Survey for Chytridiomycosis in Terrestrial Salamanders of the San Francisco Bay Area

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Abstract Chytridiomycosis is a fungal infection thought to be the cause of amphibian die-offs world wide. Though previously studied almost exclusively in aquatic frog populations, recent findings have provided evidence that chytrid is also present in terrestrial salamander populations including *Batrachoseps attenuatus* individuals in the East Bay (S. Weinstein, pers.comm.). Little is known about chytrid dynamics on land. This study seeks to strengthen the knowledge base of chytrid as a terrestrial disease by identifying new species harboring the fungus, comparing salamander age with chytrid intensity, and determining whether there is a correlation between dominant vegetation type and chytrid presence and intensity. Specifically, this study investigates whether there are other terrestrial salamander species that act as reservoirs for *Batrachochytrium dendrobatidis*, allowing the fungus to pass to *Batrachoseps attenuatus* late in the season. Three hundred seventy seven terrestrial salamanders in ten East Bay Regional parks were sampled in this study. Four species were represented. Surveys spanned from late December to early April. Chytrid was not detected in any individuals. It is unlikely that other local terrestrial salamander species act as a vector or reservoir for the fungus. Since parallel studies have detected chytrid in *Batrachoseps attenuatus* (S. Weinstein, pers. comm.), it is essential to continue to regularly monitor terrestrial populations in order to establish more accurate prediction and management strategies for the disease.

Introduction

Chytridiomycosis is an emerging infectious disease that is thought to be responsible for mass amphibian declines worldwide. It has been cited as a major cause of illness and death in amphibian populations in Australia (Berger *et al*. 1998), Europe (Bosch *et al*. 2001), North America (Bradley *et al*. 2002), Central America (Lips 1998), and South America (Young *et al.* 2001). First described in the late 1990s (Laurance *et al*. 1996; Berger *et al*. 1998), chytridiomycosis is a fungal infection caused by *Batrachochytrium dendrobatidis* that infects keratinized portions of the skin of amphibians. It is the only member of the phylum Chytridiomycota known to parasitize vertebrates, and it is especially common and virulent in the amphibian orders Anura and Caudata (Kriger and Hero 2006; Knapp and Morgan 2006). Frogs are susceptible to the infection at two life stages: pre- and post-metamorphosis. Mass mortalities have occurred in post metamorphic species infected with the fungal disease (Berger *et al*. 1998; Daszak *et al*. 1999). Tadpoles have been reported to become infected, though larval mortality is extremely rare (Rachowicz and Vredenburg 2004). In tadpoles, the infection is usually limited to mouthparts, the only keratinized areas premetamorphosis, which begin to lose pigment as the infection becomes more severe (Rachowicz and Vredenburg 2004; Knapp and Morgan 2006). A study by Rachowicz *et al*. (2006) indicated that individuals can maintain chytridiomycosis between the tadpole and post-metamorphic stages, despite not having contact with other infected individuals or fungal source.

The sudden spread of chytrid throughout the world has led to two theories on its emergence and spread. The first explains *Batrachochytrium dendrobatidis* as an organism that has been historically present in lakes and streams worldwide, but recently some aspect of environmental change (global warming, for example) has increased its pathogenicity (Daszak *et al*. 1999, Pounds *et al.* 2006). However due to the sudden appearance of the disease, as well as its low genetic variation (Morehouse *et al*. 2003), it is also widely hypothesized that *Batrachochytrium dendrobatidis* was spread by anthropogenic means from a single source area. According to Weldon *et al*. (2004), the disease probably originated in Africa, and was disseminated via trade of frogs used for food, scientific study, and pets.

Until recently, chytrid research has focused exclusively on amphibians that live in aquatic environments. However, recently the fungus has been shown to survive outside of these aquatic zones (Johnson and Speare 2005), and thus its effects and geographical limits may be underestimated. Based on the current understanding of its lifecycle, the chytrid fungus is believed to be entirely dependent on water to survive and reproduce, which explains the prevalence of chytridiomycosis in largely aquatic amphibian species (Longcore *et al*. 1999). *Batrachochytrium dendrobatidis* progresses from a zoospore to the growing organism called a thallus which produces one zoosporangium. The zoosporangium acts as a container for new zoospores, and thus continues the lifecycle of the chytrid fungus (Berger *et al*. 2005). A complete lifecycle of *Batrachochytrium dendrobatidis* has been shown to take about five days, with zoospores living for less than 24 hours (Longcore *et al*. 1999). While it is clear that the chytrid needs some form of moisture to survive and reproduce, the problem of chytridiomycosis infecting terrestrial organisms is increasing (Cummer *et al*. 2005, Johnson and Speare, 2005). To date, little research has been done to address the possibility of chytrid in terrestrial systems, and thus the strong association between chytrid and aquatic environments might be due in part to sampling bias.

