Chytridiomycosis and the Mountain Yellow Legged Frog; studies of physiological factors that influence disease in *Rana muscosa*.

by

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Declines in *Rana muscos*a within the last ten years have been so severe that this species is threatened with extinction. The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) has emerged as a major contributor to declines in the already small and fragmented populations of this species. Adults, juveniles and larvae continue to be found within affected populations from year to year, albeit in smaller numbers. The work presented within this dissertation asked whether immune responses could be enhanced against Bd and examined the effect of physiological factors on Bd prevalence and related mortality in *R. muscosa* in lab and field caught animals. I studied the possibility of protecting individuals against Bd using immunizations and examined the beneficial effects of sub-clinical Bd infections and survival between life stages. My work also included examining interactions between seasonal changes in endocrine function related to reproduction and prevalence of Bd.

In laboratory studies I found that immunizations with killed Bd prior to live exposure in juvenile *R. muscosa* frogs was not protective. No difference in how quickly animals became infected nor in mortality between groups, was found. However, exposure to Bd during the larval stage, at sub-infectious doses, was protective in those same animals when re-exposed to Bd as sub-adults. This work provides two points for further investigation; infectious dose may be a determining factor in the pathogenesis of Bd and protective responses developed in larvae that are capable of resolving or building a memory response to infection with Bd.

Within field populations of *R. muscosa* I detected seasonal fluctuations in Bd prevalence and the hormones involved in the stress and reproduction, corticosterone and testosterone. I found no association between either hormone and Bd prevalence within and between populations This finding refutes the immunocompetence handicap hypothesis claim that these two hormones are immunosuppressive in all vertebrates and that disease and breeding are positive correlated with each other The strongest predictors of infection

prevalence and intensity were month and site of collection or population sampled. This supports work showing Bd is constrained by temperature within the environment but further suggests that factors unique to sites are important in modulating disease.

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Introduction

Conservation medicine has been described as the study of interactions between pathogens, diseases, species and ecosystems (Tabor, 2002). This goal of this relatively new field is to evaluate the ecological health of a species within its habitat and the factors that contribute either to its health or disease. It encompasses a number of study areas such as veterinary medicine, wildlife and forest management, epidemiology and conservation biology. Disease and host responses are incorporated into a larger picture of how disease operates between the host and the environment. Eco- immunology takes this concept further, examining the immune response of animals to particular diseases in light of trade offs between an individuals' fitness and life history traits for the species.

The incorporation of molecular techniques and examination of ecological or physiological factors that may contribute to diseases can reduce the "noise" in SIR (Susceptible, Infected, Recovered) models that use empirical data. Model noise is generated by an endless combination of factors that exist in natural populations; age and sex related variation in susceptibility, asymmetric transmission from pathogens that infect multiple species and seasonal changes in host, pathogen or vector biology and population densities. Techniques such as ELISAs have proven useful in monitoring species and population differences in antibodies to a number of diseases such as West Nile virus (Figuerola et al 2008), Rift Valley Fever (Evans et al, 2007) and Hantavirus (Jay et al 1997). This data provides information on exposure to pathogens in animal populations but can be used as a predictive measure for animal populations most likely to experience outbreaks and to isolate reservoirs of infection. Seroprevalance of individuals to disease can also be used in quantitative models to determine age dependency in infections and risk factors that contribute to the maintenance of disease within a population (Muma et al, 2007). This type of data can also be used in force of infection models to clarify the probability of disease within and between populations (Heisey et al 2006). These measures could be helpful in determining how infectious a pathogen is and in applying appropriate treatment and intervention methods.

Monitoring changes in host ecology by tracking population density fluctuations, age structure and host migrations and ranges in relation to disease prevalence has helped explain the seasonal periodicity in several wildlife diseases (Hosseini et al, 2004, Jolles et al, 2006 and Williams et al, 2002). The inclusion of the physiological mechanisms that regulate breeding, stress and hibernation (i.e. hormone measurements) and its relation to wildlife disease research is relatively new with only a few examples in natural populations (Ross et al, 1993 and Lindstrom et al, 2005). However, many researchers have suggested that hormones can make important contributions to disease based on studies that have found correlations between immune function and hormone titers (Owen-Ashley et al, 2004, Martin et al, 2007 and Mills et al, 2008). This type of multidisciplinary approach to wildlife disease research is particularly important in the study of emerging diseases, the incidence of which has increased dramatically within the last ten years (Daszak et al, 2001). Chytridiomycosis is one such disease that has become the focus of many research groups studying amphibian declines.

First reported in 1998 and linked to episodes of mass mortality in amphibian communities in Australia (Berger et al, 1998), Central and South America (Lips 1999 and Lips et al, 2006) chytridiomycosis has become a focus of research in amphibian declines. While the field has

expanded tremendously, there are still many questions to answer in Bd research and a number of enigmatic findings that need clarification.

For example, this disease exhibits characteristics of a new emerging pathogen and one that has been part of amphibian communities for some time. Historical specimens have documented infection in anurans with this pathogen dating back to the 1930's in Africa (Weldon et al 2004) and in California as early as 1961 (Padgett-Flohr et al, 2009). This suggests that Bd has been present in populations for much longer than would be anticipated for an emerging disease and that it is only recently that researchers have begun examining Bd in anurans. Genetic analysis of Bd isolates from Africa, Australia and the Sierras of Northern America have provided evidence for Bd as both an endemic and epidemic disease in infected populations (Morgan et al, 2007). More recent work has suggested that the genetic analysis suggesting both endemic and epidemic origins found in Bd isolates is through a recent mitotic recombination event and that Bd is a new disease in at least some populations (James et al, 2009).

One recent observation in Bd research is that some species like the American Bullfrog, *Rana catesbeiana*, appear resistant to disease with high doses of Bd (Daszak et al, 2004) while others, like *R. muscosa* can succumb quickly to infection (Rachowicz et al, 2006). This differential outcome in Bd infections is also seen in the phylogenetically distant family Pipidae ; *Xenopus laevis* is resistant to disease (Woodhams et al, 2006) while *Xenopus tropicalis* are susceptible to infection (unpublished data). The wide distribution of this disease, the differential mortality linked to Bd between species and the risk Bd poses to amphibians like *Rana muscosa* warrant a better understanding of factors that influence the pathogenesis and host immune response to infection.

In California, Bd has emerged as a major contributor to declines in populations of the Mountain Yellow Legged Frog, *R. muscosa*, across the Sierra Nevadas (Rachowicz et al, 2006). Declines in *R. muscosa* have occurred historically with the introduction of non native fish in the Sierra Nevadas (Knapp et al, 2007) with the first report of Bd in *R. muscosa* larvae in 1998 (Fellers et al, 2001). Later surveys have found Bd in populations across the Sierras associated with mortality and abrupt population declines (Rachowicz et al, 2006). Chytridiomycosis has now become an established disease in many anuran communities, with *R. muscosa* included in this group. A portion of Bd research has shifted toward examining contributions various ecological and physiological factors make to Bd disease in anurans (Parris et al, 2004 and Voyles et al, 2009). The work contained in this dissertation belongs in this category. A combination of lab and field work was used to explore ways to artificially promote immunity to Bd, examined innate protective responses in individuals and the relationship of breeding to infection prevalence and intensity.

In chapter one, I assessed the efficacy of Bd immunizations to prevent disease in sub-adult *R. muscosa* following exposure with live Bd. This study found that immunizations were not protective against developing disease; no difference in mortality, infection prevalence, intensity or rate at which individuals became positive for Bd was found between groups. This work was initiated by the suggestion that the severity of chytridiomycosis was due in part to low antigenicity in Bd and failure of the immune system to recognize and clear infections (Berger et al, 2005). Recent work suggests that adaptive immune responses are not an effective way to combat Bd in *R. muscosa*; Andre et al (2008) found that housing temperature significantly impacted mortality in infected animals and Harris et al, (2008) found that innate immune

responses initiated by commensul bacteria was protective in animals exposed to disease. Adaptive immune responses in amphibians, while complex, are slow to develop (J. Roberts, personal communication) and can be dampened with cooler temperatures (Cooper et al, 1992). Using adjuvants to provoke an immune response to Bd in this experiment was unsuccessful but provides direct evidence that adaptive responses may not as protective innate immune responses. Adaptive immune responses in anurans are long lived and exhibit memory to pathogen which make them ideal, in theory, for resolving chytrid infections given the persistence of this pathogen within a population. This study was a direct test of this response against Bd and the result, while negative, supports existing work and suggests that recognition of disease in anurans by adaptive responses requires a more robust signal

In chapter two I compared mortality rates in sub adult *R. muscosa* that received larval exposure to Bd with those that were exposed only to broth culture media as larvae. Larval exposure was protective in sub adults when re-exposed to live Bd. This finding is significant to Bd research for two reasons. First, it suggests that amphibians can transition from larvae to adult frogs in the presence of Bd. Previous work had found survival of infected larvae through metamorphosis was very low in *R. muscosa* exhibiting clinical symptoms (i.e. mouthpart depigmentation) seen in larval Bd infections (Rachowicz et al, 2006). Metamorphosis is a period of immunosuppression and coincides with alterations in cellular protein expression and drastic morphological change. The major difference between my study and Rachowicz et al, (2006) was the severity of infection these individuals had prior to metamorphosis. The animals in my study were exposed to a high number of zoospores but had none of the clinical signs of Bd infection prior to metamorphosis. The second reason this particular study is important to Bd research is that it suggests that primary larval exposure primed some physiological response so that Bd was less pathogenic and virulent in sub adults.

In chapter three I conducted a two year field study examining associations between hormonal cycles in *R. muscosa* and disease prevalence in four populations. I was able to detect hormone cycles in corticosterone and testosterone in three out of the four populations but found no correlation with the prevalence or severity of Bd within each. Several studies have found both positive and negative correlations between these hormones and disease in vertebrates. This study was the first of its kind in ectotherms and the first to incorporate measurements of an infectious disease with hormone cycles and to suggest that a correlation between either may not exist in all vertebrates. We did find that the month of collection, temperature and collection site were significant predictors of Bd prevalence and disease severity. This finding is important evidence that Bd is regulated by environmental variables and could be used to support the claim that climate change has been instrumental in the emergence of this disease in anurans.

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Chapter One

Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*.

Mary J. Stice and Cheryl J. Briggs

Abstract
The fungal pathogen *Batrachochytrium dendrobatidis* (Bd), the causative agent of chytridiomycosis, has been implicated in amphibian declines worldwide. It has been hypothesized that low inherent immunogenicity in Bd may be related to the high rates of morbidity and mortality that are associated with Bd-infected anuran populations. To test this idea, juvenile Rana muscosa (mountain yellow-legged frogs) were immunized with adjuvants in combination with a formalin killed Bd culture to determine if it is possible to stimulate a protective immune response when challenged with a live inoculum of B. *dendrobatidis*. Three groups of juvenile R. muscosa (6 mo postmetamorphosis) were immunized with saline, Freunds Complete (FCA) and Incomplete Adjuvant (FIA), or the adjuvants in combination with a formalin-killed culture of B. *dendrobatidis*. The effects of immunization were modeled using survival analysis and a proportional hazards model. No significant differences were found between the groups in overall mortality, time to infection, infection prevalence, or intensity. While this study suggests that immunizing anurans against chytridiomycosis will not alter rates of infection or mortality among individuals, it does raise several questions regarding the attenuation and efficacy of anuran adaptive immune responses and whether they may be protective against this disease.

Introduction
Since *Batrachochytrium dendrobatidis* (Bd) was first described in association with amphibian declines in Australia in 1993, this fungal pathogen has been documented on almost every continent and linked to anuran declines worldwide (Berger et al 1998, Rachowicz et al 2005, Weldon et al, 2004, Bosch et al 2007). In recent years chytridiomycosis has become an important contributor to declines of *Rana muscosa* (mountain yellow-legged frogs), a once common amphibian in the Sierra Nevada Mountains of California. The first report of Bd in *R. muscosa* was published in 2001 (Fellers et al 2001) but analysis of preserved *Bufo canorus* specimens suggests that the fungus has been present at least since the 1970's in California (Green et al 2001). Surveys of several hundred *R. muscosa* populations in the southern Sierra Nevada from 2002 to the present have linked the presence of Bd with declines of *R. muscosa* throughout its range (Rachowicz et al 2006). All life stages of *R. muscosa*, with exception of egg masses, have been found to be susceptible to infection with Bd (Rachowicz et al 2004) but only metamorphosing and adult animals die in response to infection. Disease progression in field caught and lab infected larvae found that in both groups individuals succumbed to infection with Bd shortly after metamorphosis with mortality rates as high as 96% in the field (Rachowicz et al, 2006). Mortality rates in Bd exposed adult *R. muscosa* have also been recorded as high as 95% under laboratory conditions (Andre et al 2008).

While the mechanism behind the pathogenesis and mortality in chytrid infections is yet to be fully determined, recent work has shown that severe chytrid infections in *Litoria caerulea* are associated with decreased levels of sodium, potassium and chloride ion in blood plasma (Voyles et al 2007). Chytrid infections are localized to the outmost layer of the amphibian epidermis, the stratum corneum, which may interfere with the uptake of ions across the skin and basic osmotic requirements in blood and lymph fluid.

The number of extant uninfected *R. muscosa* populations has been decreasing each year and the threat that chytridiomycosis poses to remaining populations of *R. muscosa* has raised concerns. Prevalence of Bd in Sierran populations was found to increase rapidly following

arrival of the pathogen at a site, with rates of mortality approaching 100%, in both juvenile and adult frogs (Unpublished data). In contrast, other species of amphibians appear to be resistant to developing disease following Bd exposure (Hanselmann et al 2004). These findings have led to questions about preventing infection and mortality from Bd in amphibian communities and demonstrate the need for more additional work on protective immune responses.

What makes declines in *R. muscosa* and other species so difficult to understand is that anurans have immune systems with near the same complexity of other vertebrates. Amphibians have immune responses found in mammals; innate and adaptive responses capable of recognizing a broad spectrum of pathogens. This includes specific responses to pathogens through cell mediated responses (Rau et al 2001) and antibody isotype variability (Du Pasquier et al 2000). To date, there has been no evidence to suggest either a robust inflammatory response from the skin (Nichols et al 2001) or an innate immunity and antimicrobial peptide release that limits Bd infections or disease (Rollins-Smith et al 2006). The current study was motivated by the suggestion that low antigenicity of Bd allows infection and resulting disease to progress before an effective immune response develops (Berger et al 1999). This immunologic response during vaccination may be promoted with an adjuvant and these have been successfully used in frogs to increase the immunogenicity of haptens (Pross et al 1976).

While innate responses (i.e. antimicrobial peptides) have been shown to be efficacious at killing Bd in *R.muscosa* (Rollins-Smith et al 2006), primary responses in general are short lived, have a finite limit to clearing an infection, and exhibit no memory to pathogens. An adaptive immune response is more specific, can be sustained for longer time periods and mounts a memory response to antigens upon re exposure.

In this study, we tested the assertion that low immunogenicity in Bd infections leads to high rates of infection and mortality in juvenile *R. muscosa*. We hypothesized that immunization with a killed chytrid adjuvant mixture would lead to lower rates of infection and mortality when animals were subsequently exposed to live cultures of Bd.

Materials and Methods

Rana muscosa egg masses were collected from Sixty Lake Basin in Sequoia Kings National Park in the summer of 2002 and raised in the Office of Laboratory Care facility at the University of California at Berkeley. Animals were housed at 17 C for the duration of their care and all experiment procedures. Tanks for tadpoles were cleaned twice weekly and tadpoles were fed Purina Rabbit Chow ad libitum at each cleaning. At front leg emergence (FLE) metamorphs were switched to tanks with approximately 250 mls of water. At Gosner stage 45, tank water was reduced again to 150mls and a folded stack of unbleached paper towels measuring 15x12x0.5cm was added as a substrate. Tanks for post-metamorphic frogs were changed weekly. Beginning at Gosner stage 46, juvenile frogs were fed 15-20 crickets and dusted with calcium carbonate once weekly. Juvenile frogs were maintained until 6 mo post-metamorphosis before beginning any experimental procedures. At the termination of this study animals were assigned a health score based on the following criteria; 1= jumping and righting responses normal and no accumulation of shed skin in tank, 2= jumping and righting responses normal, accumulation of shed skin in tank and 3= abnormal jumping and righting responses and accumulation of shed skin in tank. All animal identification was coded prior to the termination of the study to prevent bias in this score.

Animals were randomly assigned into three groups at the start of the experiment and housed individually for its duration. Adjuvants used in this study have been used successfully in

fish and other anurans (Olivier et al 1985) and in promoting the recognition of haptens by the anuran immune system (Pross et al 1976).
Control animals received a saline immunization prior to exposure to live chytrid. The adjuvant only group received a 1:1 immunization of saline to Freunds Complete Adjuvant (FCA) and 1 mo later received a 1:1 of saline with Freunds Incomplete Adjuvant (FIA). The final group received a 1:1 of formalin killed chytrid in FCA and 1 mo later received a formalin killed chytrid preparation in FIA. All injections were restricted to 0.05cc and were administered into the dorsal lymph sac.

Cultured Bd (LJR 119) originally isolated from one larval *R. muscosa* collected in 2002, was used for immunizations and live exposures in all three groups. Animals in each group received exposure to the same number of live zoospores, 10^5 zoospores on the same day as counts were performed. This one time exposure occurred 1 mo after the final immunization with either saline, adjuvant only, or adjuvant and Bd. Plates were flooded with 1ml of filtered water (the same water source as that used for animal care) and allowed to sit for 20 min. Zoospores were pooled and counts were determined according to methods in Daszak et al. (2004). For the adjuvant and Bd immunizations an aliquot containing 10^5 zoospores was dispensed into eppendorf vials followed by 1ml of 10% formalin (Fisher Scientific, Pittsburgh, PA) and allowed to sit at room temperature overnight. Vials were spun for 10 min at 15, 996 x G. All but 50 µl was removed from vials after centrifugation and 1 ml of saline (Abbott Laboratories, Abbott Park, IL) was added and vials were centrifuged for 10 min at 7,000 x G. This step was repeated twice more to remove the formalin from the aliquot of Bd. After the third wash all but the last 50 µl was removed and to this 50 µl of FCA or ICA was added and used for injections. Bd and adjuvant combinations were mixed via syringe ten times prior to their administration. An aliquot delivering 10^5 zoospores was administered to each animal in all groups. The inoculum was mixed by inversion twice before being drawn and delivered to the next animal. Animals remained in tanks for 24 hr after the inoculum was added and before fresh water was replaced in the tanks. Each individual was monitored every week for infection intensity (Bd load) with swabs of the ventral surface of each animal as described (Hyatt et al. 2007). Targeted areas for swabs included the drink patch (lower ventral surface of abdomen), inner portions of both hindlimbs and webbing of each foot. Swabs were processed using a real-time PCR protocol developed by Boyle et al (2004). Zoospore load determined from the real-time PCR protocol represents the total number of copies of Bd DNA on the swab. All analyses were performed using JMP 5.1 statistical software.

Results

Immunization had no significant effect on the proportion of frogs that became infected with Bd (Table 1; 1-way ANOVA between groups, $P > 0.4$) or on the time to Bd infection (Fig 1). A failure analysis was performed to determine if immunization affected the time to Bd detection in each individual, censoring for individuals who remained negative for the duration of the experiment. No significant difference was found between immunization group using either the Wilcoxon or log rank tests ($P>0.5$ for both tests).

