a higher disease load than normal-appearing tadpoles.

Table 5. Comparison of the detectability of *Bd* using histology and qPCR. Histology picked up an infected tadpole with oral disc abnormality that qPCR failed to detect, while qPCR positively identified a normal tadpole that escaped histological detection.

		Bd Presence/Absence (+/-)	
Site	Specimen	Histology	qPCR
CORE	N5	+	+
CORE	A1	+	+
CORE	A3	+	-
DATR	N5	+	+
FECA	A1	+	+
FECA	A2	+	+
ENBE	A1	+	+
DELA	N5	-	+
DELA	A1	+	+
DELA	A2	+	+
LAEA	A1	+	+
LAEA	A2	+	+

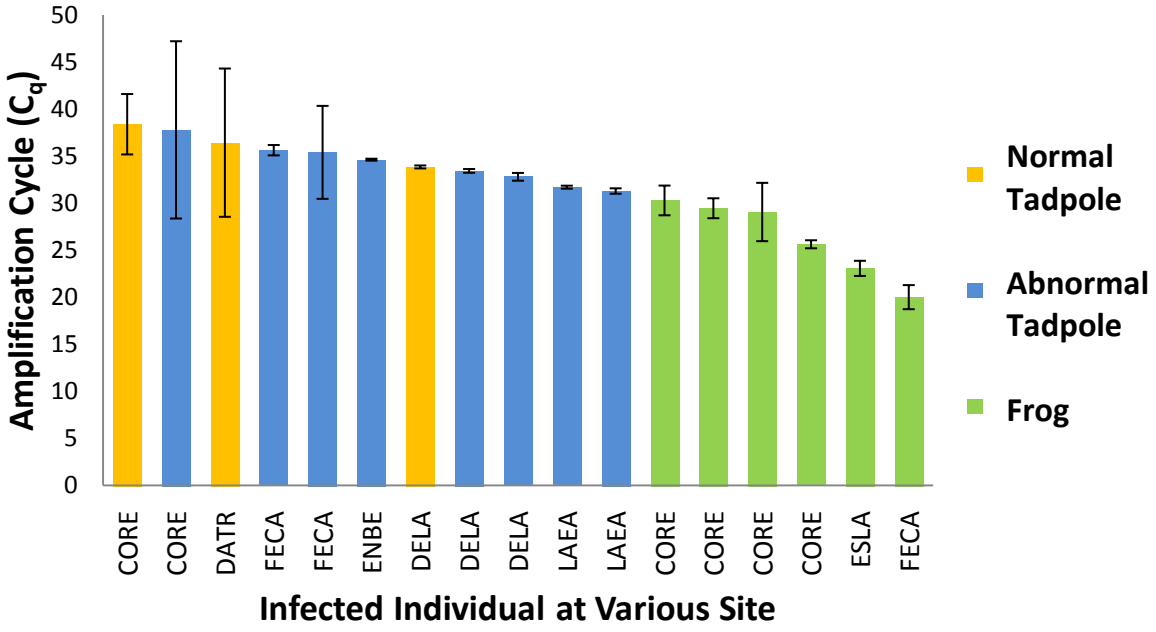

Figure 4. Relative *Bd* load of infected individuals in qPCR assays. Adult *R. aurora* tend to have a higher *Bd* load (lower C_q) than tadpoles. Abnormal tadpoles tend to be more heavily infected by *Bd* than normal tadpoles. Error bars represent the standard errors for replicate runs.

DISCUSSION

Bd Prevalence in Tadpoles

The overall prevalence of *Bd* infection in larval *R. aurora* has not changed since 2001 – 2002. A decade ago, sites with *Bd* infection had large numbers of *R. aurora* larvae, which suggests that the *R. aurora* populations were stable at that time (Nieto 2004). A decade later, there are still large numbers of larvae in areas where *Bd* occurs, suggesting that these *R. aurora* populations have remained stable with *Bd* infection. *Batrachochytrium dendrobatidis* appears to be endemic in local populations of *R. aurora* in coastal northern California.