Once in an ecosystem, chytridiomycosis can persist and spread through several different forms of biotic and abiotic transmission. Johnson and Speare (2003) gave clear evidence that the fungus has the ability to survive and spread throughout lakes and streams, regardless of the presence of a host organism. The fungus has also been shown to survive on non-living substrates, such as moist river sand and bird feathers (Johnson and Speare 2005), suggesting possible modes of dissemination beyond species to species contact. Furthermore, several studies have shown that certain species of amphibians are able to survive chytrid infection, showing few symptoms of the disease, such as *Rana catesbeiana* (American bullfrog) (Daszak *et al.* 2004) and *Xenopus laevis* (Weldon 2004). These species are suspected to be important reservoirs and vectors of chytridiomycosis (Daszak *et al.* 2004, Weldon 2004).

Because salamanders and frogs often coexist within habitat types, it is possible that salamanders might play an important role as a vector for the chytrid fungus. Moreover, unlike frogs, salamanders tend to have a distinct terrestrial phase, or even a completely terrestrial lifestyle and thus could be the means by which chytrid makes its transition from a water-based fungus to a terrestrial fungus. Salamander die-offs analogous to the massive die-offs reported in frogs have not been observed. Therefore, much less is known about their potential as a chytrid host. A study by Davidson *et al.* (2003) reported the first case of chytrid infecting salamanders in the field. Sonoran tiger salamanders (*Ambystoma tigrinum stebbinsi*) in southern Arizona were noted to have small black spots on their ventral abdomen and dorsal head surfaces, and upon further investigation were shown to be infected with *Batrachochytrium dendrobatidis*. The same study also demonstrated the possibility that salamanders can serve as reservoir hosts for chytrid, harboring the pathogen in the intervals between other host infections. Furthermore, Davidson *et al*. (2003) found clear evidence through lab experiments that transmission of *Batrachochytrium dendrobatidis* from a salamander species to a frog species is possible. While *Ambystoma tigrinum stebbinsi* has an aquatic phase, Cummer *et al.* (2005) reported the first case of chytridiomycosis in a terrestrial plethodontid salamander. The study located the fungus on a single gravid female of the terrestrial Jemez Mountains salamander (*Plethodon neomexicanus)*. The species is endemic to the relatively dry slopes of the Jemez mountains in New Mexico. While most published research has thus far found the disease non-lethal in salamander populations (Davidson *et al*. 2005, Cummer *et al*. 2005), recent laboratory research from the University of California, Berkeley has shown one hundred percent mortality in *Batrachoseps attenuatus*, the California slender salamander (S. Weinstein, unpublished data). Thus, while many salamander populations may be able to cope with the disease, acting as carriers for chytridiomycosis, still other populations may potentially suffer the same mass die-offs thus far seen only in frogs.

Even more strikingly, these results indicate that even completely terrestrial salamanders, such as *Batrachoseps attenuatus*, can become infected with the disease. Field work indicates that they do, in fact, become infected with chytridiomycosis in their natural environment, although no mortality has been noted in the field (S. Weinstein, unpublished data). Recent research has also found terrestrial plethodontid salamanders from South America harboring the disease (T. Papenfuss, pers. comm.). Consequently, it is reasonable to believe that still more terrestrial amphibian species act as reservoirs for the disease, and that the disease can occur in non-aquatic environments. It is plausible that salamanders serve as disease vectors to susceptible amphibians. To determine the possibility of salamanders serving as carriers of chytrid, in this study, terrestrial salamanders in the East Bay of Northern California were examined for the presence of chytrid. Specifically, I ask the question: “Are there other (non-symptomatic) carrier species that act as vectors, transferring the disease to *Batrachoseps attenuatus* in the late season?