Immunization had no effect on the proportion of Bd infected individuals that died prior to the end of the experiment at day 108 post-exposure (Table 1, 1-way ANOVA between treatments, $P>0.9$). A Kaplan Meier curve was generated using days survived as the response variable and grouping animals according to treatment. Data was censored for animals that were

found dead and those that were euthanized at the termination of the experiment. No significant difference was found between groups using either the log rank or Wilcoxon tests (P>0. 6 for both tests). The majority health score recorded for all animals was 2 which represented an animal that had normal jumping and righting behavior with an accumulation of shed skin in the tank at the final reading. A health score of 2 was assigned to animals in each treatment group in the following proportions; the saline only immunizations (0.9), adjuvant only (0.5) and adjuvant Bd combination (0.8). The other score assigned to animals was recorded as 1 and describes normal behavior and no abnormal shedding present in tanks. No animals in any group received a health score of 3 which denotes an animal unable to right itself, jump and has a large amount of shed skin in its tank at the time of observation.

Infection intensity (zoospore load) was measured by real-time PCR performed on weekly skin swabs from each animal. Many animals lacked a complete swab sampling record and one animal was excluded in the graphs and survival analysis because at day 0 its swab result was positive for Bd. There was no significant difference between the groups in the maximum zoospore load observed per frog (Table 1, 1-way ANOVA, $P > 0.4$). The maximum zoospore load usually occurred at the time of death. There was no significant difference between the groups in the rate of increase in zoospore load (calculated as the slope of a straight line fitted to the time trajectory of ln (Bd load) for each individual; Table 1, 1-way ANOVA).

Discussion

In this study we found no evidence that immunization against Bd, using killed Bd and/or adjuvant, protects animals against infection or mortality due to chytridiomycosis. Thus, stimulation of the adaptive immune response is unlikely to be an effective conservation strategy to protect threatened amphibian populations from this lethal disease. However, several factors may have affected these results. Our study did not include a negative control group and there is the possibility that mortality may have been related to other infections. The presence of additional mortality factors is unlikely as no individual at the termination of the study received an abnormal health score; this should have been observed if other infectious amphibian diseases were present (Densmore et al, 2007). During the course of these experiments, common amphibian bacterial infections were managed by housing animals individually and by coordinating the feeding and cleaning schedules to minimize any bacterial growth in tanks.

Given the adverse reactions associated with Freunds adjuvant (Powers et al, 2007) it was assumed that this particular adjuvant would be extremely irritating. For this reason the amount of adjuvant used was kept to a minimum (less than half of what had been used previously in anurans and had been successful; Pross et al 1976). We conducted a 60-day pilot study using varied amounts of adjuvant (25 µl to 100 µl) and observed no adverse effects. Future studies investigating tolerable amounts of adjuvant in ectothermic vertebrates may be justified as increased adjuvant concentration may promote a more protective immune response. The type of adjuvant and site of inoculation may also be important factors in facilitating an immune response in anurans and these should also be considered in future studies. Adjuvants other than Freund's, such as Ribi and Titermax adjuvants have been formulated to allow for less toxicity and more direct induction of cell mediated and humoral responses; however, these have not been tested in ectotherms.

Temperature also may have influenced our results, and in our experiments, animals were kept at 17 C. It has been shown that Bd can be eliminated in *R. muscosa* at temperatures of 22 C

(Andre et al 2008). Because 22 C is within the range of optimal Bd growth, this suggests that disease was eliminated due to a host response rather than limited growth of the pathogen. Low temperatures (4C) have been shown to cause the loss of lymphocytes in the spleen and thymus of adult *Rana pipiens* (Cooper et al 1991). Temperature also has been shown to be an important factor in susceptibility to *Bd* infections (Woodhams et al 2003). Based on work done in the most basal members of the order anura, we know they have immune systems that include both adaptive (Du Pasquier et al 1989) and innate responses (Conlon et al 2004). Work is just beginning to look at how both innate and adaptive responses are involved in natural infections, particularly those associated with amphibian declines.

There is no evidence to date that adaptive immunity plays any part in resolving chytrid infections. Histological examination of anuran skin in chytridiomycosis infections in *Dendrobatid* spp. revealed only low numbers of neutrophils and macrophages (Nichols et al 2001). Survival of Bd-infected anuran species may be most dependent on the innate immune response and antimicrobial peptide production rather than any adaptive immune response; however, Bd is an intracellular pathogen (Berger et al 1998) and anurans can mount protective adaptive responses to intracellular pathogens (Morales et al 2005).

Rana muscosa antimicrobial peptides are capable of inhibiting Bd growth in vitro (Rollins-Smith et al 2006) but the species is highly susceptible to infection with Bd. The work by Rollins-Smith suggests that innate immunity and the repertoire of antimicrobial peptides produced by *R. muscosa* may not be the determining factor in limiting Bd infections. The role of adaptive immunity in limiting this disease is not currently understood but may be important.

Our study investigated the net effect of a putative adaptive immune response on frog infection and survival, but did not look directly for increased antibody production following immunization. Existing ELISA protocols to determine antibody titers in anurans have been developed for *X. laevis* and have exhibited little or no cross reactivity in some anuran species like *R. muscosa* (Jeremy Ramsey, personal communication). Our lab is currently developing a *R. muscosa* specific ELISA protocol to measure humoral responses to chytridiomycosis. Although the results of our study do not offer any new methods for protecting *R. muscosa* and other threatened species from Bd it suggests that anurans may not become immune when exposed to inactive Bd and presents a starting point for future work on establishing effective ways to combat infections in threatened amphibian populations.

Table and figure legends.

Table 1. Effects of experimental treatment on infection prevalence and mortality. Shown is the fraction of frogs in each group that tested positive at some point during the experiment (fraction infected) and the fraction of individuals that died prior to the end of the experiment (day 108). No difference in Bd growth nor for the max zoospore load was found between groups, 1 way ANOVA($p>0.2$ and $p>0.4$ respectively).

Figure 1. Failure of immunizations to prevent the development of *Batrachochytrium dendrobatidis* (Bd) infections. Number of days post exposure to live Bd until individuals were noted as positive, measured by real time PCR. There was no difference between groups in the proportion of individuals that became positive for Bd (log rank $\chi 2 = 1.29$, $p > 0.05$).

Figure 2. **Survival analysis of immunization and subsequent mortality in *R. muscosa*.** Mortality amongst groups receiving saline, adjuvant only or adjuvant and killed chytrid immunizations is modeled using a Kaplan Meier curve. Neither difference in mortality nor the rate at which animals died was found between (log rank $\chi 2 = 0.53$, $P>0.05$).

Figure 3. Infection prevalence over time in animals receiving saline immunization, measured via qPCR. Swab results for nineteen individuals examining the rate of Bd infection represented by "zoospore load" after saline immunization. Approximately 21% of controls died before the experiment ended; one of which was found dead at the end of the experiment, at day 108 which had remained negative for Bd infection for the duration of this study. Seventy-nine percent of animals in this group were positive for Bd.

Figure 4. **Bd infection prevalence measured via qPCR, in animals receiving adjuvant immunizations.** Animals in this group received a primary immunization with Freund's Complete Adjuvant and a secondary immunization with Freund's Incomplete Adjuvant one month later. Thirty-five percent of animals either died (n=6) or were euthanized (n=1) before the end of the experiment at 108 days and 90% were infected with Bd.

Figure 5. Bd infection prevalence measured via qPCR in animals receiving adjuvant and killed Bd immunizations. Animals in this group received a primary and secondary immunization with Freund's Complete Adjuvant one month apart from each other. Forty percent of animals either died (n=6) or were euthanized (n=2) before the termination of the experiment and 75% of animals tested positive for Bd at some point during the experiment.

Table 1.

Immunization Group	Saline/Control	FCA/ FIA Saline	FCA/FIA:Killed Bd
Sample size, n	19	20	20
Fraction infected	0.79	0.9	0.75
Fraction dead	0.21	0.35	0.4
Maximum Bd load (mean ± SE)	5106 +/- 6487	17831 +/- 12922	53990 +/-78843
Bd growth rate (mean ±SE)	0.089 +/- 0.017	0.077 +/-0.036	0.078 +/-0.017

Figure 1.

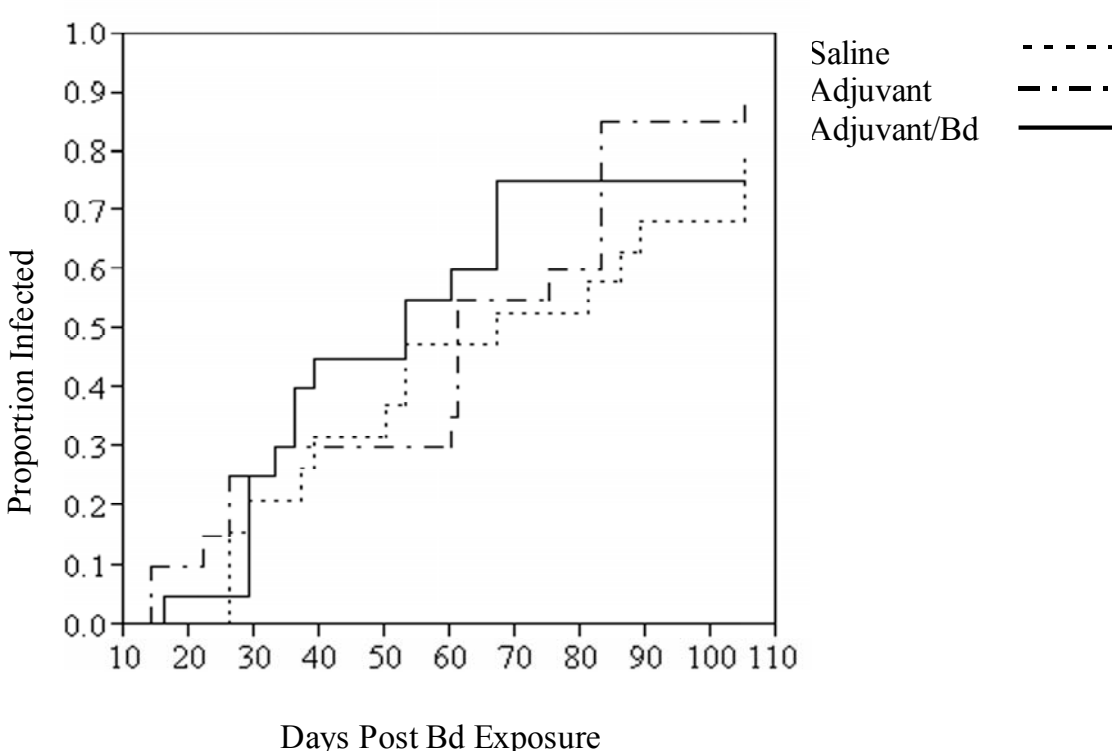
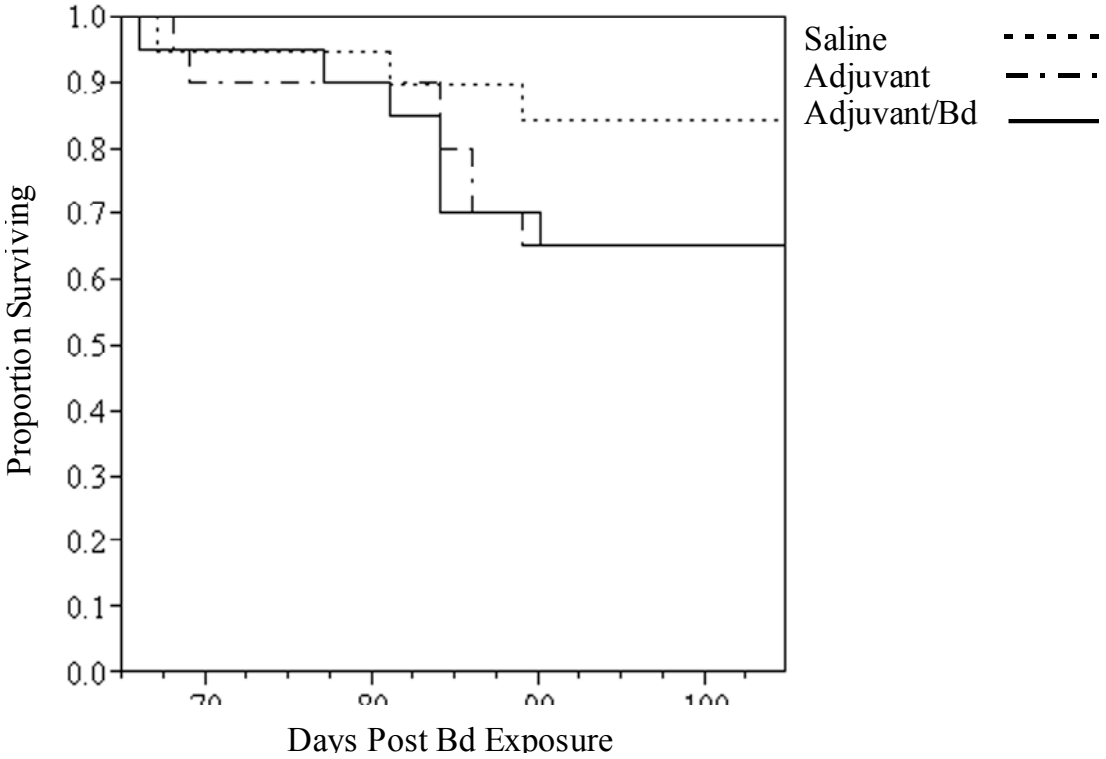

Saline
Adjuvant
Adjuvant/Bd
Proportion Infected
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10
20
30
40
50
60
70
80
90
100
110
Days Post Bd Exposure

Figure 2.


Saline
Adjuvant
Adjuvant/Bd
Proportion Surviving
1.0
0.9
0.8
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0.6
0.5
0.4
0.3
0.2
0.1
0.0
Days Post Bd Exposure

Figure 3.

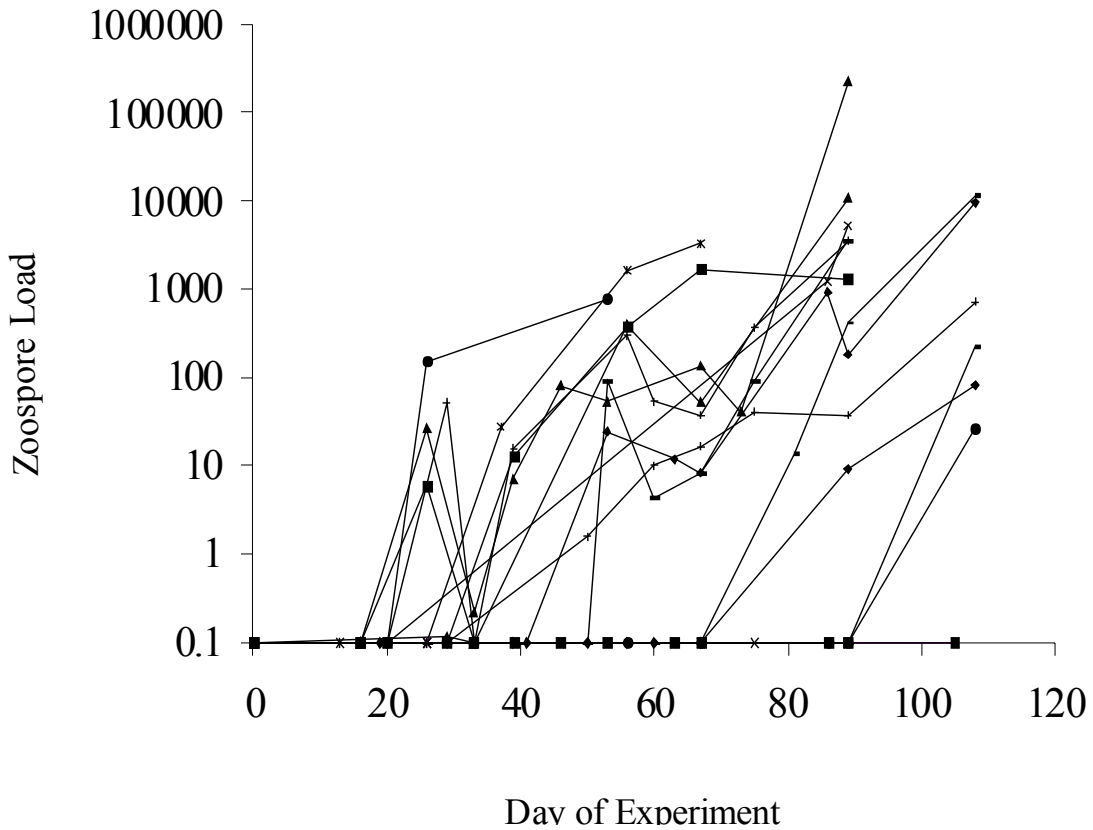
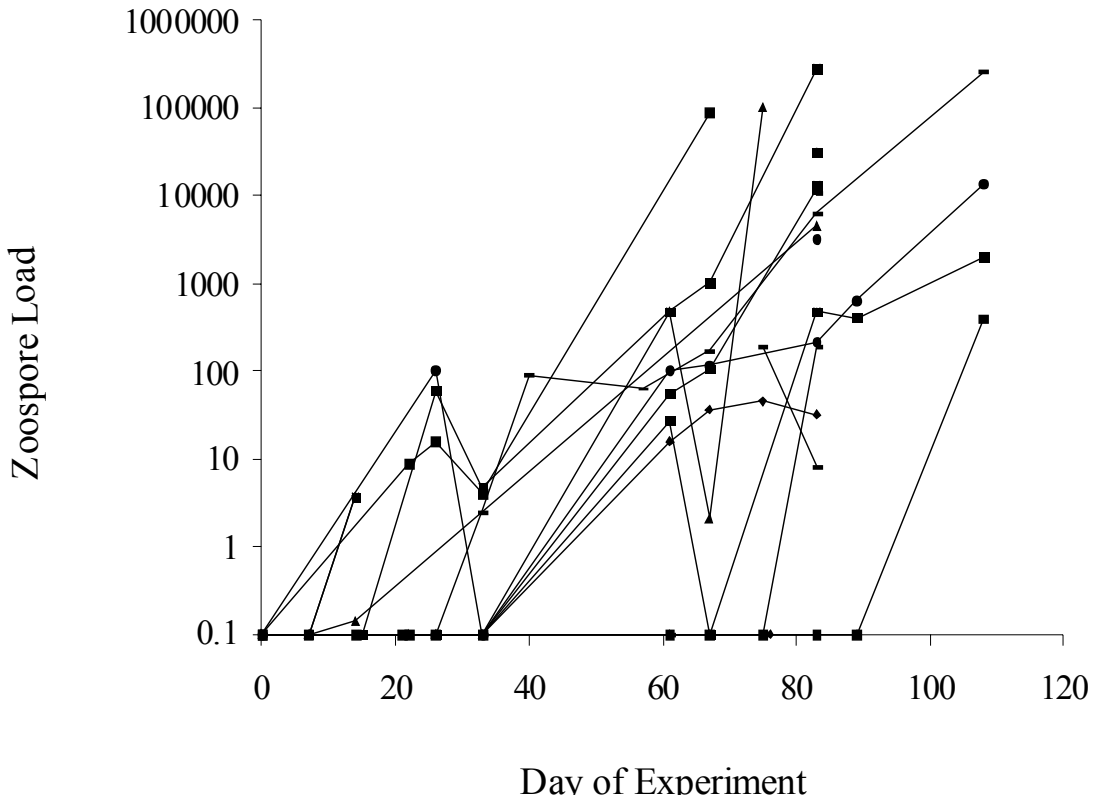

1000000
100000
10000
1000
100
10
1
0.1
Zoospore Load
0
20
40
60
80
100
120
Day of Experiment

Figure 4.