Other than Nieto et al. (2007), no other published field studies have recorded *Bd* infection in larval *R. aurora*. In Oregon, Adams et al. (2010) found no infected tadpoles in a sample of 207 larval *R. aurora* from 15 different sites. In British Columbia, Canada, Adams et al. (2007) did not find any infected *R. aurora* tadpoles out of 20 sampled. *Bd* appears to affect larval *R. aurora* minimally throughout the sampled regions. This generally low *Bd* prevalence in larval *R. aurora* suggests they are highly unlikely to serve as an effective disease reservoir in the Pacific Northwest.

Nieto (2004, Nieto et al. 2007) and the current study recorded a low, consistent, and ubiquitous *Bd* prevalence in *R. aurora* populations throughout the coastal northern California (Table 4). Together, our findings support the general consensus that *Bd* is

widespread in the Pacific Northwest (Adams et al. 2007, Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010). *Bd* infection has been present in populations of North American amphibians at least since the 1960's (Ouellet et al. 2005). Nonetheless, without an extensive retrospective museum specimen sampling of *Bd* and DNA sequencing of the local *Bd* strain(s), the results from this research cannot distinguish between the NPH and EPH, because when and how *Bd* arrived on the North Coast remains unknown.

On the other hand, the low level persistence and ubiquity of *Bd* in the area may be explained by vector and reservoir species for *Bd* (Garcia et al. 2006, Garner et al. 2006, Reeder et al. 2012) that are sympatric with *R. aurora*. For example, *P. regilla* and *L. catesbeianus* can often carry *Bd* asymptomatically and without succumbing to chytridiomycosis (Garner et al. 2006, Reeder et al. 2012). Moreover, *L. catesbeianus* had demonstrated a high chance of carrying *Bd* in both its native and introduced ranges (Ouellet et al. 2005, Garner et al. 2006, Pearl et al. 2007). These two common frog species may have helped in facilitating the spread of *Bd* throughout the study sites reported here and beyond. Nonetheless, the impact of this consistent, low *Bd* infection in local *R. aurora* populations remains unclear.

Bd Prevalence Across the Range of Post-metamorphic *Rana aurora*

Most adult *R. aurora* leave the breeding waters soon after the breeding period (Licht 1971). During the non-breeding seasons, *R. aurora* may frequent wet forested

areas that are 200 to 300 m away from water (Nussbaum et al. 1983), which explains the general difficulties of capturing adult frogs during the current data collection. Nevertheless, a large enough sample size (n = 42) of adult *R. aurora* was collected for statistical analysis.

Infection of *Bd* is regularly detected in a number of lentic-breeding frogs that are sympatric with *R. aurora* in the Pacific Northwest (Pearl et al. 2007). Surprisingly, populations of *R. aurora* are generally less likely to be infected by *Bd* than other sympatric anurans (Adams et al. 2007, Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010). For example, *Bd* was frequently detected in *R. pretiosa* (57.1%) and *L. catesbeianus* (31.2%) from Oregon (Pearl et al. 2007). Yet *Bd* was much less commonly found in post-metamorphic *R. aurora* from the same general region: two (20.0%) infected frogs in 10 sampled from British Columbia, Canada (Adams et al. 2007); five (4.0%) infected frogs in 125 sampled from Oregon (Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010, D. Green, unpublished data [www.bd-maps.net]); one (100%) infected frog in one sampled from northern California (Adams et al. 2010). These previous studies showed post-metamorphic *R. aurora* exhibited an overall *Bd* prevalence of 5.2% (7/135) in the Pacific Northwest (Adams et al. 2007, Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010, D. Green, unpublished data). This value is considerably lower than the 14.3% (6/42) overall *Bd* prevalence observed in northern California in the present study. Interestingly, the one infected frog recorded by Adams et al. (2010) was the only specimen they collected within California, and it also happened to come from the southernmost study site of the present study (CORE; Figure 3), where a 40.0% (4/10)