Chytrid has already been shown to occur in *Batrachoseps attenuatus* in the area, making a study of this geographic range important (S. Weinstein, pers. comm.). Species that co-exist with

Batrachoseps attenuatus were sampled for chytrid, including *Ensatina eschscholtzii xanthoptica* (Yellow-eyed Salamander) and *Aneides lugubris* (Arboreal salamander). Together with *Batrachoseps attenuatus,* these species are the only entirely terrestrial salamander species that are known to occur locally. Also sampled was *Taricha torosa* (California newt) which is of special interest because it coexists with these salamanders, but also has an aquatic phase (Stebbins 2003) and traverses great distances (Stebbins 2003), potentially acting as an important carrier of the disease. To answer determine whether sympatric salamander species act as vectors for the fungal infection, salamanders in ten transects were sampled over the course of the year. Other environmental elements were also investigated in order to establish correlations if the disease was detected.

Within salamander populations, it is possible that the disease is not evenly distributed. Older salamanders have had more opportunity to come into contact with and to cultivate the fungus. Since size can be used as a surrogate for salamander age, which is very difficult to determine accurately, this study asks whether chytrid presence and intensity is correlated with salamander snout-vent length.

Little is known about which habitat types are best suited to harboring *Batrachochytrium dendrobatidis*. While research has examined the occurrence of chytrid across several aquatic environments (Kriger and Hero 2007b), similar associations have yet to be determined in terrestrial environments. Is there a correlation between dominant vegetation type and chytrid presence? Furthermore, the spatial distribution of individuals within a habitat type could play an important role in the persistence and spread of the fungus. Is there a correlation between chytrid intensity and cohabitation of one individual with another? Currently, most research is concerned with tracking the chytrid fungus within populations where massive die-offs have occurred, but it is just as important to identify and understand potential host populations and fungal spread that may not be lethal.

Methods

Field

The study was conducted at ten locations throughout the East Bay Regional Park District (Alameda and Contra Costa Counties, CA; see Figure 1). At each site, a 1km stretch of trail-side habitat was surveyed, turning all visible downed wood. The transects were chosen by randomly selecting a point in each park using ArcGIS technology (ESRI, Redlands, CA) and then locating the trail closest to that random point to sample. Since terrestrial salamanders tend to have extremely small home ranges (Staub *et al.* 1995), trail conditions were not thought to influence salamander activity or behavior. Furthermore, since studies have shown that there is more downed wood in trailside areas, and thus more suitable habitat for salamanders (Olson *et al.* 2002), it was thought that a trailside study would allow for higher density sampling. To determine if a relationship between chytrid infection in salamanders and dominant vegetation type exists, locations representing several dominant vegetation types were selected. Oak, laurel, pine, redwood, blackberry, eucalyptus, and grasslands were represented.