1000000
100000
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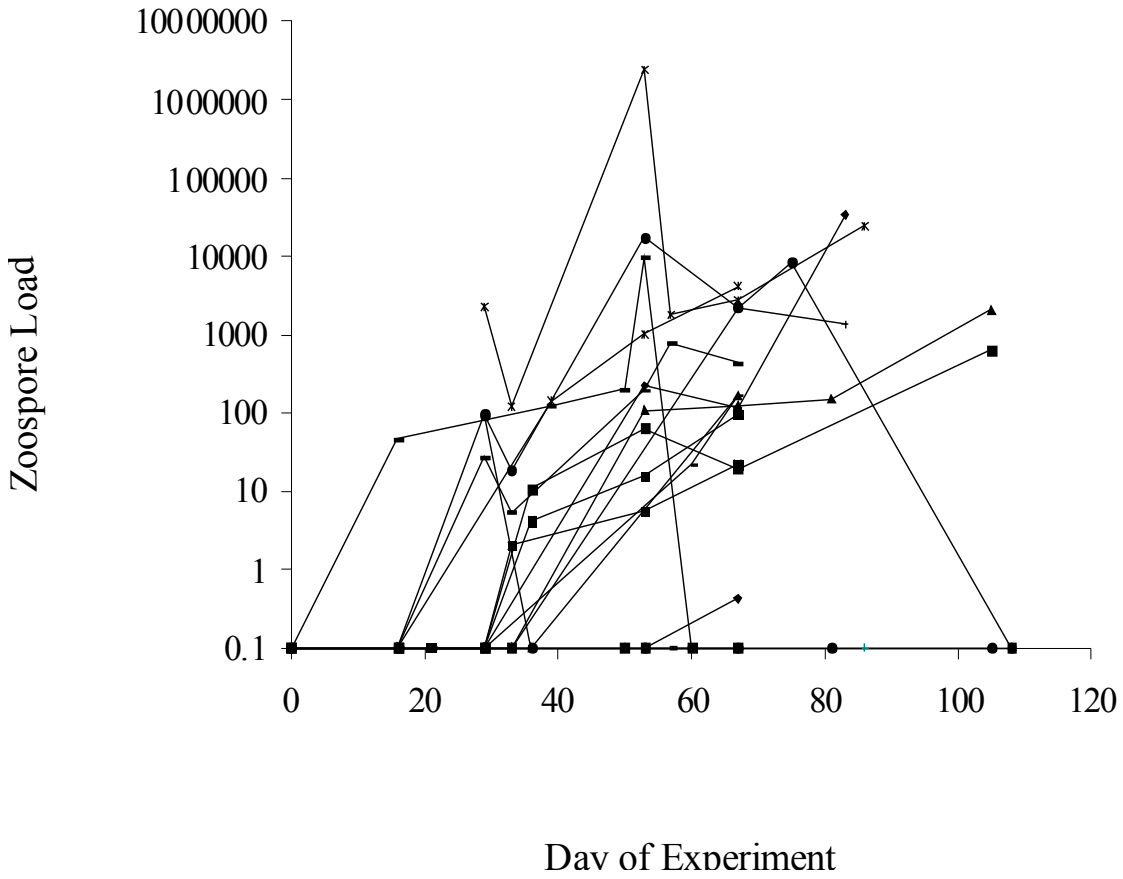
1000
100
10
1
0.1
Zoospore Load
0
20
40
60
80
100
120
Day of Experiment

Figure 5.


10000000
1000000
100000
10000
1000
100
10
1
0.1
Zoospore Load
0
20
40
60
80
100
120
Day of Experiment

Chapter Two

Protective effects of exposure to *Batrachochytrium dendrobatidis* during larval development in *Rana muscosa* sub adults.

Mary Stice, Eleanore Sternberg, Tyrone Hayes and Cheryl Briggs

Abstract: *Rana muscosa* tadpoles were exposed to high doses of live zoospores from two strains of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), and followed through metamorphosis. No animals were diagnosed as positive for Bd infection neither during the larval stage nor throughout metamorphosis and no mortality was observed in animals during either developmental period. Tadpole mouthpart inspections were used to monitor for Bd infections in larvae. Behavioral and clinical symptoms characteristic of Bd infections in *R.muscosa* were used to diagnose infections in animals during metamorphosis.. Those same individuals were re exposed as sub adults to the same Bd strain they had been as larvae (experimental group) and monitored along with controls with no previous larval exposure to Bd using qPCR. Following the sub adult exposure experimental animals had significantly lower rates of mortality (logistic regression $\chi 2 = 8.84$, $p < 0.05$) compared to control animals but infection prevalence measured by qPCR did not differ between experimental and control groups (contingency table $\chi 2 = 7.53$, $p > 0.05$). However, infection rates were significantly different between animals exposed to either Bd isolate (contingency table $\chi 2 = 6.84$, $p < 0.05$). Survival analysis revealed significant differences in the rates at which animals died between groups (log rank $\chi 2 = 9.11$, $p < 0.05$) and time until Bd infections were detected (log rank, $\chi 2 = 10.84$, $p < 0.05$) Blood smears from surviving control and experimental animals were examined for differences in number and type of white blood cells. We found a three fold elevation in the number of neutrophils between experimental and control animals but were unable to detect any significant differences in white blood cells between animals exposed to either isolate. Mortality results suggest that larval exposure to sub-infectious doses of Bd can be protective against developing fatal Bd infections as young adult frogs. We suggest that related future work include molecular methods to measure the effects of quantitative Bd dose variation, repeated exposures and innate and adaptive immune responses between life stages in anurans to confirm and clarify the results of this study.

Introduction:

Chytridiomycosis has been implicated in declines of amphibian populations and species in Australia (Berger et al, 1998), Europe (Bovero et al, 2008 and Bosch et al, 2000), North (Frias-Alvarez et al,2008 and Rachowicz et al, 2006) and South America (Lips et al, 1999 and Lips et al, 2006). The Mountain Yellow Legged frog, recently split into two species, *Rana muscosa* and *Rana sierrae* (Vredenburg et al. 2007), are at particular risk of extinction from Bd. Historically, *R. muscosa* was the most abundant amphibian species encountered at high elevation lakes throughout the California Sierra Nevada. Dramatic declines have been noted in this species first from the introduction of trout for sport fishing (Knapp et al, 2000 and Finlay et al, 2007) and now from the emergence of Bd (Rachowicz et al, 2006). Bd has been found in populations of *R. muscosa* across the Sierra Nevada and this amphibian species is particularly vulnerable because of its life history traits which favor infection and maintenance of this disease (Woodhams et al, 2008); a long aquatic larval stage, temperatures which promote its growth and the overwintering of all stages in deep lakes. Bd is an aquatic fungus transmitted between anurans through an infectious motile zoospore stage (Longcore et al, 1999) with infection localized to keratinized tissue in both larvae and metamorphosed frogs. Bd can grow in a wide range of temperatures (Pitrowski et al, 2007), all of which fall within the mean for many lakes where *R. muscosa* is found. At sites where Bd has become an established disease there is little opportunity for animals to avoid contact with Bd either from each other or the water in which they are found. Yet, despite the dramatic reduction in populations with Bd and environmental factors that strongly favor its

growth and persistence, individuals of all life stages are found between years at most sites (unpublished data). This means that some individuals are surviving as tadpoles, persisting through metamorphosis, reaching maturity and reproducing even in the presence of Bd (Briggs et al, 2005). This suggests that there are variables that can influence the outcome of infection and may even enhance survival within infected populations. This study three of these potential influences; the effects of variable Bd exposure between anuran developmental periods and the role adaptive immune responses in the pathogenesis and mortality associated with Bd infections in *R.muscosa*.

The influence of developmental period on infectious diseases in amphibians is important in the larger context of amphibian declines because each life stage coincides with variable immune function. Larval amphibians are known to have adaptive immune responses but they are much less complex than in adult frogs (Du Pasquier et al, 1979). It is also known that anurans go through a period of immune suppression as self antigens change expression patterns transitioning from larval to adult tissues during metamorphosis (Rollins- Smith,1998). Metamorphosis is associated with high rates of mortality and morbidity in Bd infected *R.muscosa* (Rachowicz et al, 2006). High rates of mortality in newly metamorphosed frogs is common enough in anurans that the term post metamorphic death syndrome (PDS) has been used to describe the observed mortality associated with this developmental period. Multiple infectious causes are associated with PDS and mortality associated with this life stage is likely influenced by this transient immune suppression (Wright et al,2001). PDS mirrors many characteristics of infectious diseases, particularly those of Bd infected *R.muscosa* populations with outbreaks being linked to cooler temperatures and involvement in some but not all species at outbreak locations (Wright et al, 2001). It is unclear how mortality from infectious causes in newly metamorphosed frogs influences population estimates over long periods of time. Given the immunological constraints of anurans during metamorphosis, infection with Bd at this period in the life cycle may be a major factor in declining amphibian populations.

A strong argument for the recognition and resolution of Bd infections in *R.muscosa* and other amphibians can easily be made. Amphibians have immune systems that are capable of complex responses similar to other vertebrates in many respects; humoral immunity, isotype variability, CTL and T helper cell responses (Du Pasquier et al, 1989). They are highly pathogen specific and capable of resolving infections (Maniero et al, 2006). Anurans also have memory responses that persist from the larval period through metamorphosis (Rollins-Smith, 1998). Innate immune responses have been shown to be important in clearing Bd infections; antimicrobial peptide release and colonization with commensul skin bacteria have been shown to reduce growth of Bd in vitro (Rollins-Smith et al, 2006) and even clear Bd infections (Harris et al, 2009) in *R.muscosa* Because so little is known about adaptive responses in anurans to Bd we chose to measure the number and type of white blood cells between experimental and control animals. The use of blood smears in amphibians has been used to ascertain the effects of temperature (Raeffel et al, 2006), stress (Davis et al, 2008) and toxic compounds (Garavaini et al, 1978) and is a reliable measure of detecting differences in adaptive responses (Wright et al, 2001). The goal of this study was twofold; to test for protective effects against Bd infection and mortality from a primary larval exposure and determine if adaptive immune responses between individuals varied accordingly

Materials and Methods:

All *R.muscosa* used in this study were reared in the laboratory from egg masses collected from one lake in Sixty Lake Basin, Kings Canyon National Park in 2003. Larvae were housed individually and tanks were changed once weekly through metamorphosis, after which tanks were changed twice weekly. Larvae were fed approximately 5 grams of Purina Rabbit Chow on the day prior to tank changes. Metamorphosing frogs were fed 5-10, 1-2 week old crickets dusted with calcium carbonate. Following metamorphosis animals were pooled into tanks separated by Bd isolate to which they had been exposed as larvae. Animals were maintained in these communal tanks for two months prior to the sub adult Bd exposure. Sub adults were housed individually, in 250mls of filtered tap water for one month prior to the second inoculation.

***Batrachochytrium dendrobatidis* culture:** Two Bd cultures were used for both the larval and sub adult exposures, JAM 11 and JAM 68. JAM 11 was originally cultured from a wild caught *R. sierrae* tadpole from Mono Pass, Yosemite National Park in 2003, and JAM68 from a *R. muscosa* tadpole collected from Hitchcock Lake, Sequoia National Park in 2003. Bd zoospore doses for all larval exposures, were of liquid Bd culture and quantified by methods described in Daszak et al 2004. Sub adult exposures were done using Bd that had been grown on 1% tryptone agar plates for approximately two weeks prior to exposure. For both larval and sub adult exposures inoculations occurred the same day as counts.

Primary Larval Bd exposure: Each isolate was used to inoculate a total of 40 animals, 20 animals received a single exposure, and 20 animals received repeated exposures over a five day period. One control group of 10 animals was exposed to 1% tryptone broth alone; five animals were exposed to a one time 1 ml dose of tryptone broth and five animals were exposed to a 1 ml dose of 1% tryptone broth, each day, over a five day period. The same number of zoospores, $2.5x10^6$, was used for exposures for both isolates (JAM11 or JAM68) and exposure scheme (one time exposure or five day exposure). Animals receiving a one time exposure received $2.5x10^6$ zoospores on one day and those receiving the repeated dose received approximately $5x10^5$ zoospores once a day for five days.

One month after the final exposure and once a month thereafter a subsample of ten animals from each group was monitored for infection by examining mouthparts and scoring pigmentation loss. Tadpole mouthpart inspections occurred for two months. The upper and lower beaks and toothrows were examined and pigmentation status was scored as fully pigmented, partially pigmented or fully depigmented. Because PCR methods were not yet readily available, weekly mouthpart inspections of tadpoles were used to identify Bd infections following larval Bd exposure. This method is less sensitive than the qPCR protocol (Boyle et al, 2004) now used by many labs to diagnose chytrid infections in amphibians. Nonetheless, this method is able to provide a fairly accurate diagnosis of Bd infection in *R. muscosa* (Knapp et al, 2006). We monitored larvae for infection using this technique up until Gosner stage 42.

Animals at Gosner stages 42-46 were monitored diagnosed for Bd infection based on characteristic clinical signs seen in captive animals infected with Bd (Nichols et al, 2001 and Parker et al,2002). These signs included noting an accumulation of shed skin in tanks, lethargy and the inability to right themselves after handling. These observations were made every three to four days once an individual was recorded at Gosner stage 42 and lasted on average for two months for each individual. When metamorphosis was complete, Gosner stage 46, the first stage of the experiment was ended and animals were put into communal tanks separated by Bd isolate

to which they had been exposed as larvae. Animals were maintained in communal tanks until the sub adult Bd exposure occurred.

Secondary Sub adult exposure: One to two mls of Bd broth cultures were plated onto 1% tryptone agar plates and grown for 13 days before live exposures. Counts were performed the same day as exposures by methods described in Daszak et al 2004. The control groups for the sub adult exposure were animals from the same egg masses and cohort that were also housed communally prior to the second phase of the experiment. Control groups had 14 and 13 individuals in either isolate group. Experimental groups with previous larval exposure to either JAM11 or JAM 68 had 14 and 15 individuals respectively. Each animal was exposed to 1x 10^5 zoospores once by placing the inoculum directly onto the back of each animal. Tanks were not changed for three days after inoculations were performed. Animals were monitored after the secondary sub adult exposure for 87 days.

qPCR; Quantitative (real-time) PCR and methods described in Boyle et al, 2004 were used to determine infection prevalence from skin swabs prior to and following the secondary sub adult exposure to Bd. A sub sample of animals in each group was swabbed prior to the secondary sub adult exposure; JAM11 control: 10 swabs, JAM11 experimental: 10 swabs, JAM68 control: 10 swabs, JAM68 experimental: 5 swabs.

One month after the sub adult Bd exposure individuals were swabbed once a month. Animals having one positive Bd swab, designated by a ge (genomic equivalent) score greater than 0, at any point during the experiment were categorized as positive for the analysis. Each positive result obtained from qPCR was multiplied by 80 to account for the portion and dilution of DNA swab extractions used in qPCR reactions.

Statistical Analysis*:* SPSS 17.0 and JMP 7.0 software was used for all statistical analysis. Mortality amongst each group was analyzed using binary logistic regression. Individuals were coded 0 if they died during the experiment and 1 for animals euthanized at the termination of the study. All but one animal in the JAM68 experimental group was euthanized at the termination of this study. This animal suffered a seizure during an observation and was euthanized. Although the inclusion of this animal in the mortality analysis introduces some uncertainty into the final measurement we decided to include it in our final analysis. Infection prevalence between isolates and within both isolate groups was examined using a contingency table. Individuals were recorded as being Bd positive if they had at least one positive Bd swab at any time during the study. All ge scores were log transformed to satisfy assumptions of normality. The maximum Bd load by group was used as a measure of disease intensity and analyzed using ANOVA. The rate at which animals died during the experiment were compared between experimental and control groups using survival analysis. The rate at which individuals became infected was examined using survival/failure analysis. Individuals that were Bd positive but lost the infection during the experiment were censored and coded 1.

Blood smears; Blood was collected from remaining animals at the termination of the experiment and was used to make blood smears for each individual. Blood smears were stained using a 1% o -dianisidine solution and Wright Giemsa for contrast. Slides were read at 100x and the type and number of white blood cells were noted in a minimum of 5000 erythrocytes from each slide. Samples were pooled according to the exposure group to which the individuals belonged, either controls (n=4) or experimental animals (n=16). Blood smears were also analyzed for animals grouped according to whether they tested positive or negative for Bd for the duration of the

experiment. The Bd positive group consisted of two JAM 11 control animals and four JAM 11 experimental animals. The Bd negative group consisted of one JAM 68 control, three JAM 11 and eight JAM 68 experimental animals.

Results:

Infection prevalence and mortality following larval Bd exposure:

No larvae were diagnosed as chytrid positive based on mouthpart readings and pigmentation loss after the larval Bd exposure and were followed for. Mortality in larvae (Gosner stages 31-41) was low between all groups, reaching a maximum of 20% in the JAM11 experimental group receiving repeated Bd exposure. No mortality nor Bd infections were noted in individuals belonging to any of the four groups throughout metamorphosis (Gosner stage 42-46) No individual displayed the clinical symptoms associated with Bd infections for this portion of the study. The swabs taken from a subsample of individuals, prior to the second Bd exposure in both experimental and control groups were likewise negative for Bd.

Mortality following sub adult Bd exposure:

Mortality rates (figure 1) were significantly elevated in individuals in both control groups which did not receive a larval exposure to Bd (logistic regression $\chi2 = 8.84$, $p < 0.05$). The following proportion of animals were found dead in control and experimental groups; 57% for JAM11, 62% JAM68 and 29% JAM 11 and 27% JAM68 respectively. A survival analysis (figure 3) examined the rate at which individuals within groups died during the study and found significant difference between control and experimental animals (log-rank, $\chi2 = 9.83$, $p < 0.05$.) The exclusion of the JAM68 experimental animal, euthanized on day 74 because it experienced a seizure while being examined, did not affect the significance of this measurement. Survival rates amongst individuals positive for Bd followed the same pattern as seen for all individuals (figure 5); the JAM68 control group experienced significant mortality more quickly and the JAM11 experimental group more slowly than the other two treatment groups (log rank $\chi2 = 9.74$, $p < 0.05$). No difference in the rate at which animals died was found for Bd negative animals in each group, figure 6 (log rank $\chi2 = 2.51$, $p > 0.05$).

Infection prevalence prior to secondary sub adult exposure;

qPCR was used to screen a subset of animals in each group prior to the sub adult exposure. All available swabs of sub adults taken prior to the second Bd exposure were negative for Bd. All swabs from animals chosen for the control groups were also negative for Bd prior the secondary sub adult exposure.

Infection prevalence following sub adult exposure: The proportion of individuals that became infected differed between experimental groups with larval exposure and control groups for each culture used in this study. Significant differences were found in infection prevalence between animals by Bd isolate ($\chi2 = 6.84$, $p < 0.05$) but not between experimental and control groups exposed to either isolate ($\chi2 = 7.53$, $p > 0.05$). The following proportion of animals were Bd positive within each group; 86% of JAM 11 control and experimental groups were positive for Bd and the JAM 68 control and experimental groups had infection rates of 62 and 47% respectively. There was no difference in infection intensity measured using the maximum zoospore load from individuals between all groups (two way ANOVA, $p > 0.05$). . A survival analysis of time to infection (figure 4) did show a difference in the rate animals became infected between groups (log-rank $\chi2 = 20.9$, $p < 0.05$).