prevalence in adult *R. aurora* was detected. Four of the six total infected frogs came from CORE, which may have inflated the estimate for overall *Bd* prevalence in frogs. Both adult and larval *R. aurora* appeared in high abundance during field surveys at CORE. However, this suggests that the local *R. aurora* population occurring at CORE may be at a greater risk than the populations occurring further north, although a detrimental effect of *Bd* to any population of *R. aurora* has not yet been observed. Similarly, *Bd* infection of *R. draytonii* was found in 65.5% of the sites (Marin County, CA) where *R. draytonii* occurred, yet no *Bd*-related declines had been observed since 1993 (>1000 site visits) (Fellers et al. 2011). Nevertheless, there is an energetic cost associated with *Bd* infection in amphibians (Padgett-Flohr 2008). Excessive skin sloughing was observed in *R. draytonii* and other amphibians infected by *Bd* (Voyles et al. 2007, Padgett-Flohr 2008). This may create an energy allocation in the infected animal that leads to a depletion of fat reserved for reproduction (Padgett-Flohr 2008). Consequently, limited breeding efforts could result a decrease in recruitment rate (Padgett-Flohr 2008).

Finally, my frog sampling results have connected the *R. aurora* sampling range from British Columbia, Canada (Adams et al. 2007), Oregon (Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010), and now throughout the coastal freshwater bodies of northern California, where Adams et al. (2010) had previously sampled a single specimen.

Host-Pathogen Dynamics

Although it possible that intervening years saw notable increases or decreases in prevalence, it is most reasonable to assume that *Bd* prevalence in North Coast *R. aurora* populations has stayed at a low level since 2001. This assumption, combined with no differences in *Bd* prevalence between life stages, and apparently stable *R. aurora* populations over the past decade, suggests two alternative hypotheses for the history of *Bd* in these populations.

The first hypothesis is that *Bd* was never lethal to *R. aurora*, due to a combination of low ambient temperature, life history traits, and the effective antimicrobial skin peptides found in *R. aurora*, which synergistically suppressed potential chytridiomycosis outbreaks from happening. For instance, the cool ambient temperature in the study areas may have contributed to the low prevalence and pathogenicity of *Bd*. *Batrachochytrium dendrobatidis* thrives between 17°C and 25°C and growth stops below 4°C (Piotrowski et al. 2004). Redwood National Park and its immediate areas consistently experience an average annual temperature of 10.2°C (www.noaa.gov) that generally ranges between 4°C and 15°C (www.weather.com/weather/wxclimatology/monthly/USCA0928). These temperatures are below the optimal ranges for *Bd*. In the neotropics and Australia, where high *Bd* prevalence and major chytridiomycosis outbreaks have been recorded, there are generally no long periods of cool climate that may stunt *Bd* growth (Berger et al. 2004, Pounds et al. 2006, Kriger and Hero 2007). Furthermore, the more terrestrial habits and a relatively short larval period (typically 3 – 4 months) of *R. aurora* may have also

decreased their exposure to *Bd*, compared with those highly aquatic species (e.g., *R. pretiosa*) or those with longer larval periods (e.g., *L. catesbeianus*) (Pearl et al. 2007). Lastly, the antimicrobial skin peptides found in *R. aurora* can effectively reduce *Bd* infection, which may also help to prevent the development of chytridiomycosis (Conlon 2011).

The second hypothesis is that chytridiomycosis swept through these frog populations upon *Bd*'s initial arrival, and *R. aurora* has since evolved a level of resistance that allows it to co-exist with *Bd* (Longcore et al. 2007, Voordrouw et al. 2010). A mixture of host evolution, extrinsic factors, and the innate defense of *R. aurora* could have allowed both *Bd* and *R. aurora* to move towards a low level, sublethal equilibrium in their host-pathogen interactions. This equilibrium is what we would predict in an endemic pathogen situation. Briggs et al. (2010) developed a model to describe the pattern of *Bd* invasion into a naïve frog population, using data from *R. sierrae*, which may help to explain the current phase of enzootic dynamics of *Bd* here locally: 1) *Bd* arrives at a naïve population and infection load and prevalence in susceptible individuals increase rapidly, especially in areas of high frog density, small non-flowing water bodies, temperature favoring *Bd* growth, etc., 2) once *Bd* load reaches a certain threshold, population extirpation occurs, 3) the surviving amphibian populations after the first epidemic then persist with low infection intensity in a new endemic state. Based on this model, these local *R. aurora* populations may now persist with a stable and endemic *Bd* infection since as early as the initial observation of *Bd* in 2001.