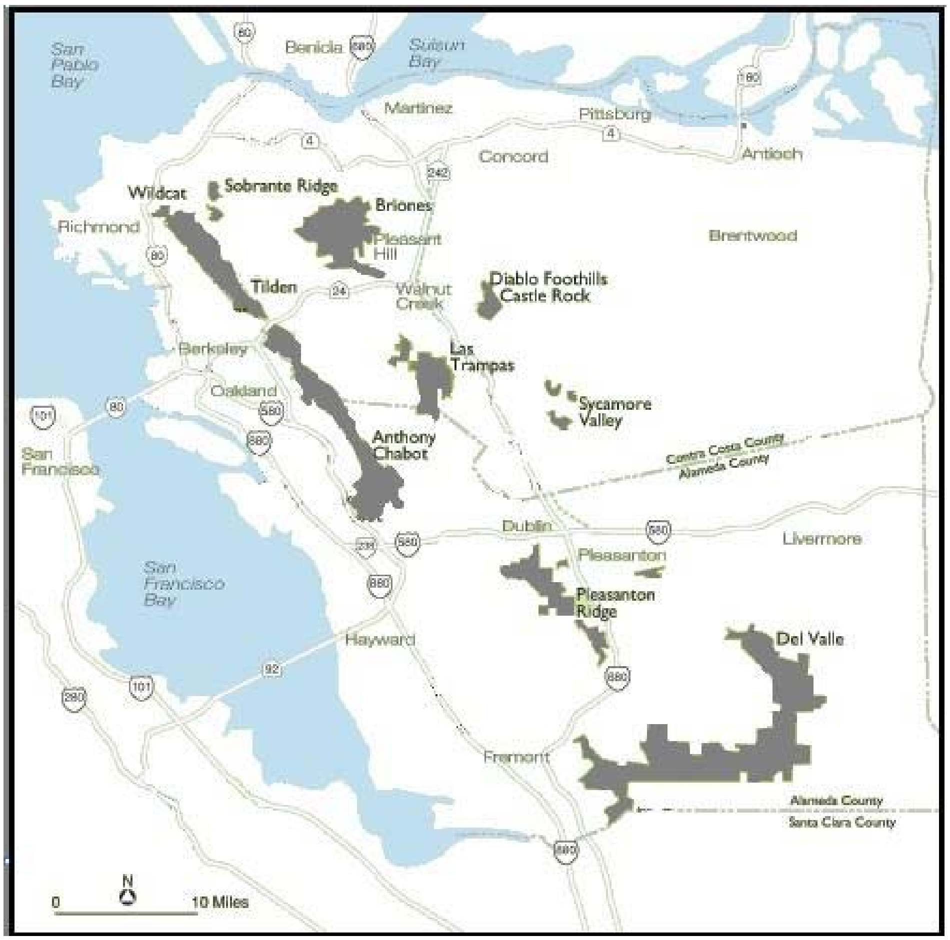

Figure 1. Survey efforts were carried out in ten East Bay Regional Parks, shaded above.

Since salamander activity is highly dependent on the weather, rain was a necessary condition of beginning research. All sites were surveyed three times, with one survey conducted December 5th to December 19th, another from January 31st to February 27th, and a final survey completed

March 18th to April 1st. Each transect was investigated thoroughly for terrestrial salamanders during daylight hours. All woody debris was turned over, as it provides ideal salamander habitat. Three hundred seventy seven salamanders in total were caught by hand and collected in clean bags to prevent cross-contamination. They were then swabbed for 30 seconds on the ventral side in order to remove chytrid DNA. Swabbing methods followed the protocol established by the Briggs NIH research group. A snout-vent length measurement was taken to help approximate the salamander's age (Heyer 1994), and it was recorded if the salamander was a gravid female. After swabbing, salamanders were released. Dominant vegetation type within three meters of the individual was noted. Species type and notes on the animal's condition were also recorded.

Laboratory

The swabs were collected, labeled, and taken to the lab for analysis. In the past, chytrid infection has been detected on salamanders using histology (Davidson *et al.* 2003), but more recent research has favored real-time PCR methods in detecting chytridiomycosis since this method requires only swabs of DNA rather than skin samples and allows large numbers of samples to be processed more quickly (Annis *et al.* 2004). Therefore, in the lab, DNA from each swab was extracted and run through a real-time PCR machine, which not only detects presence/absence of *Batrachochytrium dendrobatidis* but also determines the number of *Batrachochytrium dendrobatidis* zoospores present, a direct correlate to infection intensity.

Results

Three hundred and seventy seven salamanders were found in all, with four different species represented (Table 1). The most salamanders were encountered during the second sampling effort, which took place in late February. The fewest were found in the final sampling effort in late March and early April. *T. torosa* individuals were only found in the sample transects during the final survey (see Table 2). Two *Hyla regilla* (Pacific tree frogs) were also found during the course of the sampling efforts but they were not included in the study.

Salamanders were found in all transects sampled and were present in a variety of habitat types. Not only was *Batrachoseps attenuatus* the most prominent species in the study, it also occupied the most habitat types, occurring in areas with dominant vegetation ranging from oak and laurel to grassland. *Aneides lugubris, E. eschscholtzii xanthoptica,* and *T. torosa* were not nearly as abundant, and were found in fewer uniform habitat types.

Table 1. East Bay Regional Parks survey dates and dominant vegetation types in sample transects. Species found during each sampling effort are signified with superscripts above survey dates (A – *Aneides lugubris*, B – *Batrachoseps attenuatus*, E – *Ensatina eschscholtzii xanthoptica*, T – *Taricha torosa*).

Park	Survey One	Survey Two	Survey Three	Dominant Vegetation Types
Anthony Chabot	Dec. 10^{BE}	Feb. 2^{BE}	Mar. 20^{B}	Buckeye, Eucalyptus, Oak, Redwood
Briones	Dec. 7^{B}	Feb. 5^{B}	Mar. 21^{BT}	Grass, Laurel, Oak, Pine
Del Valle	Dec. 19^{BE}	Feb. 27^{BE}	Mar. 31^{B}	Grass, Oak, Pine
Diablo Foothills	Dec. 17^{ABE}	Feb. 8^{AB}	Mar. 25^{B}	Grass, Oak
Las Trampas	Dec. 14^{B}	Feb. 9^{ABE}	Apr. 1^{BT}	Oak
Pleasanton Ridge	Dec. 18^{B}	Feb. 25^{BE}	Mar. 27^{B}	Buckeye, Grass, Oak
Sobrante Ridge	Dec. 12^{AB}	Feb. 3^{B}	Mar. 25^{B}	Grass, Laurel, Oak
Sycamore Valley	Dec. 18^{B}	Feb. 27^{B}	Mar. 31^{B}	Grass, Oak
Tilden	Dec. 11^{BE}	Feb. 1^{BE}	Mar. 18^{B}	Pine, Oak
Wildcat Canyon	Dec. 5^{BE}	Jan. 31^{B}	Mar. 21^{B}	Blackberry, Oak