Blood smears; The number of individuals surviving in the control groups (n=8) and the number of slides that were able to be used in this portion of the analysis from them (n=4) prohibited any statistical tests to look at differences between groups. One experimental animal had a hundred fold higher count of heterophils and was excluded in all blood smear analysis. Experimental animals had a three fold higher proportion of neutrophils than controls but the elevation in neutrophils was no longer present when counts were examined with animals divided in Bd positive and negative groups.

Discussion;

We found that re exposing adult *R.muscosa* to the same isolate of Bd that they had been exposed to as larvae was associated with lower rates of mortality when compared to control individuals with no Bd larval exposure. This was the most significant finding of this study and opens up the possibility that the larval exposure was somehow protective when these individuals were re exposed to Bd as sub adults. We also saw significant differences in the rate at which animals died during the experiment and the rate at which individuals became infected between groups. There is evidence that control groups became infected and died more quickly than experimental animals. It is unknown from this work if the reduced mortality and infection rate were mediated by the variation in Bd dose between the larval and sub adult exposures or if an innate or adaptive immune response was ultimately protective. These are areas which remain questionable in this work and within Bd research as a whole. Immune responses and the effects of quantitative variation of Bd inoculum should be explored to potentially explain the survival we saw under the lab conditions used in this study and those that occur in natural populations of *R.muscosa*.

At each developmental stage in anurans there exists the capacity to mount immune responses to infections. Amphibian immune responses can be considered restricted because of their dependence on temperature (Cooper et al, 1992) but are capable of resolving infections nonetheless (Maniero et al, 2006). In terms of studied and successful immune responses to Bd infections, innate immunity appears to be protective in *R.muscosa* (Harris et al, 2009 and Rollins-Smith et al, 2006). However, it is unknown if the duration of these innate responses, which as a rule are typically short lived, are capable of protecting animals throughout the year. There are several more questions to explore involving these innate responses. First, it is known that antimicrobial peptide expression becomes detectable at metamorphosis in *X. laevis* (Reilly et al, 1994) but it is unknown if this is also true of ranid frogs. It is also unknown how commensul skin bacteria populations change over time in individuals and between species at the same diseased site. Similarly, how commensul bacterial populations may change over time on an individual, between life stages and populations may potentially be important areas to study. Populations of *R.muscosa* continue to become infected every year and remain infected from year to year so the role of innate responses being ultimately protective against mortality is unclear.

Our measure of adaptive immunity found an elevation in the number of neutrophils between the control and experimental groups but because of small sample sizes this requires further investigation. The role of amphibian neutrophils in infections is unknown but Wright et al 2001 has suggested that these cells may in fact be a type of senescent or immature monocyte or granulocytic cell. In other vertebrates monocytes and granulocytes are known to have phagocytic cell properties and responsive to chemical mediators at the site of infection and in circulation. Valuable information about infections can be gathered from blood smear analysis and its use should be considered in future work. Examining the composition and number of white blood

cells between groups does not allow us to rule out the possibility that specificity in these cells may differ among groups and have a role in the lower rates of mortality seen in the experimental group. More specific assays to measure specificity from adaptive immune responses, specifically to Bd infections, are sorely needed.

There is also the possibility that the dose of Bd early in larval development may be important in generating resistance later on. Lower mortality in experimental animals may be related to the number of zoospores that were used in the larval exposure. Experiments examining the transmission of Bd in different species typically use a one time exposure to a high number of zoospores in adults. This type of exposure probably does not reflect conditions animals are exposed to in the wild; Bd growth is influenced by temperature (Piotrowski et al 2004) and it appears to grow on a number of substrates besides amphibians (Johnson et al, 2003). As temperature and biomass fluctuate throughout the year it is likely that the number of zoospores that an individual will be exposed to will vary as well. In addition, there is evidence that the duration of exposure (Tunstall, 2007) and temperature's effect on host responses (Andre et al, 2008) influence the rate and intensity of infections in R.muscosa. Both of these laboratory studies mirror conditions animals are likely to encounter in natural populations; ponds with stagnant water and favorable temperatures are likely to increase the number of zoospores an individual comes in contact with, the duration of exposure as well as the hosts' immune response to infection. A one time exposure also does not account for the increase or fluctuation of zoospores released by other infected individuals within their communities. Similar work may want to consider the inclusion of multiple Bd dose exposure groups; from a high exposure that is associated with high rates of infection and mortality to low Bd exposures. Variable schedules should also be explored, from one time to repeated exposures to Bd.

That mortality and infection outcomes may vary between life stages in anurans presents some challenges to this type of work but could also be beneficial to our understanding of the dynamics of Bd infections within amphibian communities more thoroughly. For an animal to remain infected with Bd from the larval to juvenile stage, there must be a severe enough infection so that the pathogen can maintain itself through metamorphosis. The larval period in *R. muscosa* can be as long as four years and in populations where Bd has become endemic this ensures that animals are exposed to Bd for at least some portion if not the entirety of their larval development. The amount of pigmentation loss in larval anurans is proportional to the amount of Bd DNA detected in PCR reactions (Knapp et al, 2006). We did not see a significant loss of pigmentation in any individual after the larval exposure to Bd which could suggest that the animals in our study had either no or mild infections before they began metamorphosis. It is likely but unknown if mortality from Bd is related to the severity of infection prior to the immunosuppression during metamorphosis in infected anurans. Rachowicz et al, 2006 found high rates of mortality through metamorphosis in animals that had significant mouthpart pigmentation loss as larvae. The lower mortality we observed in experimental groups may have been related to the clearance or low level Bd infections animals had prior to metamorphosis. The lab and field studies conducted by Rachowicz et al examining mortality in Bd infected *R.muscosa* larvae suggest this is true but more work is required to clarify the relationship. This should be explored as it would allow researchers to target treatment to a specific age class that has enormous potential to stabilize population losses.

Recent work helps to explain the differences in infection prevalence between control and experimental groups for the Bd cultures we used in our work. It is known that Bd isolates with different passage rates can be associated with variable mortality rates (Berger et al, 2005) and so is not unusual that we found a similar variation of Bd virulence in this study. Recent work has shown that the pathogenicity in different Bd isolates is also related to genetic variation and differences in protein expression (James et al, 2009). As the methods advance for this type of work in Bd research it will be interesting to determine what allows a Bd isolate to cause more severe disease than another in an animal.

Our study showed that a species thought to be highly susceptible to infection with Bd, *R.muscosa*, had higher survival rates following prior larval exposure to Bd. While the findings of our work are promising, future studies need to replicate this relationship in order to validate the protective effects we saw between larval and sub adult exposure to Bd. Our work is suggestive that susceptible individuals and species may not be defenseless against Bd. Conditions that guard against infection and mortality may exist, either within environmental or physiological constraints that promote survival within Bd infected communities in nature.

Table and Figure Legends.

Table 1. White blood cell composition between groups with and without larval exposure to Bd. The average number and type of white blood cells were noted from blood smears taken at the end of the study for control animals receiving no larval exposure and experimental animals with larval exposure to Bd. For the analysis by infection status, Bd positive (2 control and 4 experimental animals) or negative (1control and 11 experimental animals) animals were found to have very similar proportions of all cells. No qPCR results were available for two animals and so they were excluded from this analysis.,

Figure 1: Mortality differences amongst individuals with and without previous exposure to Bd. Re exposure to the same Bd isolate as sub adults produced significant lower rates of mortality in Rana muscosa sub adults (Logistic regression ,chi square = 14.42, $p < 0.05$). Individuals with no previous exposure to Bd had higher rates of mortality with exposure to both Bd isolates.

Figure 2: Infection prevalence amongst sub adult *Rana muscosa* exposed to Bd. Significant differences were found between Bd cultures (JAM 11 or JAM 68)in the proportion animals infected (One way ANOVA, $F = 6.3$ $p < 0.05$). No significant difference was found between animals that received larval exposure and those with no previous exposure in the proportion of individuals that became infected within either Bd culture group, JAM 11 and JAM 68, (One way ANVOA, $F = 2.1$, $p > 0.05$).

Figure 3. Survival analysis of Bd positive individuals with primary and secondary exposure to Bd . The rate of mortality in animals that tested positive for Bd in each group was examined over the duration of the experiment, 120 days. A log rank and Wilcoxon test showed no significant differences between groups ($p= 0.08$ and 0.3).

Figure 4. Survival analysis of Bd negative individuals with primary and secondary exposure to Bd. The rate of mortality for animals negative for Bd was examined by group and no difference was found over the duration of the experiment, 120 days (log-rank, Wilcoxon $p = 0.9$ and $p =0.8$).

Figure 5. Survival analysis of Bd positive individuals post sub adult exposure. Significant differences in the rate at which animals died between groups was found following the sub adult Bd exposure. Those animals in the JAM68 control group died more quickly and those animals in the JAM11 experimental group more slowly than the other two control groups (log rank, $\chi 2 = 9.74$, $p < 0.05$).

Figure 6. Survival analysis of Bd negative individuals The rate of mortality for animals negative for Bd was examined by group and no difference was found over the duration of the experiment (log-rank $\chi 2 = 2.51$, $p > 0.05$).

Table 1.

%	Lymphocytes	Neutrophils	Azurophils	Basophils	Heterophils	Monocytes
Control n=4	95.7	1.3	0.9	1.8	0.3	0
Experimental n=16	93.2	3.9	1.1	1.3	0.5	0.3
Bd Positive Animals n = 6	92.8	3.9	1.5	1.2	0.5	0.1
Bd Negative Animals n = 12	93.3	3.3	1.0	1.6	0.5	0.3

Figure 1.

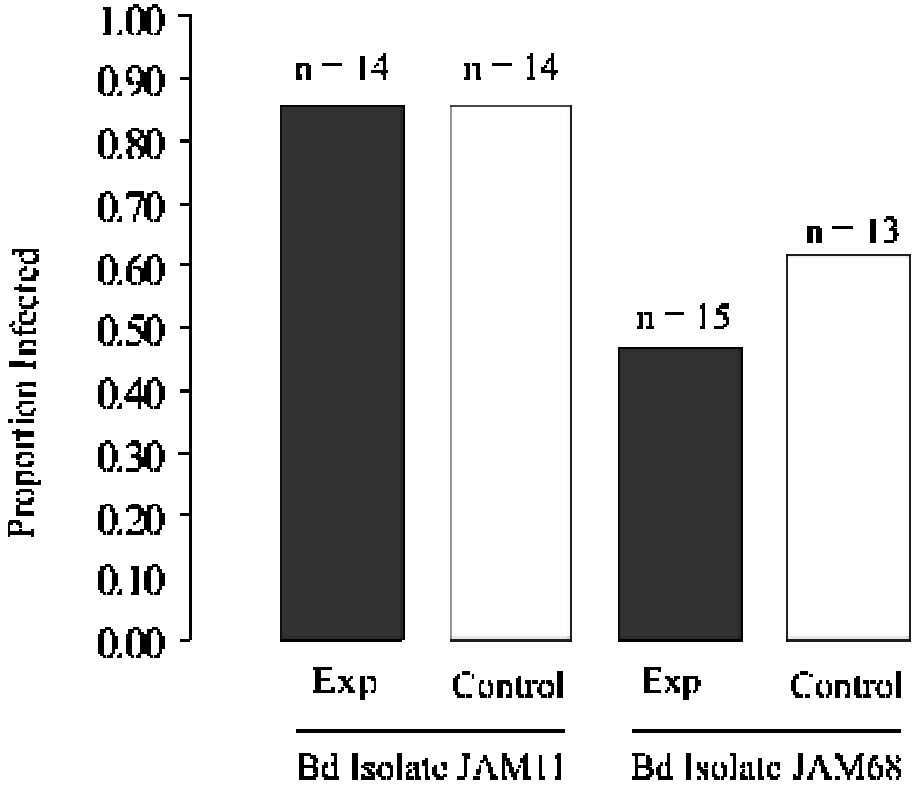

Proportion Infected
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
n = 14
n = 14
n = 15
n = 13
Exp
Control
Exp
Control
Bd Isolate JAM11
Bd Isolate JAM68

Figure 2.

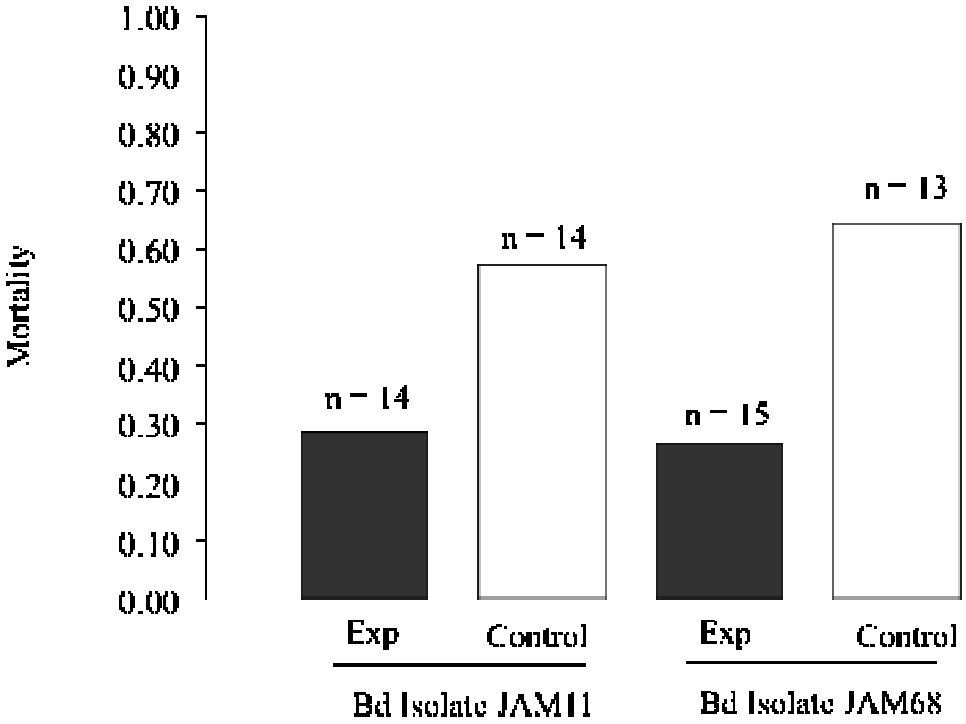

Mortality
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
n = 14
n = 14
n = 15
n = 13
Exp
Control
Exp
Control
Bd Isolate JAM11
Bd Isolate JAM68

Figure 3.

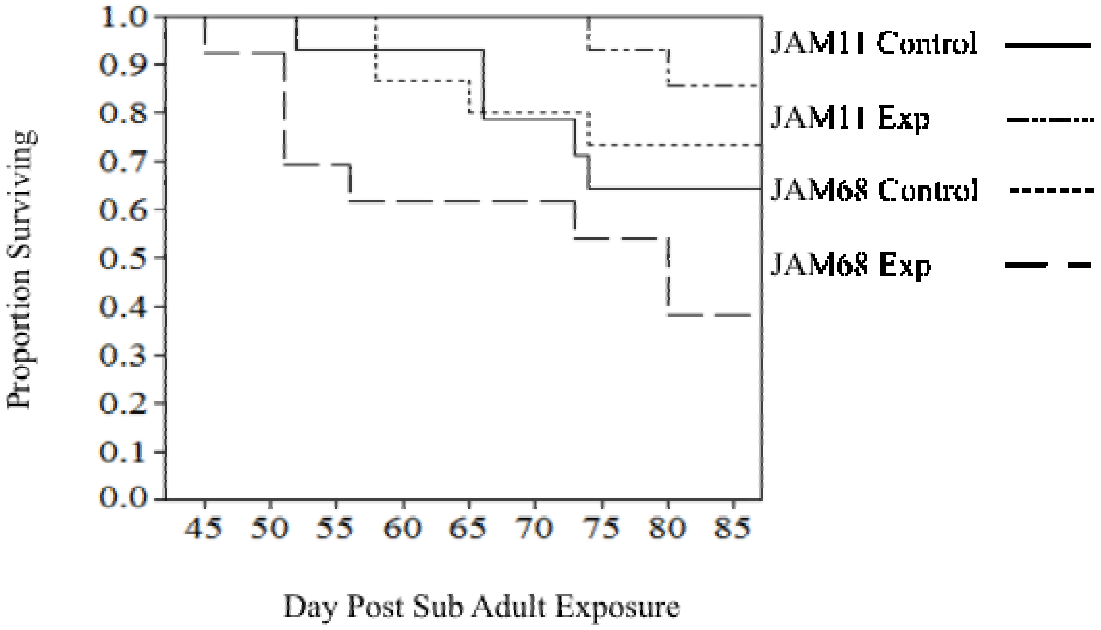
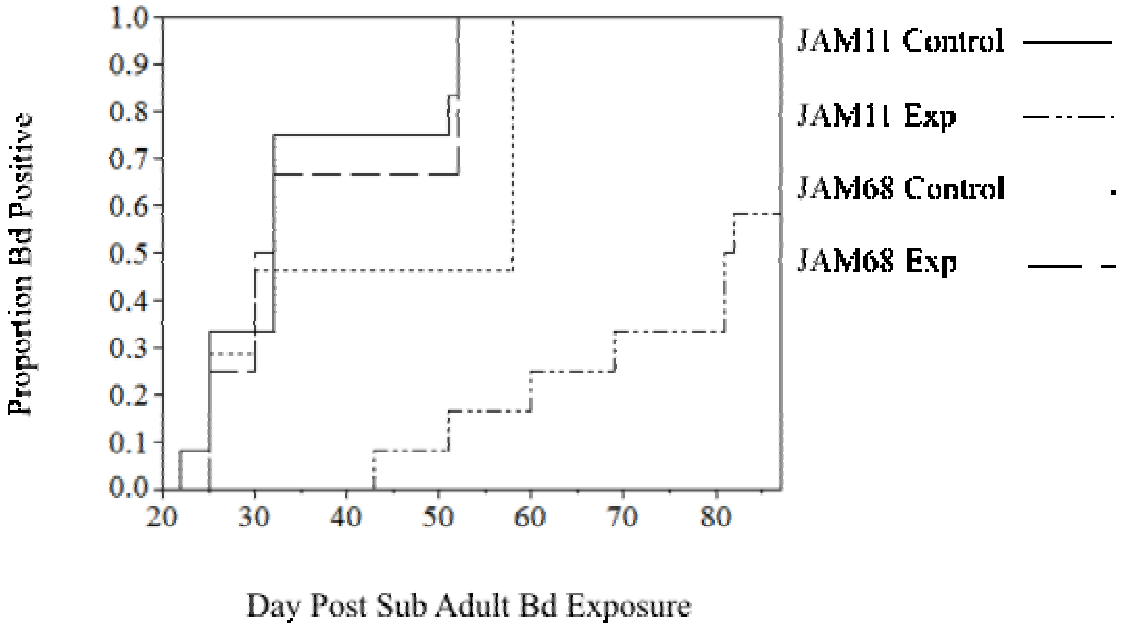

Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
45
50
55
60
65
70
75
80
85
Day Post Sub Adult Exposure
JAM11 Control
JAM11 Exp
JAM68 Control
JAM68 Exp

Figure 4.