Since the initial arrival of *Bd* to the North Coast, it is unlikely that this highly infectious agent has remained completely non-lethal to *R. aurora* populations for a prolonged period of time. For at least during the past decade, *Bd* likely has experienced some favorable growth conditions. For instance, during breeding seasons of *R. aurora*, increased *Bd* transmission can occur among breeding frogs through physical contacts in water during amplexus. The uncharacteristic warm weather spurts may have also occurred on the North Coast for a temporary increase in *Bd* virulence and optimum growth. Therefore, selection of higher *Bd*-resistant *R. aurora* populations seems more likely to have happened. Regardless of the past history, however, this research does not find evidence that is currently threatening these local *R. aurora* populations.

Mouthpart Abnormality in Larval *R. aurora* as an Indicator of *Bd* Infection

Oral disc abnormalities in anuran larvae are not only caused by *Bd* infections. Mine tailings, dichlorodiphenyl trichloroethane (DDT) and glucocorticosteroid exposure can also cause abnormalities in the tooth rows, upper jaw sheath, and anterior maxilla in anuran tadpoles (Hayes et al. 1997). There are no known mine tailings near to the study sites on the coast of northern California. No snout (mandibular) fenestrations or a complete loss of the upper jaw sheath in tadpoles that would be associated with DDT or corticosterone exposure (Hayes et al. 1997) were observed. A long-term exposure to low temperatures by overwintering in frozen ponds can also induce oral depigmentation in *R. sierrae* larvae (Rachowics 2002). However, no overwintering behavior has ever been

documented in larval *R. aurora*. Consequently, the mouthpart abnormalities were likely caused by either the infection of *Bd* or by other previously unidentified process(es). Mouthpart anomalies are generally not a reliable indicator of *Bd* infection in most tested species, for reasons unknown (Blaustein et al. 2005, Padgett-Flohr and Goble 2007). Nevertheless, no other studies but Nieto et al. (2007) had previously tested larval *R. aurora* to see if oral anomalies correlate with *Bd* infection.

Both Nieto et al. (2007) and the current study found mouthpart abnormalities to be a significant predictor of *Bd* infection in *R. aurora*. Therefore, the morphology of abnormal mouthparts can be useful when infection is common in *R. aurora* tadpoles. With little training and a hand lens (10X), anyone can diagnose potential *Bd* infection in the field. This may be an ideal diagnostic technique in *R. aurora* populations where *Bd* prevalence is low, when screening is done specifically for those orally abnormal larvae. On the other hand, rarity of tadpoles with oral anomalies would make it likely that *Bd* prevalence is low in that population. Between the two studies a combined total of 10,730 *R. aurora* tadpoles were examined and only 54 (0.5%) of those individuals had mouthpart anomalies. Of these 54 abnormal tadpoles, 20 (37.0%) were infected with *Bd*, which was significantly higher than the 5.0% (9/180) overall *Bd* prevalence exhibited by the normal-appearing larvae.

As expected, larval *R. aurora* with mouthpart anomalies generally have a higher disease load than normal-appearing individuals (Figure 4). The presence of oral depigmentation should indicate a higher disease load relative to normal-appearing

mouthparts. Pigment loss is likely a manifestation of the darkly pigmented keratin cells being destroyed by colonies of *Bd* zoospores and zoosporangia (Figure 5). On the other hand, a cryptic infection (i.e. normal-appearing mouthparts yet infected; Figure 6), likely a precursor to abnormal mouthparts, is expected to have a lighter disease load (Figure 4).

Both Nieto (2004) and the current study noted that *Bd* was most likely to infect the upper jaw sheath of larval *R. aurora* as opposed to the lower jaw sheath and tooth rows. Interestingly, *Bd* had also shown an extremely high affinity to infect the upper jaw sheath of larval *R. sierrae* (Knapp and Morgan 2006). Due to the likelihood of *Bd* infection occurring on the upper jaw sheath of larval *R. aurora*, future researchers should focus their attention on the upper jaw sheath during visual examination.