Lab analysis of the swabs taken during the field surveys did not show a significant amount of *Batrachochytrium dendrobatidis* zoospores in any of the individuals sampled. Minor florescences were noted in many samples when analyzed with real-time PCR, but none reached the threshold for detection. Any florescences can thus be attributed to sampling error in the real-time PCR machine and not the presence of *Batrachochytrium dendrobatidis* zoospores. Therefore, anticipated analysis of size, habitat associations and co-habitation relationships with chytrid were not performed as no chytrid was present.

Table 2. Individuals found in each sampling effort of ten parks. For survey dates, refer to Table 1.

Species	Number of Individuals			
	Survey 1	Survey 2	Survey 3	Total
B. attenuatus	97	168	91	356
A. lugubris	2	3	0	5
E. eschscholtzii	8	6	0	14
T. torosa	0	0	2	2
Total	107	177	93	377

Discussion

No chytridiomycosis was detected in terrestrial salamander populations during the course of this study. Previously, only one published study had noted a terrestrial salamander to carry the disease (Cummer *et al*. 2005) and locally, unpublished research has also identified infected *Batrachoseps attenuatus.* A project undertaken concurrently with this study confirmed chytrid in nearby terrestrial salamander populations, with detection beginning in early April, in a minority of the *Batrachoseps attenuatus* sampled (four of 30 individuals) (S. Weinstein, pers. comm.). This study suggests that despite detection of *Batrachochytrium dendrobatidis* in nearby local salamander populations, the fungus does not appear to be common or widespread in East Bay terrestrial habitats. While this study suggests that the fungus is not widespread terrestrially, it does offer some interesting possibilities for future research into the dynamics of terrestrial spread of the infection with the continued monitoring of the populations surveyed.

For example, it is possible that there are temporal patterns in chytridiomycosis prevalence that could account for negative chytrid results in this study. Previous studies have shown that chytridiomycosis has seasonal properties (Kriger and Hero, 2007), with chytrid prevalence increasing toward the beginning of spring. This study was concluded at the beginning of April, around the time chytrid is most prevalent in aquatic populations (Kriger and Hero, 2007). It is possible that the populations surveyed had simply not yet been affected by the fungus and later sampling may have detected the presence of chytridiomycosis. However, by looking at the dramatic drop in salamanders found between the second and third survey (Table 1), it is clear that conditions were becoming inhospitable for salamander populations. It is likely that many of the salamanders would have retreated underground in the very near future, at which point they would no longer serve as important vectors for the disease.

Another possible explanation for the absence of chytridiomycosis in the populations examined in this study could be innate immune defenses to the fungus. Previous studies have confirmed patterns of specific amphibian species declines in areas where other amphibian species do not decline (Lips 1999, Kriger and Hero 2006). This is most likely due to an innate resistance in certain species. Woodhams *et al.* (2007) suggest that multiple mechanisms are involved in innate resistance to chytridiomycosis, placing an emphasis on skin peptide defenses activated by pathogen exposure. It is also possible that innate defenses are not the only factors that keep individuals from becoming susceptible to chytridiomycosis. Carey *et al.* (1999) also tied factors like ambient temperature, man-induced stress, and psychosocial stressors (i.e. higher density populations) to modulating amphibian pathogen resistance. Thus, while no chytridiomycosis was found in the study populations during the one year survey conducted for this study, it is possible that in years of more intense environmental stress the disease could be more prevalent.

In a recent study by Kriger and Hero (2007b), it was suggested that future research focus on amphibians that breed in permanent bodies of water. This study confirmed that chytridiomycosis is not widespread in terrestrial environments at the present time. However, should research continue to focus solely on aquatic environments, it is clear that a piece of the puzzle would be missing. Several studies indicate that it is possible for amphibians breeding away from permanent water to become infected with *Batrachochytrium dendrobatidis* (Waldman 2001; Lips *et al.* 2003). However due to the much higher prevalence of chytrid in aquatic environments (Kriger and Hero, 2007b), these results have not been given as much weight as they should have.