Proportion Bd Positive
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0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
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20
30
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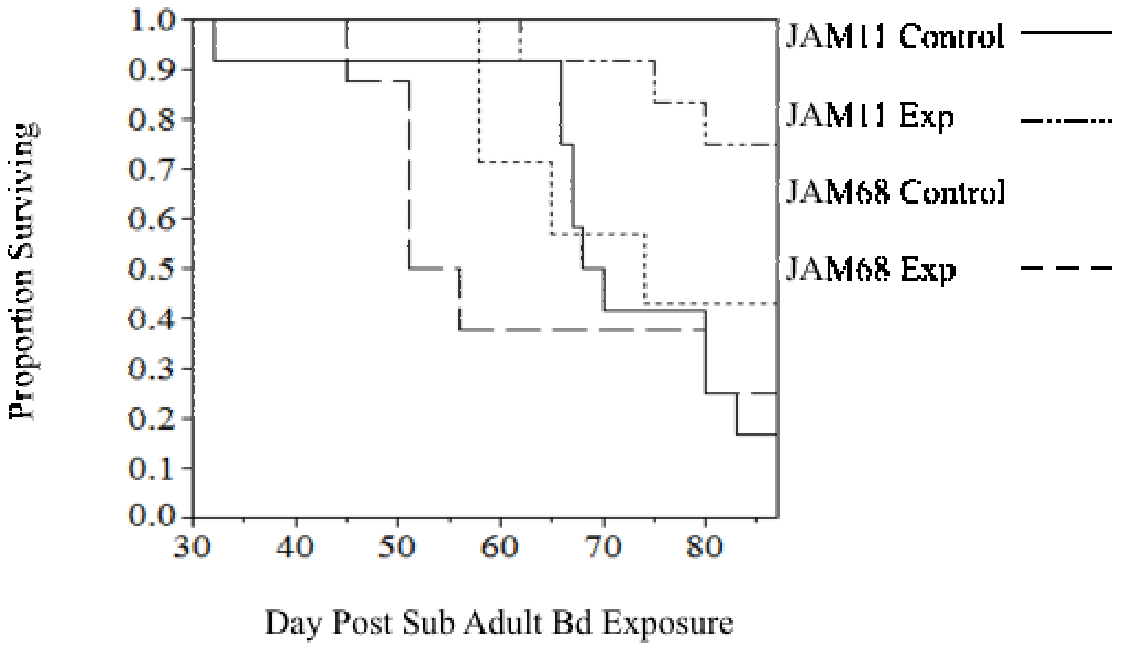
60
70
80
Day Post Sub Adult Bd Exposure
JAM11 Control
JAM11 Exp
JAM68 Control
JAM68 Exp

Figure 5.


Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
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30
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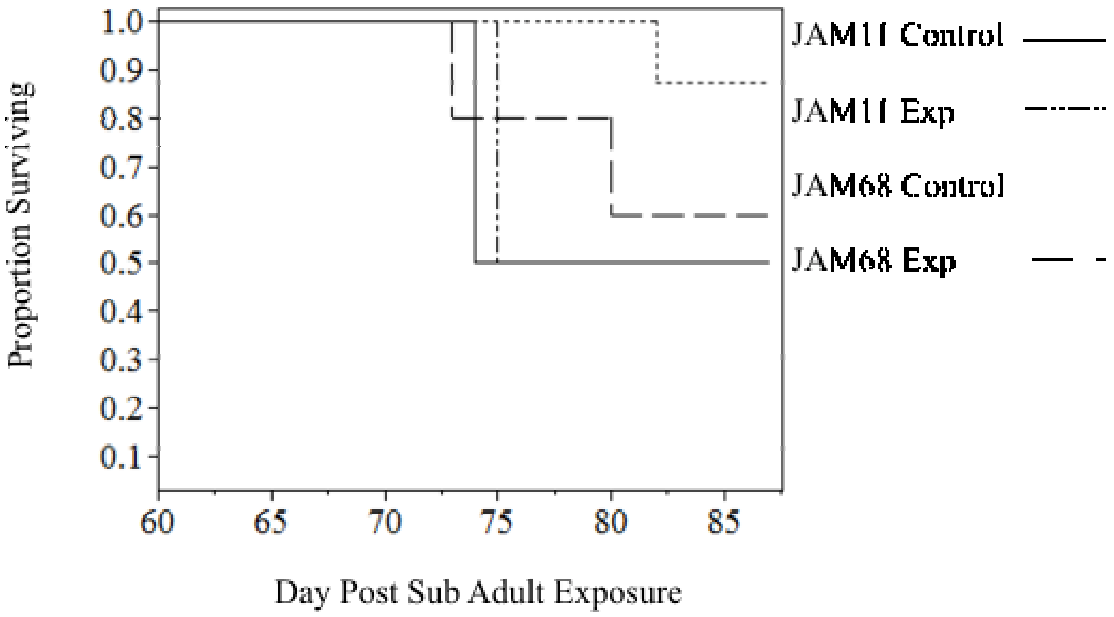
70
80
Day Post Sub Adult Bd Exposure
JAM11 Control
JAM11 Exp
JAM68 Control
JAM68 Exp

Figure 6.


Proportion Surviving
1.0
0.9
0.8
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0.2
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85
Day Post Sub Adult Exposure
JAM11 Control
JAM11 Exp
JAM68 Control
JAM68 Exp

Chapter Three

Endocrine influences of seasonal disease in the Mountain Yellow Legged Frog, *Rana muscosa*; testing associations between disease, stress and reproduction in a threatened amphibian species.

Mary Stice, Eleanor Sternberg, Natalie Reeder, Tate Tunstall, Vance Vredenberg, Paul Falso, Sherrie Gallipeau, Tyrone B. Hayes and Cheryl J. Briggs.

Abstract: The prevalence, spread and severity of wildlife diseases can be influenced by a number of variables; habitat structure, seasonal temperature changes and population density fluctuations. Recent work has also suggested that hormones involved in reproduction and stress for a number of vertebrates, corticosterone and testosterone, may have secondary effects on the immune system and its response to disease. We investigated the relationship between both of these hormones in four populations of the temperate amphibians, *Rana muscosa* and *Rana sierrae*, infected with the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). Surveyed sites across the California Sierra Nevadas included three populations where Bd has been present for at least six years and now appears to be endemic. Another site experiencing a newly defined Bd epidemic was also included in our study. We hypothesized that there would be a significant positive correlation between disease load in individuals and hormonal titers, and that this relationship would vary seasonally. Changes in disease prevalence were successfully predicted by month (and collection site but not for all sites and not in the expected pattern for month of collection. There was no significant global correlation between corticosterone, testosterone and disease load in individuals sampled across all four populations. There were increases in disease intensity, prevalence and corticosterone from the one collection site experiencing a Bd outbreak. However, from the lack of correlation between hormones and disease amongst all sites we conclude the dynamics of this disease are influenced by factors outside the physiological and environmental variables included in this study. From temperature data available from two collection sites we did see significant differences between temperature and disease intensity. Higher disease intensities in animals were obtained at higher temperatures (21 and 24C). Month, collection site and temperature were the three factors that influenced Bd prevalence and intensity most often in our study, suggesting that temperature regulates Bd growth and disease. Our data detected no contribution from the endocrine system to disease. That collection sites were seen as significant predictors of infection, even between sites where Bd has been consistently maintained for several years, and may indicate that there are unique contributions at a collection site which influence the dynamics of Bd in addition to temperature.

Introduction:

The immunosuppressive effects of glucocorticoids in vertebrates have been researched in a number of molecular, clinical and ecological contexts. These disciplines have posed questions examining the contributions that stress and glucocorticoids make to infection and chronic disease in both humans and animals. Lab work in a variety of vertebrate taxa, including amphibians, has shown that glucocorticoids can alter immune cell trafficking, regress the size of immune organs (Zapata et al, 1992), and contribute to progression of disease (Belden et al, 2005). Stressors that amphibians experience in nature have been modeled in lab studies and linked to both increases in glucocorticoids and negative effects on immune function. Temperature fluctuations (Cooper et al, 1992 and Maniero et al, 1997), overcrowding, density changes (Dare et al, 2006 and Koprivnikar et al, 2007), and habitat changes related to urban development and pollution (Brodkin et al, 2003 and Christin et al, 2004) can raise glucocorticoids and alter the prevalence and severity of diseases in amphibians. Endogenous glucocorticoid increases during amphibian metamorphosis have also shown a profound interaction between corticosterone and the immune system; inducing thymus and spleen regression and lymphocyte apoptosis (Rollins-Smith, 1998) with diminished antigen recognition (Du Pasquier et al, 1989).

More recently, researchers have also found that testosterone has secondary effects on the adaptive immune response in a variety of animals; birds (Evans et al, 2000, Buchanan et al, 2003, Owen-Ashley et al, 2004), mammals (Hughes et al, 2001, Martin et al, 2007) and reptiles (Varas et al 1992, Berger et al, 2005, Mills et al, 2008). Negative interactions between androgens and immune responses are a more recent finding, proposed first by Zuk (1990), which became the foundation of the immunocompetence handicap hypothesis (ICHH) developed by Folstad and Karter (1992). Folstad and Karter (1992) assert that the relationship between androgens and infection is negative; secondary sex traits in males enhance fitness but come at a cost. According to Folstad and Karter there is a trade-off between the ability to mount effective immune responses and the development of secondary sexual characteristics and behavioral traits which enhance reproductive fitness. Because the development of male specific traits and mating behaviors are under the control of androgens it is this class of hormones which initiates the change in immune responses.

Laboratory and field studies have tested this hypothesis by measuring parasite loads, humoral responses to immunizations and inflammatory responses to antigens in breeding males and after exogenous testosterone administration. The majority of the work supports the correlation between immunosuppression and an elevation in androgens but there have been discrepancies within and between taxa (Roberts et al, 2005). There have been few studies examining this relationship in amphibians. Those studies that have been done looked at the relationship between disease and testosterone in anurans by looking at disease prevalence in breeding frogs and found no significant interaction (Tinsley, 1990, Forbes et al, 2004 and Dare et al, 2009). To date, few studies have tested this hypothesis in animals experiencing more virulent disease with endogenous hormone cycles.

Many temperate animals breed seasonally and experience fluctuations in the hormones involved in reproduction. Seasonally reproducing vertebrates, anurans included, experience a concomitant rise in glucocorticoids and androgens at the height of the breeding season (Romero, 2002). Seasonal cycles of disease transmission and prevalence are found in human and wildlife populations and have been related to increases in population density, frequency of contact between individuals as well as variation in the availability of resources and host health (Andersen et al, 1991 and Altizer et al, 2006). A portion of work in this area has focused primarily on the influence of host health and considers the seasonal changes in disease a reflection of seasonal changes in immunity regulated by the physiological costs involved with reproduction, growth and thermoregulation (Lochmiller and Deerenberg, 2000). The study of these relationships is beginning to be incorporated into various fields like eco immunology and conservation medicine; examining prevalence of diseases over long periods of time in the context of the hosts' environment and physiology (Lee, 2006 Aguirre et al, 2002). Glucocorticoid measurements have been used by several studies to evaluate stress in animals experiencing dramatic habitat changes (Munson et al, 2005) and captivity (Wielebnowski et al, 2002) but little is known about the association of glucocorticoids with disease in animals reproducing or experiencing regular cycles of stress. Examining disease transmission in the context of endogenous hormone cycles may help elucidate the relationship between infection and normal physiological processes. In animals undergoing reproduction, the impact of these hormones on prevalence and severity of disease is also unknown. It is also true that the density and frequency of contact between individuals increases during reproduction but it is unclear how

the prevalence of disease may respond during this period. We are also uncertain if testosterone has immunosuppressive effects on all vertebrates or if the relationship is restricted to amniotes as the literature suggests. Being able to study these relationships in animals currently experiencing outbreaks of virulent disease allows researchers to test these hypotheses.

The amphibian fungal disease chytridiomycosis and its causative agent *Batrachochytrium dendrobatidis* have been implicated in the declines of several amphibian species, including mountain yellow-legged frogs (*Rana muscosa* and *Rana sierrae*) (Rachowicz et al, 2006). Since the first description of this disease in Australia in 1993 (Berger et al, 1998) it has been reported from almost every continent (Lips et al, Weldon et al 2004, Collins et al, Garner et al, 2005). Amphibian declines related to this disease have become an area of intense interest and every year there is more information on the prevalence and dynamics of this disease in different species. Some factors that are beginning to be incorporated into our understanding of Bd transmission include evidence that the prevalence of this disease is enhanced by cooler temperatures, rainfall and latitude (Krieger et al, 2004, Pounds et al, 2007) as well as life history traits and behavior (Woodhams et al, 2008). Studies have also found seasonal variation in disease with Bd prevalence peaking at certain times of the year (Krieger et al, 2006) proposed to be related to the temperature requirements of Bd growth. The emergence of this disease in *R. muscosa* and *R. sierrae* has been followed by declines in populations across the Sierra Nevada mountains of California(Rachowicz et al, 2006). There is genetic evidence in support of Bd as both an endemic pathogen and as a newly introduced disease in populations of *R. muscosa*/*R. sierrae* (Morgan et al, 2007). The genetic analysis mirrors the epidemiology of Bd in *R. muscosa*/*R. sierrae* populations in the Sierras; since 2002 when surveys began for Bd there have been populations that have become positive and then maintained the infection from year to year, while other populations have gone extinct rapidly. What remains unknown about this disease in populations of *R. muscosa*/*R. sierrae* and other anurans experiencing declines from Bd is how the disease is influenced by normal physiological processes like reproduction and acute stressors triggered by environmental changes.

R. muscosa/*R. sierrae* live at high elevations in alpine lakes in California. Once one of the most abundant vertebrate species encountered in the California Sierra Nevada it has become increasingly rare due to fish stocking of lakes (Knapp et al, 2007) and more recently because of Bd (Rachowicz et al, 2006). Larvae may take as long as three years to metamorphose because they experience short warm periods between June and August and overwinter with juveniles and adults in deep alpine lakes. Many of the sites we included in this study maintain at least partial snow cover until July and by September the temperatures have cooled dramatically making animals scarce. It is unknown if *R. muscosa*/ *R. sierrae* experiences seasonal changes in corticosterone and testosterone that have been found in other anurans. Seasonal changes in hormone titers have been found in hylids (Girgenrath et al, 2003), bufonids (Itoh et al, 1990) but the most extensive work has been done on populations of *Rana catesbeiana*, living at low elevations in California's central valley (Licht et al, 1983 and Mendoca et al, 1985). Any comparison to *R. muscosa*/*R. sierrae* must be scrutinized because hormone titers vary with the reproductive cycle of populations which are known to vary by location even within the same species (Mendoca et al, 1985). The populations of *R. muscosa*/*R. sierrae* chosen in our study were based on Bd prevalence for the years prior to the initiation of this work in 2005. The positive sites were based on at least one year of Bd identification prior to beginning this study.

The control population, negative for Bd in 2004, experienced an outbreak of Bd the first year of the study and remained positive for the duration of our surveys. Our hypothesis was that the hormones testosterone and corticosterone would vary positively with the prevalence and severity of Bd in populations of *R. muscosa*/*R. sierrae*.

Materials and Methods:

Sampling Design and Study Sites; Four populations of mountain yellow-legged frogs, restricted to high elevation sites along the Sierra Nevada, were sampled based on Bd prevalence prior to 2005. The species designation of all frog populations was *R. muscosa* at the time of our study, but the species has since been split into two closely-related sister species, *R. muscosa* and *R. sierrae* (Vredenburg et al. 2007). The Sixty Lake Basin population of *R. muscosa* is located in Kings Canyon National Park and was considered a control site, negative for Bd, prior to initiating this work in 2005. Unicorn Basin is located within Yosemite National Park and its *R. sierrae* population has been Bd positive site since at least 2004. Ebbetts Pass is located within Grover Hot Springs State park and its *R. sierrae* population had been positive for Bd since 2003. Milestone Basin is located within Kings Canyon National Park California and its *R. muscosa* population has been Bd positive site since 2003. Two sites, Unicorn Basin and Ebbetts Pass were visited at three time points throughout the season for both years for a total of six surveys and two sites, Sixty Lake Basin and Milestone Basin, were sampled at least once for each year. The approximate elevations for the sites are as follows; Sixty Lake Basin (3353 m) Unicorn Basin (2900 m), Ebbetts Pass (2590 m) and Milestone Basin (3300 – 3800 m).

Blood collection; Blood was collected via cardiac puncture and handling time was kept between five to ten minutes. In *Litoria ewingi*, artificial increases in glucocorticoids were obtained when captivity exceeds three hours under anesthesia (Coddington et al, 1995). Attempts at collecting blood were abandoned if not obtained within ten minutes and these samples were not included in analysis. Blood was centrifuged in the field and kept on ice until transported to UC Berkeley where it was stored at -20C. A total of 246 blood samples were collected from the four populations over the two year sample period and used in the analysis of corticosterone and Bd disease load. A subsample of 86 blood samples from male *R. muscosa*/*R. sierrae* were used to examine the relationship between testosterone and Bd disease load from all four populations.

ELISA Measurement of Corticosterone and Testosterone. Corticosterone and testosterone ELISA kits (Assay Designs) were used to measure hormone concentration from blood samples. Twenty five microliters of plasma was extracted using diethyl ether and stored at -20C prior to ELISA analysis. Diethyl ether was used for all plasma extractions and samples were assayed within a maximum of one month after extraction. When 25 microliters was not available for extractions a volume 5 microliters from each sample was spiked with a 60pg corticosterone standard and corrected for volume and supplementation for hormone analysis. Extraction efficiency in spiked control samples for corticosterone and testosterone ELISA kits ranged between 71 and 84% respectively. Pooled *R. catesbeiana* and *R. muscosa*/*R. sierrae* plasma control samples were used to examine inter assay variation between plates and averaged 10%. Intra assay variation between replicates averaged 5%. If samples differed by more than 5% they were re-extracted and re -run in additional ELISAs.

qPCR Measurement of Zoospore Load. Real time PCR using methods described by (Boyle et al, 2004) to measure Bd infection in individuals. Swabs of individuals in the field were used to assess disease load, designated as "zoospore load" and represent the total Bd DNA from the

swab of each individual. Each ge (genome equivalent) has been multiplied by 80 to account for the fraction of DNA extraction and dilution used in the real time assay.

Statistics

JMP 7.0 and SPSS 17.0 were used to analyze data. All Bd readings, corticosterone and testosterone titers were log transformed to satisfy assumptions of normality. Planned comparisons using one way ANOVAs were made examining hormone and disease fluctuations by month, year, location and sex. Linear regression was used to examine the association between Bd disease load and either corticosterone or testosterone. Only animals that tested positive for Bd were included in the linear regression comparing disease load (zoospore load) and either corticosterone (n = 83) and testosterone (n = 35).Comparisons of the proportion of diseased individuals between sites and year of study were examined using chi square tests. A binary logistic regression examined associations between disease prevalence by site, month of collection and year (n = 359). Binary logistic regression was also used to examine associations between disease prevalence by site, corticosterone titer, site, year, sex and month of collection (n = 246). Testosterone titer was also included as a predictor in binary regression models examining association between disease prevalence at study sites. Multivariate regression models were run using the same set of predictors to examine relationships between disease intensity (zoospore load) in individuals predicted by corticosterone titer, site, month of collection, year and sex (n = 246). The analysis was also repeated using testosterone titer as a predictor (n = 86). Sixty Lake Basin and the month of July were used as reference variables in all binary logistic regression and multivariate models. Temperature data was consistently available only for two sites from collections that occurred in 2006 (n= 104). One way ANOVA was used to compare disease intensity with temperature and site.

Results

Correlation between corticosterone, testosterone and disease; A bivariate plot between animals positive for Bd, designated zoospore load, and either corticosterone (figure 1) or testosterone titers (figure 2) revealed no significant association between these variables. The correlation coefficient between zoospore load and corticosterone or testosterone was R^2= 0.005 and 0.009 respectively. The average hormone titers and disease load in individuals from each site, figures 10-13) also suggest no relationship between these two variables. Hormone levels and disease intensity are often in opposition to our predictions at the beginning of this study ; when disease load is elevated there is often a decrease or no change in corticosterone or testosterone titers.