Sensitivity of Detection Methods

Swabbing the mouthparts of a tadpole can be an effective non-lethal detection technique for *Bd* (Rachowicz and Vredenburg 2004). Detection via swabbing improves significantly in heavy infections, with up to a 90% agreement rate between swabbing and histology (Retallick et al. 2006). The results from the two diagnostic techniques had an 83.3% agreement rate, which suggests that 1) non-lethal swabbing is an effective alternative to the lethal diagnosis, 2) the infected tadpoles had relatively high disease loads, and 3) Nieto's (2004) histological data are likely comparable with other studies that employed qPCR in *Bd* sampling of tadpoles. Notably, Retallick et al. (2006) used wooden toothpicks instead of the sterile cotton swabs (Medical Wire & Equipment

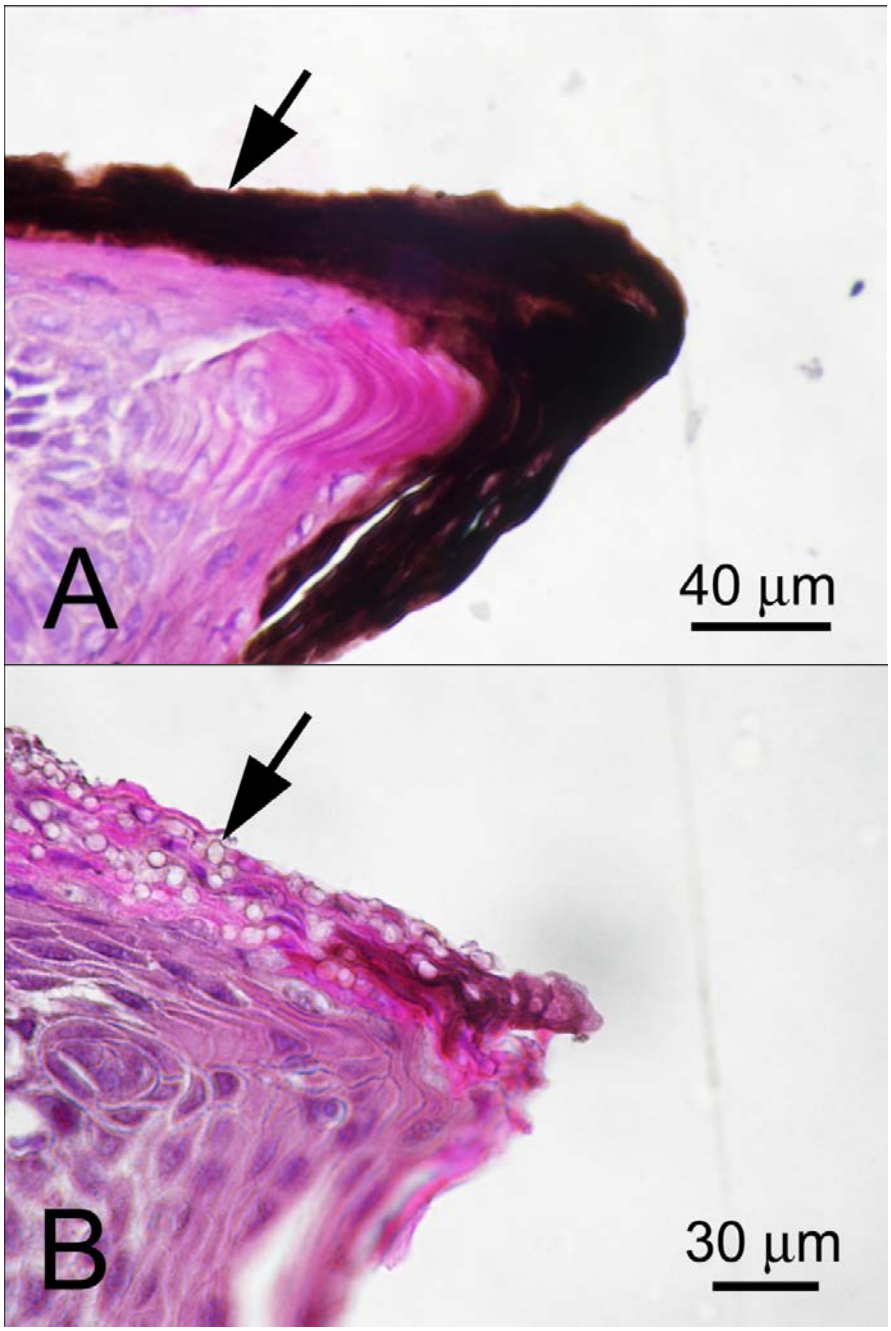

Figure 5. A) Sagittal section of the jaw sheath of an uninfected normal tadpole. Notice the thickly layered keratinized jaw sheath (arrow). B) Sagittal section of the jaw sheath of an infected abnormal jaw sheath. Note that the colony of *Bd* has destroyed layers of keratinized epidermal tissue, and caused depigmentation of the jaw sheath. A single *Bd* zoosporangium is indicated by the arrow. Tens of *Bd* zoosporangia are visible in that infected region.

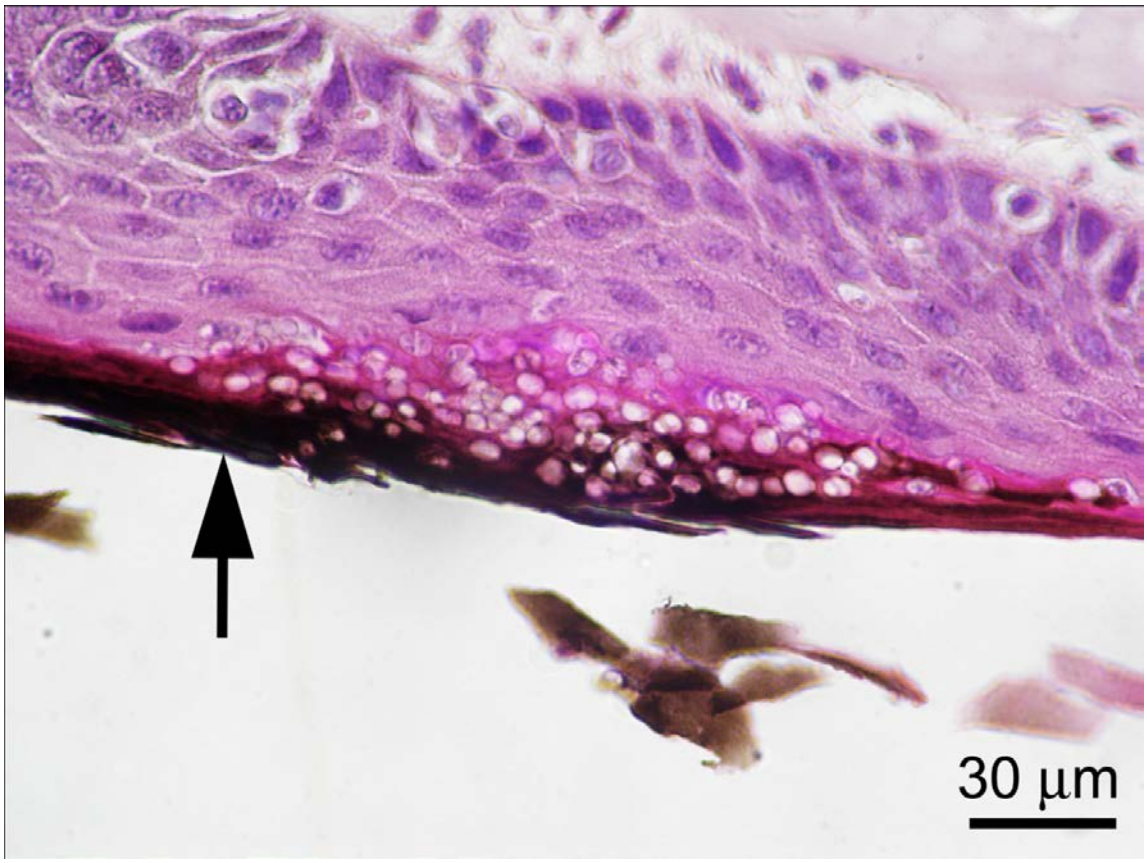

Figure 6. A macroscopically cryptic infection of *Bd* viewed in sagittal epidermal section. Notice that the infected area is covered by layers of thickly keratinized normal-appearing upper jaw sheath (arrow).