Previous studies have shown that chytrid cannot survive desiccation (Johnson *et al.* 2003). However this ignores the fact that terrestrial salamanders also depend on soil moisture for survival. Thus, infected salamanders are almost never in completely dry microhabitats, and thus the fungus may also have the moisture it needs to persist. However it is possible that the fungus requires higher levels of moisture than do the salamanders, which might explain its disappearance between the end of the rainy season and the early rainy months the next year (Kriger and Hero, 2007). As the rainy season continues, the fungus is more successful in terrestrial environments and infections begin to be noted in sampling efforts.

Until quite recently, the life cycle of *Batrachochytrium dendrobatidis* was argued to be quite simple, with no noted findings of resting spores (Berger *et al.* 2005). Pounds *et al.* (2006) argued that global warming acts as a proximate cause of amphibian declines by encouraging outbreaks *Batrachochytrium dendrobatidis.* However recent findings have taken the global warming argument a step farther by adding a more complex stage to the *Batrachochytrium dendrobatidis* life cycle. Di Rosa *et al.* (2007) observed spherical, thick-walled unicellular organisms attached to the skin of frogs in their study. They suggest that these organisms were an encysted form of *Batrachochytrium dendrobatidis.* These findings support the global warming hypothesis, providing evidence that chytrid can exist in a non-pathogenic form in the environment before outbreaks are observed.

The study also raises important issues regarding the spread of the fungal infection to terrestrial communities. A thick walled, encysted form of *Batrachochytrium dendrobatidis* might offer some explanation as to how the fungus is able to survive in terrestrial environments. More research is needed to determine whether this is indeed the case. A model created by Mitchell *et al*. (2008) suggests that the persistence of *Batrachochytrium. dendrobatidis* outside the amphibian host greatly increases the probability of host extinction. The model only accounted for aquatic habitats, but as chytrid is observed in more widespread and numerous terrestrial habitats, the problem can only become more serious. This study indicates that at the present time chytrid is not widespread in terrestrial environments of the San Francisco East Bay Area, but if chytrid is able to rest in a dormant encysted form, the likelihood that it can spread into new environments is increased.

Quite clearly, the problem of chytridiomycosis is incredibly widespread both in terms of geography and diversity of aquatic habitat types. This study suggests that while several studies have found the fungus in terrestrial salamanders (Cummer *et al.* 2005, Weinstein, pers. comm.), it has not yet become a widespread issue. However close and consistent monitoring of the terrestrial environment is essential to predicting and managing the spread of the disease. We have seen chytrid cause rapid and tragic affects in aquatic frog populations. Now that several examples of chytrid in the terrestrial environment exist, monitoring numerous and geographically widespread populations will help us in earily detection. It is likely that chytrid will continue to spread, and if it does become more common in terrestrial populations new control measures will need to be sought. This study is only the first step in establishing routine surveys of terrestrial populations to better detect the disease in all the environments that can support it.

Further research should focus on identifying other infected species in different geographical areas. While no individuals infected with chytridiomycosis were observed in my study, species sympatric with semi-aquatic species should perhaps be looked at most carefully, since they might provide clues to die-offs in seemingly isolated ponds. Furthermore, investigating the dynamics between terrestrial and aquatic populations would provide a useful basis for further claims of possible vectors for *Batrachochytrium dendrobatidis*. This study suggests that chytridiomycosis is not widespread in the San Francisco Bay Area. However it does not discount the fact that chytridiomycosis might be found in the same areas in the future. With a changing climate and new discoveries regarding the lifecycle of the chytrid fungus, the dynamics of this deadly disease are still mysterious. The study of chytridiomycosis in a terrestrial environment is emerging, and it is important that future research continues to fill in the many gaps still left open.

Acknowledgements:

I would like to thank Craig Moritz, David Wake, Shelly Cole, Pete Oboyski, Sara Weinstein, and Ted Papenfuss for their comments, guidance, and support. Thanks also to The ASUC Academic Opportunity Fund and the Environmental Sciences department of the University of California, Berkeley for funding necessary elements of this project. I would also like to extend my thanks to my many field assistants: Derek Pillay, Nora Downs, Marianne Balin, Aviva Lipkowitz, Ryk Shepphard, Katarina Morici, Ariel Schneider, Annie Chang, Jeffrey Doker, Gadi Garfinkel, and Christy Fox.

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