Corticosterone and infection status; No significant difference was found between Bd infection status and corticosterone titers (Figure 3; one way ANOVA, $F = 0.022$, $p > 0.05$). Averaged corticosterone titers categorized by site and year of study in figure 5 (n = 246) were significant for both collection site (one way ANOVA, $F = 7.0$, $p < 0.05$) and year of collection (one way ANOVA, $F = 31.7$, $p < 0.05$). However, when the averaged corticosterone titers collected from just individuals positive for Bd, figure 6, (n=83) were analyzed by site and year, significant differences were only found between years (one way ANOVA, $F = 6.99$, $p < 0.05$).

Disease prevalence by site; The number of individuals testing positive for Bd by site and year of study is summarized in table 1. Total Bd prevalence by site was significantly different (chi square = 10.2, $p < 0.05$) but not when the proportion of positive and negative samples was pooled and analyzed by year of study (chi square = 0.787, $p > 0.05$). No differences were found

in the proportion of positive and negative samples for each site between years (chi square = 0.787, $p > 0.05$). Collection site and month made significant contributions to predicting infection status of individuals in the binary regression model (table 3) and the inclusion of these variables increased the models predictive ability from 60 to 66 %.

Bd Intensity by site; The average intensity of Bd by site for all individuals ($n = 359$) for both years of the study (figure 4) were not significantly different from each other (one way ANOVA, $F = 0.555$, $p > 0.05$). However, disease intensity at Sixty Lake Basin and the year of study was significantly higher in two smaller data sets examined, figures 5 and 6. Temperature and disease intensity shared a positive relationship, higher zoospore loads were seen at higher temperatures. Differences in zoospore load and temperature were also significant (one way ANOVA, $F = 27.0$, $p < 0.05$).

Post hoc planned comparisons; Seasonal changes in corticosterone, testosterone and zoospore load by year and sex (table 2) were found for all sites but these fluctuations varied by location. Small sample sizes for some of these comparisons prohibited some comparisons from being conducted, particularly for Sixty Lake Basin and Milestone Basin in 2006. Variations in corticosterone and zoospore load or Bd intensity were found for both sexes in Sixty Lake Basin during 2005 but not for 2006. Bd intensity varied significantly in females during 2005 and for all individuals in 2006 at Unicorn Basin but no increases or decreases in either hormone were found for either year. Hormone titers varied during both years in Ebbetts Pass in males or females but Bd intensity did not between and within either year.

Predicting infection from hormone titer, sex, site, month and year of collection. Binary logistic regressions were used to predict infection using the predictors corticosterone (table 4), testosterone (table 5), sex, collection site, month and year of study. Collection site made a significant contribution to predictive ability of the model when corticosterone was included in the binary logistic regression model (table 4). No significant contributions were made to prediction of infection status when testosterone was included as a predictor.

Discussion;

Chytridiomycosis has been implicated as a major contributor to amphibian declines in Australia (Berger et al, 1998), Europe (Bovero et al 2008 and Bosch et al 2000), North (Rachowicz et al, 2006 Muths et al 2003 and Green et al, 2002) and South America (Lips et al 2006). Reports of Bd in amphibian communities have increased ever since its recent description (Longcore et al, 1999) but the investigation of variables that influence Bd infections in amphibians is just beginning to occur. This is especially important because locations that first recorded in amphibians , Australia, North and South America, all continue to report its persistence within populations. It appears as though Bd is maintained in populations either as a low level infection or by an unknown reservoir. The areas where more research is needed about Bd include examining how populations change in response to infection; how species richness within communities is altered, how remaining communities can be protected and how the disease is influenced by physiological responses. This study fits into the later and examined relationships between hormones involved in regular physiological processes in an amphibian threatened by a virulent disease and found no association. We were able to detect seasonal fluctuations in both disease and endogenous hormone cycles in our chosen populations but the two were often in conflict with each other and contradict the hypothesis that there would be positive relationships between them. The one study site experiencing an apparent epidemic of Bd, with no history of

infection, had the highest disease loads and the lowest corticosterone titers compared with the other populations included in this work. The first year of recorded Bd in Sixty Lake Basin saw an increase in disease prevalence, intensity and in corticosterone titers but we believe the relationship is coincidental. The overall findings in this work document no connection between higher corticosterone nor testosterone and the presence of Bd within *R.muscosa* populations. Seasonal prevalence of disease did not increase during periods of active breeding further suggesting the increased contact between individuals dose not contribute to increases in disease. The strongest predictor of whether an individual was infected and the intensity of infection was site and month of collection. Our findings provide further evidence that there is no relationship between endocrine function and disease in all tetrapods. Research has found a connection between disease and metamorphosis in amphibians related to corticosterone increases but this may be the only period in the amphibian lifecycle where the two systems interact.

The concentrations of corticosterone and testosterone reported in this work for *R. muscosa* are significantly lower than reported in studies with other anuran species. The average concentration of corticosterone in *R. muscosa* populations ranged from 57 to 500 pg/ml and testosterone ranged from 215 to 1000 pg/ml. The two fundamental endocrine studies examining seasonal hormonal cycles in *Rana catesbeiana* reported much higher concentrations for both hormones; one found a peak of both corticosterone and DHT during April, averaging 8 ng/ml and 50 ng/ml respectively (Mendoca et al, 1985) and the second found elevated corticosterone levels in the month of April through August with a range of concentrations ranging from 4-29 ng/ml (Licht et al, 1983). There are three potential causes we believe are related to the differences obtained from *R. muscosa*/*R. sierrae* ; time until blood draws occurred, environmental and habitat differences between species and technical issues with the method we employed in this work. The amount of time until blood collection occurred in this work is significantly shorter at ten minutes than the majority of work done in other anuran which typically ranges within hours of capture (Romero, 2002). Capture stress has been considered less of a problem for glucocorticoid hormones in ectothermic species because metabolic processes are so much slower than in endotherms but there are studies that have found elevations in plasma corticosterone in fish in as little as 3 minutes after capture (Manire et al, 2007). A pilot study we conducted found elevations in corticosterone with the anesthetic MS222 with handling times of 20-30 min prior to blood draws in *R. muscosa*/*R. sierrae* (unpublished data). To date this is also the only study to measure hormone titers in an amphibious species living at high elevations. Amphibians that are subject to colder temperatures may experience diminished metabolic processes in hormone production when compared to species living at lower elevation.

We also used a relatively new method of measuring hormones from blood samples, a commercially available ELISA kit as opposed to the radioimmunoassay (RIA) which has been used in many similar studies with amphibians. A recent study has suggested that these ELISA kits are better at detecting qualitative differences between samples because there was more than 10% variation in samples between both methods (Sink et al, 2008). Elevated testosterone titers were seen in Unicorn Basin during active breeding but not at the other populations when breeding and the presence of egg masses were recorded. Additional work in this area could help clarify the concentrations that are typical for species living at high elevations experiencing short summer months and to verify endogenous sex hormone cycles that are seen in other amphibians during breeding. The use of ELISA kits to measure hormones in amphibians is relatively new.

Future studies may want to validate their use with the more standard RIA before processing a large number of samples. The smaller concentrations of corticosterone and testosterone may be related to the temperature constraints that *R. muscosa* has evolved in but further work is required in both *R. muscosa/ R.sierrae* and other amphibians living at high elevations to clarify this finding.

Temperature appears to influence Bd disease dynamics within amphibian communities more strongly than any other variable to date. Successful elimination of Bd has been seen in amphibians housed at warmer temperatures (Woodhams et al, 2003 and Andre et al, 2008) and field data has shown fluctuations in Bd infections that follow environmental gradients toward cooler temperatures (Kriger et al, 2004 and Kriger et al, 2008). Temperature constrains Bd growth in culture (Pitrowski et al, 2004) and cooler temperatures have been shown to prolong the infectious stage of Bd in culture (Woodhams et al, 2008). Cooling daytime temperatures and warmer evening temperatures in a retrospective analysis have also been linked to mass declines of amphibians in South America (Pounds et al, 2007). We examined the relationship between temperature and disease intensity at two sites for which data was available and found significant relationships between them. In contrast to the previous work which suggests Bd grows best at lower temps we found the highest disease loads were found at higher temperatures. The majority of samples collected at the higher temperature were obtained at Ebbetts Pass, which had been positive for Bd for two years prior to this work. This effect of temperature and the finding that site was a significant predictor in Bd prevalence suggests that sites themselves make unique contributions to the dynamics of Bd disease. The potential contributions a site could make to this disease are numerous. The inclusion of variables in Bd work that examines population genetics, potential reservoirs for Bd and physical variations in habitat are sorely needed. From such studies it may be possible to determine which sites are at greater risk of extinction from Bd as it seems this disease is becoming established and may remain part of amphibian communities.

Table and Figure Legends.

Table 1. Disease prevalence by site for both years under study. Site prevalence of Bd was significantly different between populations when individuals were pooled according to location (chi square = 11.2, $p < 0.05$) but not when Bd prevalence was compared between years of study (chi square = 1.0 $p > 0.05$).

Table 2. Seasonal differences in hormones and disease. Planned comparisons were done to examine seasonal differences between sites in disease, corticosterone and testosterone titers. Significant seasonal fluctuations were found for disease, corticosterone and testosterone for most sites with the exception of Milestone Basin. Small sample sizes and too few sampling periods prohibited statistical analysis of hormone and disease differences from being conducted in Milestone Basin and for second year of the study in Sixty Lake Basin.

Table 3. Binary logistic regression predicting infection status by month, year and site of collection (n = 359). Infection status of individuals was successfully predicted by month of collection and collection site. Individuals were more likely to be infected in August and September and if sampled from Unicorn Basin in Yosemite National Park.

Table 4. Binary logistic regression predicting infection status of individuals from sampling month, collection site, year of collection and corticosterone titers (n=246). Collection site successfully predicted infection with Milestone Basin making the strongest contribution ($p < 0.05$, OR = 2904). There was also a significant interaction between the infection prevalence, year of collection and sampling in Milestone Basin ($p < 0.05$, OR = 0.015). Inclusion of all variables to the model improved prediction of infection from 62.2 to 71.2%.

Table 5. Predictions of infection status in male *R.muscosa* from sampling month, collection site, year of collection and testosterone titer (n =86). Binary logistic regression analysis found no significant contribution to model prediction of infection with the above variables, with Sixty Lake Basin and the month of July as reference points. The overall model prediction of infection did increase from 59.3 to 62.2 % when variables were included but without significant contribution to its predictive ability.

Table 6. Multiple regression analysis of the relationship of infection intensity (log zoospore load) to day, site, year and month of sample collection (n =246). The sign of the model's b coefficients indicate that the infection loads from each site outside Sixty Lake Basin were lower. This reduction of infection intensity (log zoospore load) between sites is most significant between Sixty Lake Basin and Milestone Basin ($p < 0.05$, OR= -2.695). Positive interactions between infection intensity (log zoospore load), site and year of collection were found in Ebbetts Pass and Milestone Basin.

Table 7. Multiple regression analysis of the relationship between testosterone titer to day, site, year and month of collection (n=86). Testosterone titers collected in June were significantly higher ($p < 0.05$) than other months. Seasonal differences in testosterone also failed to be detected from this model at any of the sites. An interaction between Ebbetts Pass and the month of August significantly increased testosterone titers. Testosterone titers collected from Ebbetts Pass were not significantly higher compared to other collection sites or at any other collection month

Figure 1; Bivariate relationship between infection intensity and corticosterone titers. No association between disease intensity, zoospore load, and corticosterone titers were found in individuals positive for Bd (n = 83).

Figure 2; Bivariate relationship between testosterone and infection intensity in Bd positive individuals (n = 35). No association was found between disease intensity, zoospore load, and testosterone titers in animals that tested positive for Bd (n = 35).

Figure 3. Infection status and corticosterone titers (n = 246). Corticosterone titers did not vary significantly between animals that were classified as either negative or positive for Bd (one way ANOVA $F = 0.21$, $p > 0.05$).

Figure 5. Total Bd disease intensity by site for each year of study (n = 359). All individuals testing negative or positive for Bd were included in the above analysis. No significant differences in disease intensity were found between sites nor between years (one way ANOVA, $F = 0.55$, $p > 0.05$).

Figure 6. Average Bd disease intensity by site for each year of study for individuals testing positive for infection (n = 142). Significant differences were found in the intensity of Bd infection, average zoospore loads, between sites (one way ANOVA, $F = 12.6$, $p < 0.05$) and year of study (one way ANOVA, $F = 6.6$, $p < 0.05$). Sixty Lake Basin was significantly higher in over all Bd intensity for both years of the study.

Figure 7. Averaged Bd intensity by site and year of study (n = 246). Significant differences were found in the averaged zoospore loads by site (one way ANOVA, $F = 7.0$, $p < 0.05$) and year of study (one way ANOVA, $F = 31.7$, $p < 0.05$). This analysis included individuals negative and positive for Bd

Figure 8. Disease intensity by site for individuals positive for Bd (n = 83). Significant differences in the average disease load by site and year for individuals positive for Bd were found. Sixty Lake and Milestone Basins were higher than the other three sites surveyed (one way ANOVA $F = 12.5$, $p < 0.05$ and $F = 6.6$, $p < 0.05$) respectively. Both sites were also higher in disease intensity for the second year of the study (one way ANOVA, $F = 7.0$, $p < 0.05$).

Figure 8. Average corticosterone titers by site and year (n = 83). No significant differences in corticosterone concentrations when categorized by site (one way ANOVA, $F = 1.6$, $p > 0.05$) but did vary significantly between years at each site (one way ANOVA, $F = 6.99$, $p < 0.05$).

Figure 9. Average corticosterone titers by site and year of study (n = 246). Significant differences were found in the average corticosterone levels categorized by site (one way ANOVA, $F = 7.0$, $p < 0.05$) and year of study (one way ANOVA, $F = 31.7$, $p < 0.05$). Sixty Lake Basin had significantly lower corticosterone levels than the other three populations

Figure 10. Averaged hormone and disease loads for individuals collected from Sixty Lake Basin. Increases in either hormone were not correlated to increases in disease intensity. The overall disease intensity was highest for Sixty Lake Basin and had the overall lowest corticosterone titers in the study.

Figure 11. Averaged hormone and disease loads for individuals collected in Unicorn Basin. Disease intensity at this location was highest for the last month of collection in both years, September, which was also the coolest month recorded. Breeding did occur in July for both years but an increase in testosterone was only found in the second year. The only significant increase in corticosterone was seen in September in 2006. There are no coordinated increases in either hormone and disease intensity for this population

Figure 12. Averaged hormone and disease loads for individuals collected at Ebbetts Pass. Significant changes in corticosterone and testosterone were found in both years of this study but not for disease intensity at this site. Breeding was recorded in July for both years but the increases in testosterone were seen in august for both years.

Figure 13. Averaged hormone (pg/ml) and disease loads for individuals collected from Milestone Basin. No significant changes in zoospore load nor corticosterone titers between collection years. Testosterone was not measured in males captured from this location for either year.

Figure 14. Disease intensity and temperature at time of collection. Higher zoospore loads were seen at 21 and 24C. Samples for animals at these two temperatures were both from collections that occurred at Ebbetts Pass in 2006. These two temperatures had significantly higher zoospore loads then the other recorded temperatures (one way ANOVA, $F = 27.0$, $p < 0.05$). The other three temperatures had collections from Ebbetts Pass (10C) and Unicorn Basin (12.5, 16.5 and 21C).

Figure 15. Mean Bd intensity for Unicorn Basin and Ebbetts Pass from temperature data (n=104). Ebbetts Pass had a significantly higher zoospore load than Unicorn Basin (one way ANOVA, $F = 5.21$, $p < 0.05$).

Table 1.

Site	2005	N	2006	N	Proportion of all animals positive for Bd
Sixty Lake Basin	0.28	57	0.3	30	0.29
Unicorn Basin	0.37	30	0.53	94	0.49
Ebbetts Pass	0.42	33	0.34	118	0.36
Milestone Basin	0.31	26	0.34	35	0.33

Table 2.

	Sixty Lake Basin	Unicorn Basin	Ebbetts Pass	Milestone Basin
Corticosterone				
Females and monthly differences in 2005	$F = 11.55$, $p < 0.05$	$p = 0.56$	$p = 0.41$	
Males and monthly differences in 2005	$F = 8.25$, $p < 0.05$	$p = 0.19$	$p = 0.07$	
Monthly differences in 2005	$F = 5.57$, $p < 0.05$	$p = 0.07$	$F = 4.37$, $p < 0.05$	
Females and monthly differences in 2006	N/A	$p = 0.05$	N/A	
Males and monthly differences in 2006	N/A	$p = 0.64$	$p = 0.17$	
Monthly differences for both sexes in 2006	N/A	$p = 0.68$	$p = 0.68$	
Between the years 2005 and 2006	$p = 0.31$	$p = 0.96$	$F = 13.9$, $p < 0.05$	$p = 0.64$
Testosterone				
Monthly differences in 2005	N/A	N/A	$F = 7.66$, $p < 0.05$	
Monthly differences in 2006	N/A	$p = 0.36$	$F = 43.5$, $p < 0.05$	
Between the years 2005 and 2006	$p = 0.15$	$p = 0.67$	$p = 0.08$	
Bd Intensity				
Females and monthly differences in 2005	$F = 4.99$, $p < 0.05$	$F = 19.7$, $p < 0.05$	N/A	
Males and monthly differences in 2005	$p = 0.12$	$p = 0.18$	$p = 0.24$	
Monthly differences for both sexes in 2005	$F = 6.01$, $p < 0.05$	$p = 0.92$	$p = 0.24$	
Females and monthly differences in 2006	N/A	$p = 0.14$	N/A	
Males and monthly differences in 2006	N/A	$p = 0.34$	$p = 0.30$	
Monthly differences in 2006	N/A	$F = 4.17$, $p < 0.05$	$p = 0.26$	
Between years 2005 and 2006	$p = 0.11$	$p = 0.92$	$p = 0.47$	$p = 0.34$

Table 3.

Predictor	B	X2	p	Odds Ratio	95% C.I.for Odds Ratio Lower	Upper
Year of Collection	-.358	1.620	.203	.699	.402	1.213
Month of Collection		17.306	$p < 0.05$			
June	.101	.018	.894	1.106	.249	4.907
August	1.031	13.492	$p < 0.05$	2.804	1.617	4.860
September	1.273	8.604	$p < 0.05$	3.570	1.525	8.356
Collection Site		9.165	$p < 0.05$			
Unicorn Basin	1.088	8.452	$p < 0.05$	2.970	1.426	6.186
Ebbetts Pass	.458	1.728	.189	1.581	.799	3.128
Milestone Basin	.310	.637	.425	1.363	.637	2.917

.

Table 4.

Predictor	B	Wald, $\chi 2$	p	Odds Ratio	95% C.I. for Odds Ratio	
					Lower	Upper
Month of Collection		3.472	.324			
June	2.980	.488	.485	19.686	.005	83821.836
August	3.481	3.107	.078	32.504	.677	1559.698
September	.096	.001	.980	1.101	.001	1731.532
Year of Collection	.535	.062	.804	1.707	.025	115.685
Sex	-.212	.383	.536	.809	.413	1.583
Collection Site		10.873	$p < 0.05$			
Unicorn Basin	2.987	.791	.374	19.829	.027	14349.487
Ebbetts Pass	4.096	2.018	.155	60.122	.211	17128.258
Milestone Basin	7.974	10.659	$p < 0.05$	2904.104	24.212	348328.263
Corticosterone Titer(pg/ml)	-.085	.006	.940	.919	.099	8.483
Milestone Basin * Year	-4.218	6.589	$p < 0.05$	.015	.001	.369

Table 5.