Company, UK) recommended by the standard *Bd* sampling protocols (Hyatt et al. 2007), whereas in this study, a similar sterile-tipped swab (Fisherbrand, Hampton, NH) was used. Swabbing using wooden toothpicks causes more abrasion in anuran larval mouthparts. As a result, it may be more sensitive in detecting *Bd*, but at a cost of being considerably more invasive to the specimen than using cotton-tipped swabs.

Molecular and histological techniques had identical sensitivity ($p = 1$), but both methods failed to detect an individual identified as infected with the alternative technique. Specifically, qPCR detected a tadpole with a cryptic infection that histology failed to detect. At the same time, histology identified an infected individual with oral abnormality that escaped the detection of qPCR. Nevertheless, the sensitivity of the two detection methods was identical because they both detected the same number of positive specimens, regardless of yielding slightly different results for estimated prevalence.

There are three possible explanations for the false-negative produced using histology. First, the infected area (especially if a minor infection) may have been completely removed with the swab, and thus causing a negative result in histology. This is possible, given that the qPCR assay is extremely sensitive. A single zoospore can be detected with consistency (Boyle et al. 2004). Second, I could simply have missed the infected area on the histological slide. This is also possible, especially if the infection was extremely mild, or the infection went unnoticed due to a shift to a different spot during slide processing. Lastly, section(s) containing *Bd* infection may have been lost during microtome-sectioning. It was extremely difficult to retain every section from the sectioning procedure.

Two explanations are possible where histology detected an infected tadpole with mouthpart abnormality that qPCR had missed. First, *Bd* thalli may not have been transferred onto the swab during swabbing. This is most likely, although the infected area visible on the slide was massive, superficial, and could easily be swabbed. Alternatively, it is possible that during the DNA extraction, *Bd* DNA was not extracted from that swab, even though present. This is very unlikely, given that DNA was extracted in a group where two other infected samples had turned positive.

Mouthpart swabbing in larval *R. aurora* proves to be as reliable as the highly time-consuming histology in detecting *Bd*. Swabbing also allows for re-sampling of the same individual due to its non-lethal and non-invasive nature. Finally, Nieto's (2004) histological data on larval *R. aurora* are shown to be comparable in estimating prevalence with those studies that employ qPCR.

Conclusions

Global amphibian decline is no longer arguable (Stuart et al. 2004). Many major threats to amphibian decline have been identified, but diseases, in particular chytridiomycosis, have the ability to wipe out species faster than any other major amphibian threats (Retallick et al. 2004, Schloegel et al. 2006, Lips et al. 2006). *Bd* affects amphibians differentially; at times *Bd* infection occurs without disastrous effects to amphibian populations (Fisher et al. 2009) as evidenced in the current study. But often times *Bd* can be highly pathogenic, particularly to species that are already stressed by

other negative environmental changes, such as habitat destruction/alteration, pollution, invasive species, and climate change (Pounds et al. 2006, Muths and Hero 2010, Paetow et al. 2012). Early detection and prevention of disease dispersal are the best strategies against *Bd* as opposed to dealing with the aftermath, especially when amphibian extinctions due to chytridiomycosis can happen rapidly (Lips et al. 2006, Mendelson et al. 2006). Long-term monitoring plus an understanding of the distribution and dynamics of *Bd* globally and locally are critical to amphibian conservation in the context of this pathogen.

Seasonal variation in disease prevalence in wildlife is common (Hosseini et al. 2004). Prevalence dynamics of chytridiomycosis may vary spatially and temporally, even within a single species and geographic area (Savage et al. 2011). The many contributing factors include the thermal requirements of *Bd*, variability in host immunity over different seasons or temperatures, genetic diversity of the host population, and virulence of the pathogen (Piotrowski et al. 2004, Berger et al. 2005b, Pearman and Garner 2005, Cheng et al. 2009). The genetic diversity of local *R. aurora* populations and the virulence of local *Bd* strain(s) are unknown due to the lack of studies. But the thermal requirements of *Bd* and the immunity of *R. aurora* have been well documented (Rollins-Smith et al. 2002, Piotrowski et al. 2004, Conlon et al. 2005, Conlon et al. 2011). Low temperatures can not only reduce *Bd* growth but also decrease the pathogenicity of *Bd* (Piotrowski et al. 2004). The low (10.2°C) mean annual ambient temperature here on the North Coast likely negatively affects *Bd* growth. In addition to low temperature, the antimicrobial skin peptides found in *R. aurora* is also more effective

against *Bd* at 10°C than 22°C (Rollin-Smith et al. 2002), highlighting the importance of local climate in regulating this pathogen.