Predictors	B	Wald $\chi 2$	p	Odds Ratio	95% C.I.for Odds Ratio	
					Lower	Upper
Month of Collection		1.493	.684			
Collection Site	-1.986	.515	.473	.137	.001	31.047
Year of Collection	.349	.208	.648	1.418	.317	6.354
Testosterone Titer (pg/ml)	-.927	.099	.753	.396	.001	126.965
Day of Collection	.000	.371	.543	1.000	1.000	1.000

Table 6.

Predictor	B	SE	t	p
Intercept	1.818	1.113	1.634	$p < 0.05$
Day of Collection	0.002	0.001	2.956	$p < 0.05$
Collection Site				
Unicorn Basin	-2.132	1.155	-1.846	0.066
Ebbetts Pass	-1.368	1.039	-1.316	0.189
Milestone Basin	-1.912	0.709	-2.695	$p < 0.05$
Year of Collection	-2.42	1.079	-2.243	$p < 0.05$
Month of Collection				
June	-2.393	0.859	-2.785	$p < 0.05$
August	0.281	0.891	-2.785	0.753
September	0.245	0.412	0.594	0.553
Significant Interactions				
Ebbetts Pass * Year	2.829	0.905	3.126	$p < 0.05$
Milestone Basin * Year	2.222	0.804	2.7464	$p < 0.05$

Table 7.

Predictor	n	B	SE	t	p
Intercept		2.809	0.338	8.306	$p < 0.05$
Day of Collection		-4.53E-06	2.96E-06	-1.534	0.129
Site of Collection					
Unicorn Basin	47	0.49	0.501	0.979	0.331
Ebbetts Pass	29	-0.185	0.288	-0.642	0.523
Sixty Lake Basin	10				
Year of Collection		-0.384	0.196	-1.958	0.054
Month of Collection					
June	6	2.767	1.316	2.103	$p < 0.05$
August	24	0.272	0.361	0.754	0.453
September	18	0.215	0.29	0.742	0.46
July	38				
Significant Interactions					
Ebbetts Pass* August		1.131	0.546	2.071	$p < 0.05$

Figure 1.

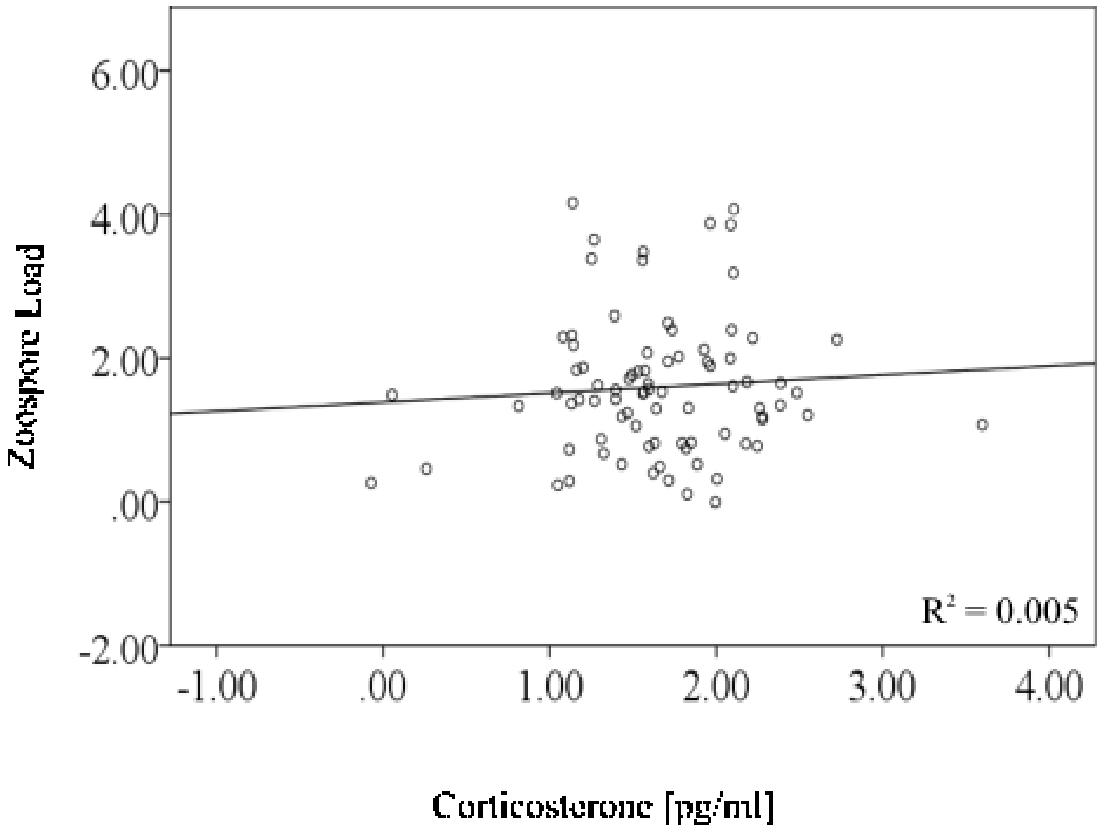

6.00
4.00
2.00
.00
-2.00
Zoospore Load
-1.00
.00
1.00
2.00
3.00
4.00
R² = 0.005
Corticosterone [pg/ml]

Figure 2.

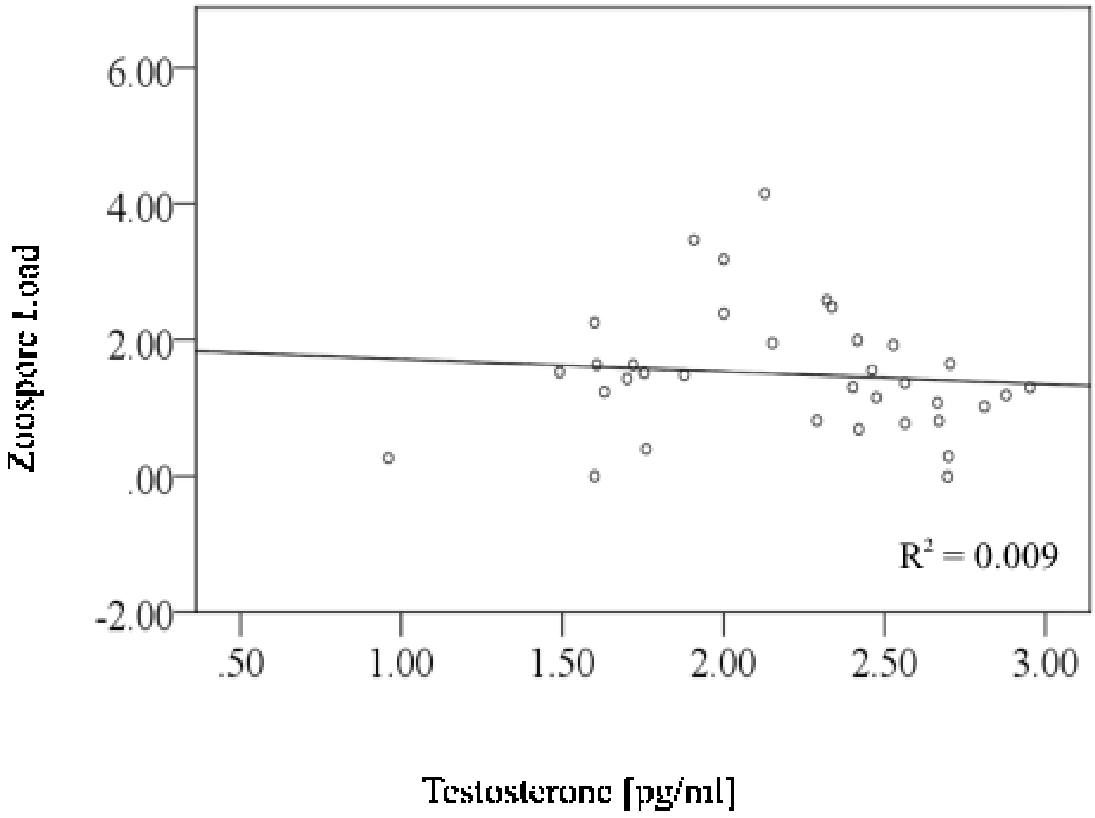

6.00
4.00
2.00
.00
-2.00
Zoospore Load
.50
1.00
1.50
2.00
2.50
3.00
$R^2 = 0.009$
Testosterone [pg/ml]

Figure 3.

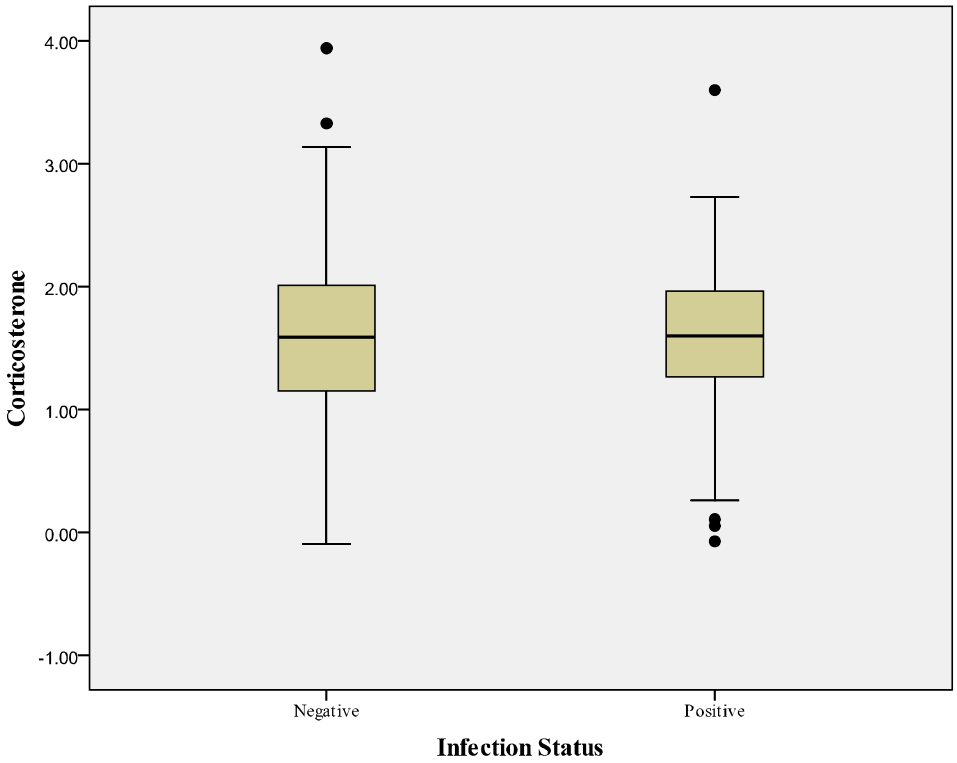

4.00
3.00
2.00
1.00
0.00
-1.00
Corticosterone
Negative
Positive
Infection Status

Figure 4.

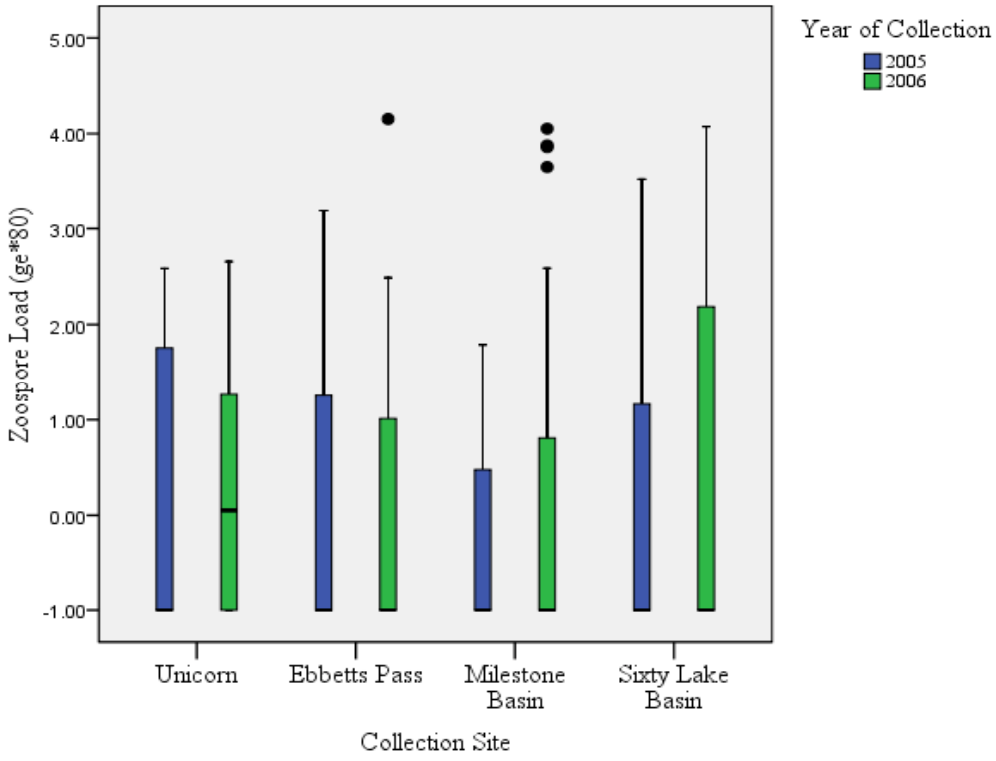

Year of Collection
2005
2006
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Zoospore Load (ge*80)
Unicorn
Ebbetts Pass
Milestone Basin
Sixty Lake Basin
Collection Site

Figure 5.

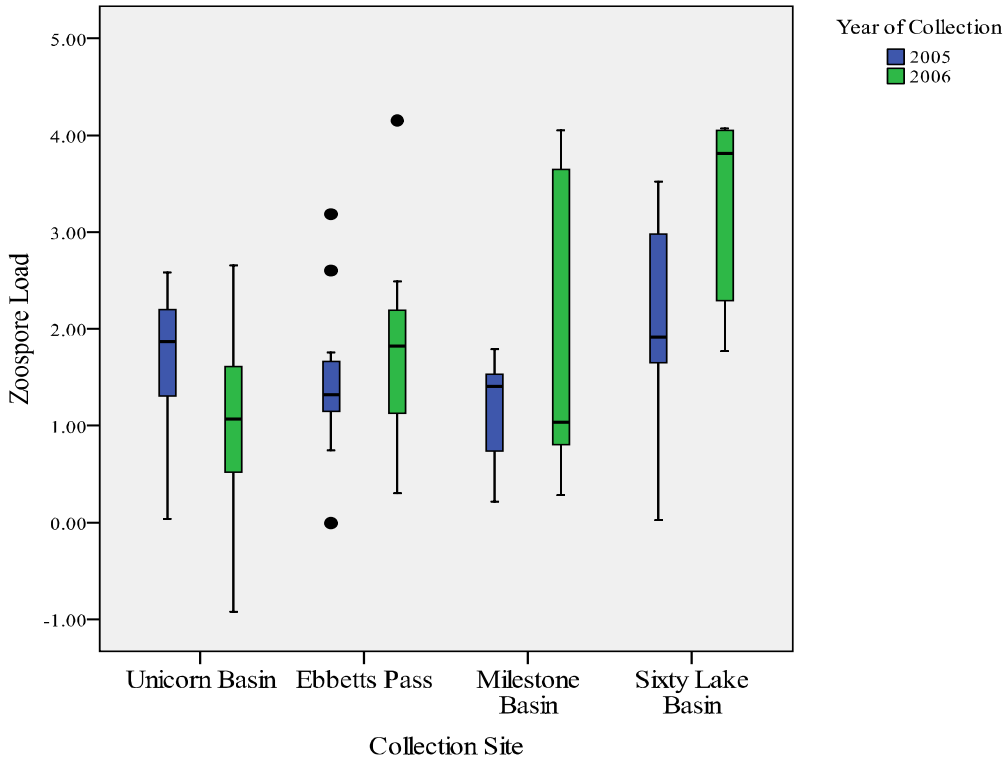

Year of Collection
2005
2006
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Zoospore Load
Unicorn Basin
Ebbetts Pass
Milestone Basin
Sixty Lake Basin
Collection Site

Figure 6.

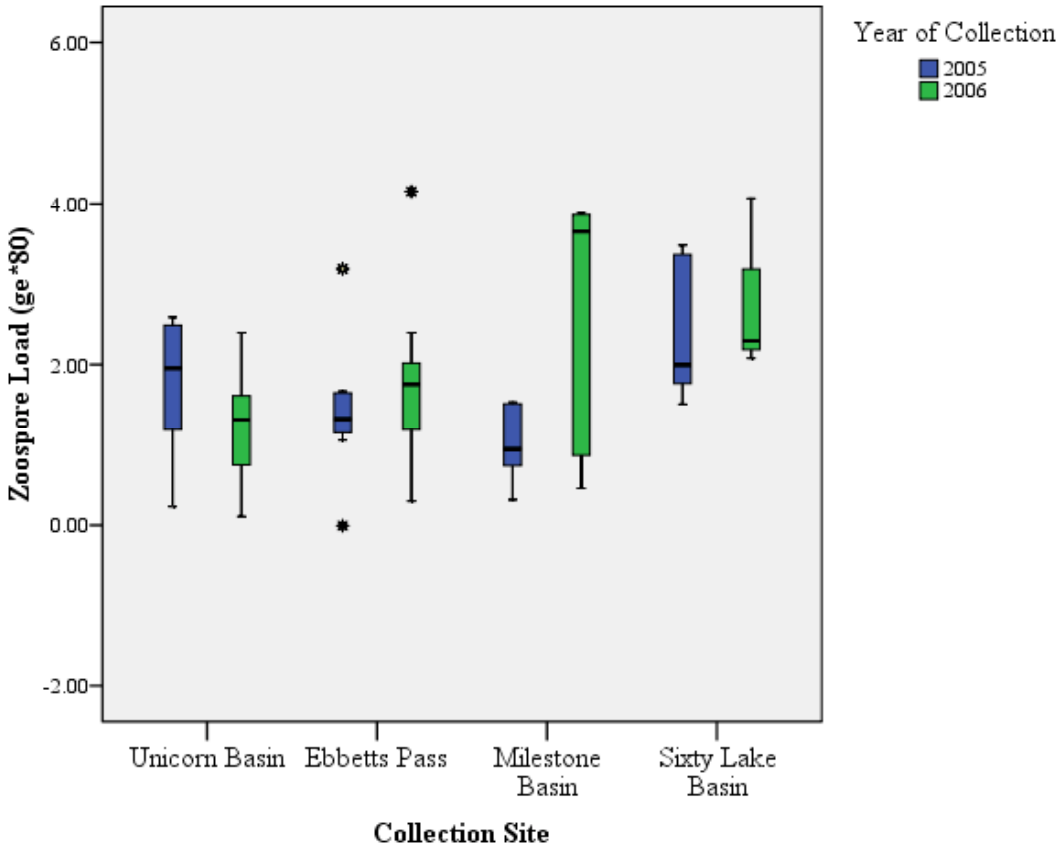

Year of Collection
2005
2006
6.00
4.00
2.00
0.00
-2.00
Zoospore Load (ge*80)
Unicorn Basin
Ebbetts Pass
Milestone Basin
Sixty Lake Basin
Collection Site

Figure 7.