My research and previous studies of *Bd* in *R. aurora* elsewhere in the Pacific Northwest have found a similarly low prevalence (Adams et al. 2007, Nieto et al. 2007, Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010). Infection by *Bd* does not seem to impact *R. aurora* populations in the Pacific Northwest. In fact, few *R. aurora* are infected by *Bd* throughout this region, despite other sympatric species frequently being infected without apparently suffering from mortalities (Adams et al. 2007, Pearl et al. 2007, Pearl et al. 2009, Adams et al. 2010). Consequently, the current study does not identify *Bd* as a significant threat to *R. aurora* populations on the North Coast of California. A combination of low ambient temperature, life history traits, and the effective antimicrobial skin peptides found in *R. aurora* may be able to synergistically suppress the virulence of *Bd*, and made it easier for our local *R. aurora* populations to reach an enzootic and benign relationship with *Bd*.

Nevertheless, a possible long-term impact from the infection of this potentially lethal pathogen should not be underestimated. For example, global climate destabilization has profound and severe repercussions on amphibians worldwide (Pounds 2001, Pounds et al. 2006, Reading 2007, Blaustein et al. 2010). Climate change can negatively affect amphibian survival, growth, reproduction, and dispersal, while altering habitat, community structure, and host-pathogen dynamics (Berger et al. 2004, Pounds et al. 2006, Bosch et al. 2007, Blaustein et al. 2010).

By 2100, the global annual average temperatures are projected to increase anywhere between 1.1°C to 6.4°C (Solomon et al. 2007). The annual average temperature may increase more than 7.5°C in the higher northern latitudes, which will be most pronounced during the winter months (December – February) of the northern hemisphere (Solomon et al. 2007). Notably, the prominent temperature increase will happen during the peak of breeding season of *R. aurora*, where major disease transmission among frogs can occur due to physical contact during amplexus. As a result, this large-scale warming would likely negatively affect populations of *R. aurora*, as well as many other local native amphibians (e.g., *R. boylii*, *Anaxyrus boreas*, *Dicamptodon tenebrosus*) in the Pacific Northwest. Increases in ambient temperature will not only decrease the effectiveness of antimicrobial skin peptides in many ranid frogs (Rollins-Smith et al. 2002), but it may also disable the thermal restrictions on *Bd*. Consequently, global warming may give *Bd* an advantage that tips the balance from a stable pathogen-host relationship to a catastrophic extirpation of *R. aurora* populations.

Future Directions

Metamorphs and juvenile frogs often exhibit a higher *Bd* load and prevalence than other age classes (Pearl et al. 2009, Russell et al. 2010, Piovia-Scott et al. 2011). Younger frogs generally have a weaker immune capacity than their adult counterparts (Briggs et al. 2005, Rachowicz et al. 2006). As a result, they tend to be more vulnerable

to *Bd* infection and chytridiomycosis. Future studies of *R. aurora* should include these vulnerable intermediate life stages in their surveys for *Bd*.

To quantify long-term effects of *Bd* in *R. aurora*, population monitoring data must be included as well. This can be done as a cooperative effort with our local state agencies (California Department of Fish and Game, etc.) to initiate a continuous monitoring program. Then we can detect whether there are changes in the population size of *R. aurora* with the presence of an enzootic *Bd* infection over time.

DNA extraction and molecular detection of *Bd* in formalin-fixed specimens can now be achieved with consistency (Cheng et al. 2011, Richards-Hrdlicka 2012). Swabbing museum specimens for *Bd* from Humboldt State University and other major amphibian collections throughout the Pacific Northwest region would be a useful approach. An extensive retrospective sampling may help us to identify when *Bd* arrived on the North Coast, the patterns of geographic spread, and map the transmission routes (Lips et al. 2008). This in turn can help management planning to focus on preventing *Bd* dispersal into new areas. In addition, the question of whether *Bd* is novel or endemic to the coast of northern California can subsequently be answered.

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