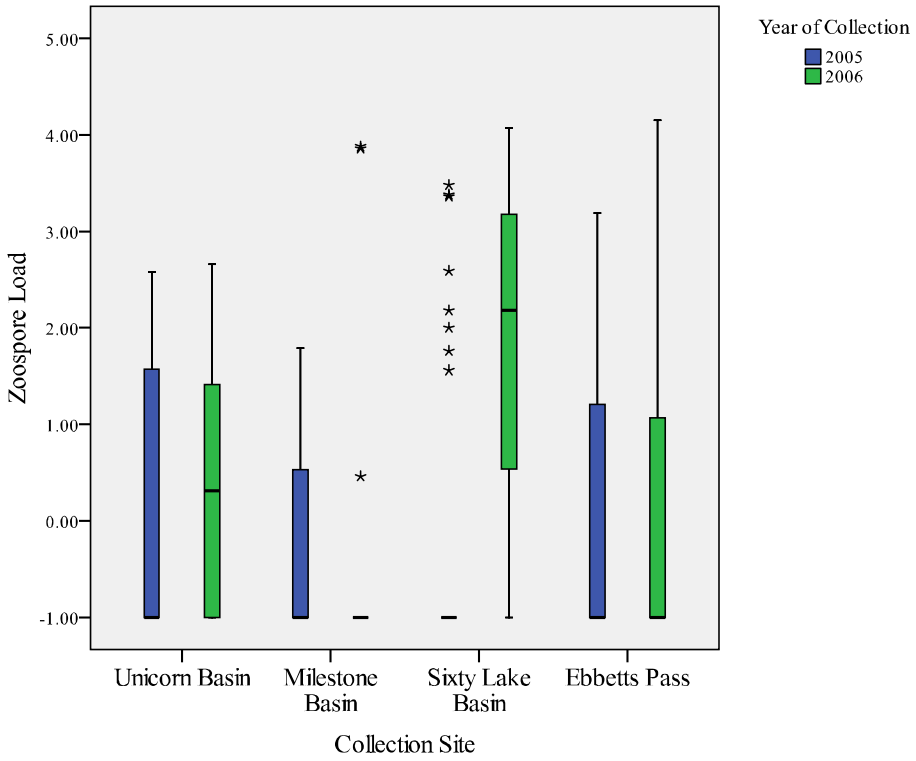

Year of Collection
2005
2006
Zoospore Load
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Unicorn Basin
Milestone Basin
Sixty Lake Basin
Ebbetts Pass
Collection Site

Figure 8.

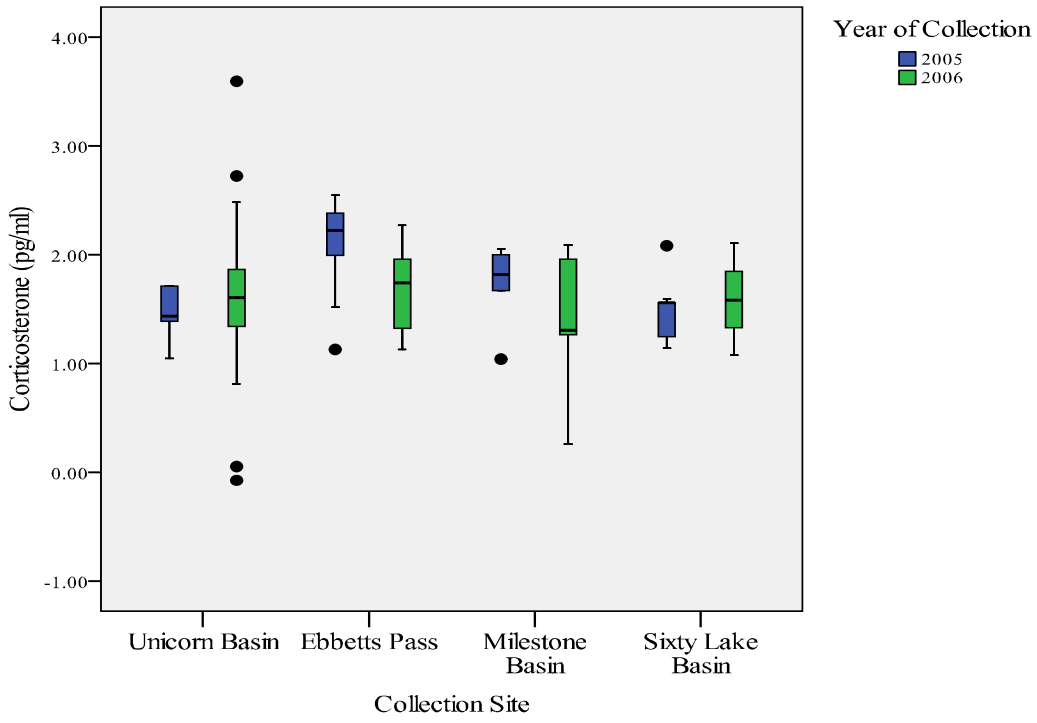

Year of Collection
2005
2006
Corticosterone (pg/ml)
4.00
3.00
2.00
1.00
0.00
-1.00
Unicorn Basin
Ebbetts Pass
Milestone Basin
Sixty Lake Basin
Collection Site

Figure 9.

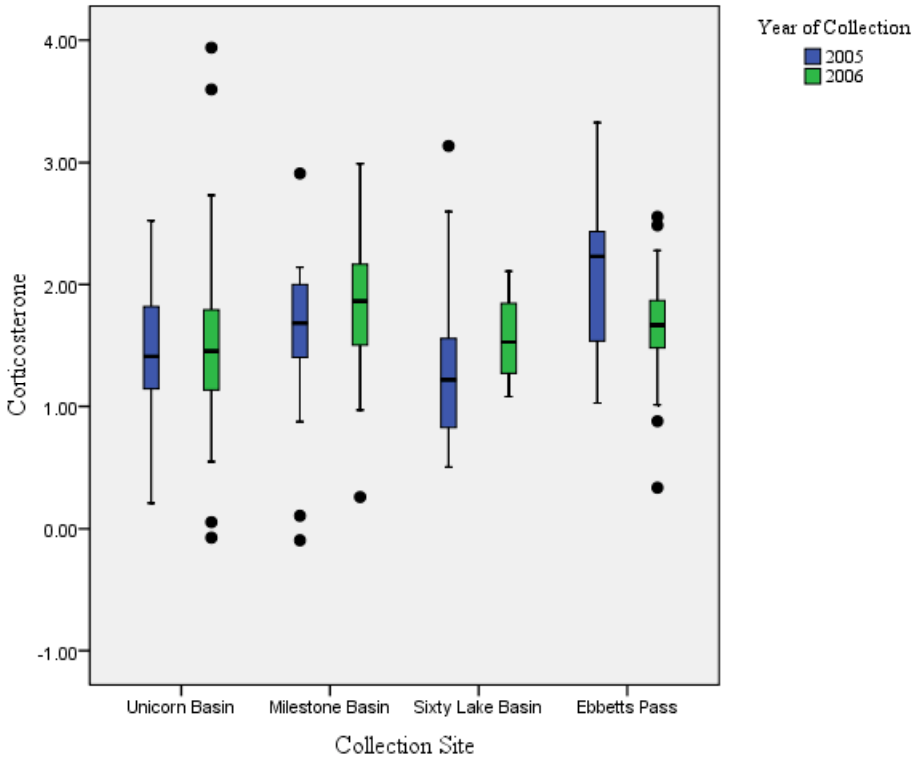

Year of Collection
2005
2006
Corticosterone
4.00
3.00
2.00
1.00
0.00
-1.00
Unicorn Basin
Milestone Basin
Sixty Lake Basin
Ebbetts Pass
Collection Site

Figure 10.

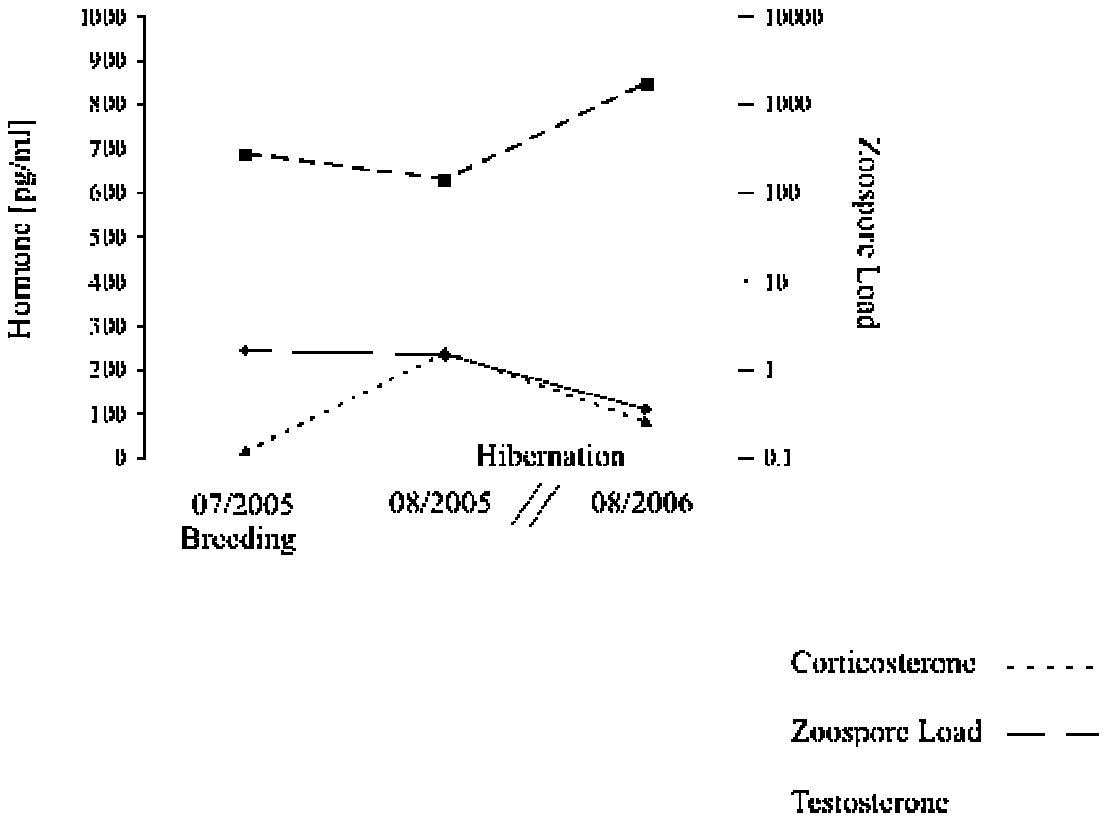

Hormone [pg/ml]
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Zoospore Load
10000
1000
100
10
1
0.1
Hibernation
07/2005
Breeding
08/2005
08/2006
Corticosterone
Zoospore Load
Testosterone

Figure 11.

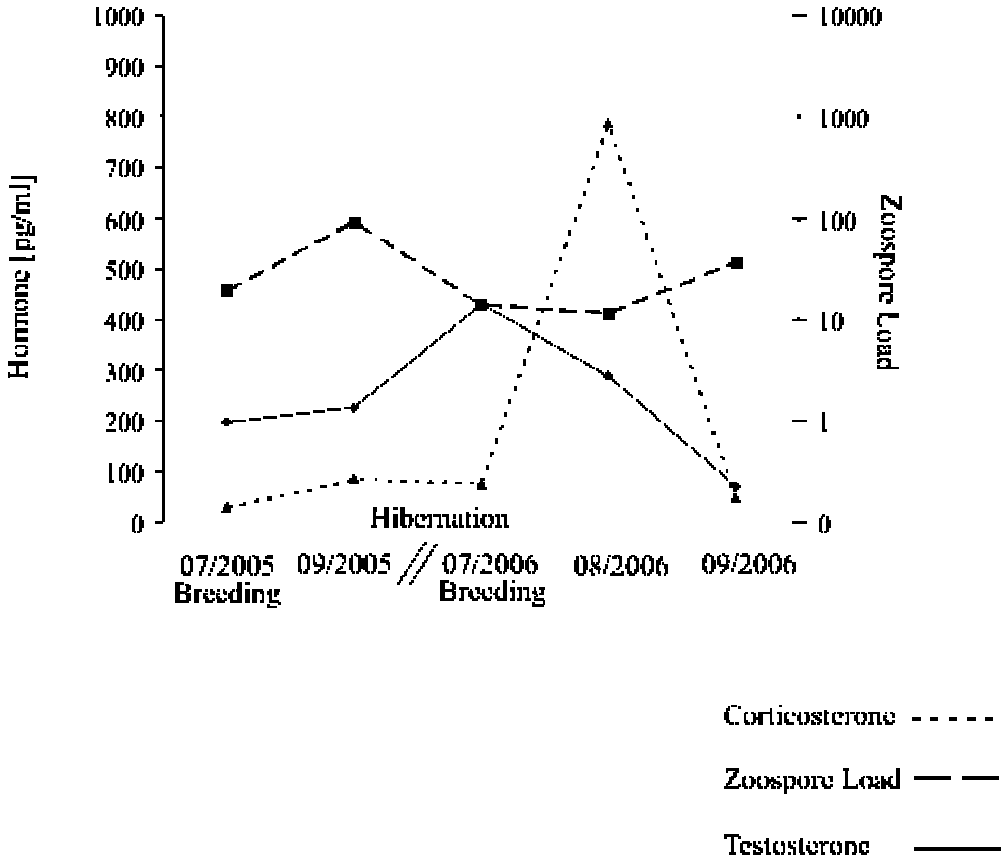

Hormone [pg/mL]
Zoospore Load
1000
900
800
700
600
500
400
300
200
100
0
10000
1000
100
10
1
0
Hibernation
07/2005
Breeding
09/2005
07/2006
Breeding
08/2006
09/2006
Corticosterone
Zoospore Load
Testosterone

Figure 12.

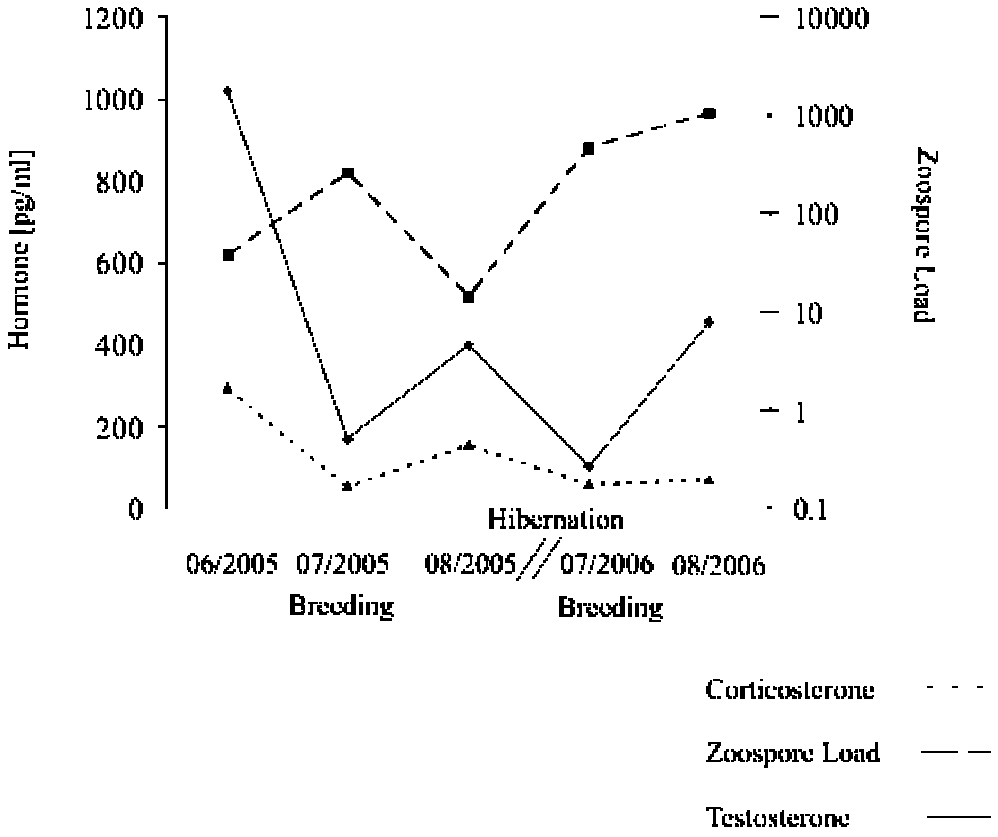

1200
1000
800
600
400
200
0
Hormone [pg/ml]
10000
1000
100
10
1
0.1
Zoospore Load
Hibernation
06/2005
07/2005
08/2005
07/2006
08/2006
Breeding
Breeding
Corticosterone
Zoospore Load
Testosterone

Figure 13.

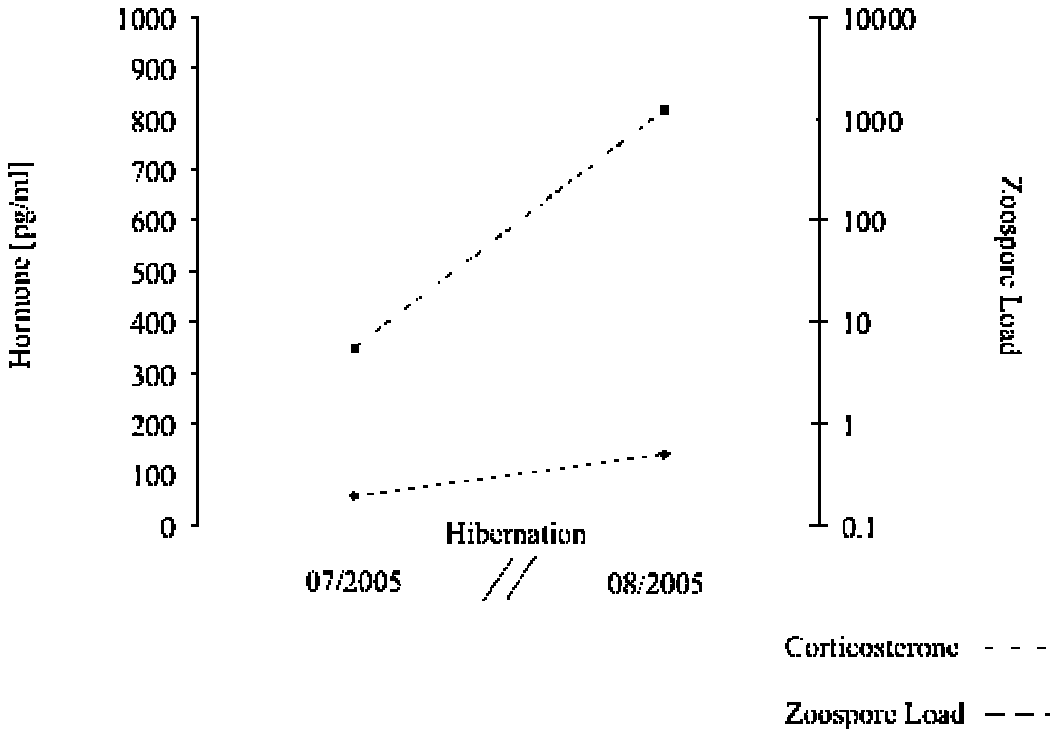

1000
900
800
700
600
500
400
300
200
100
0
Hormone [pg/ml]
10000
1000
100
10
1
0.1
Zoospore Load
Hibernation
07/2005
08/2005
Corticosterone
Zoospore Load

Figure 14.

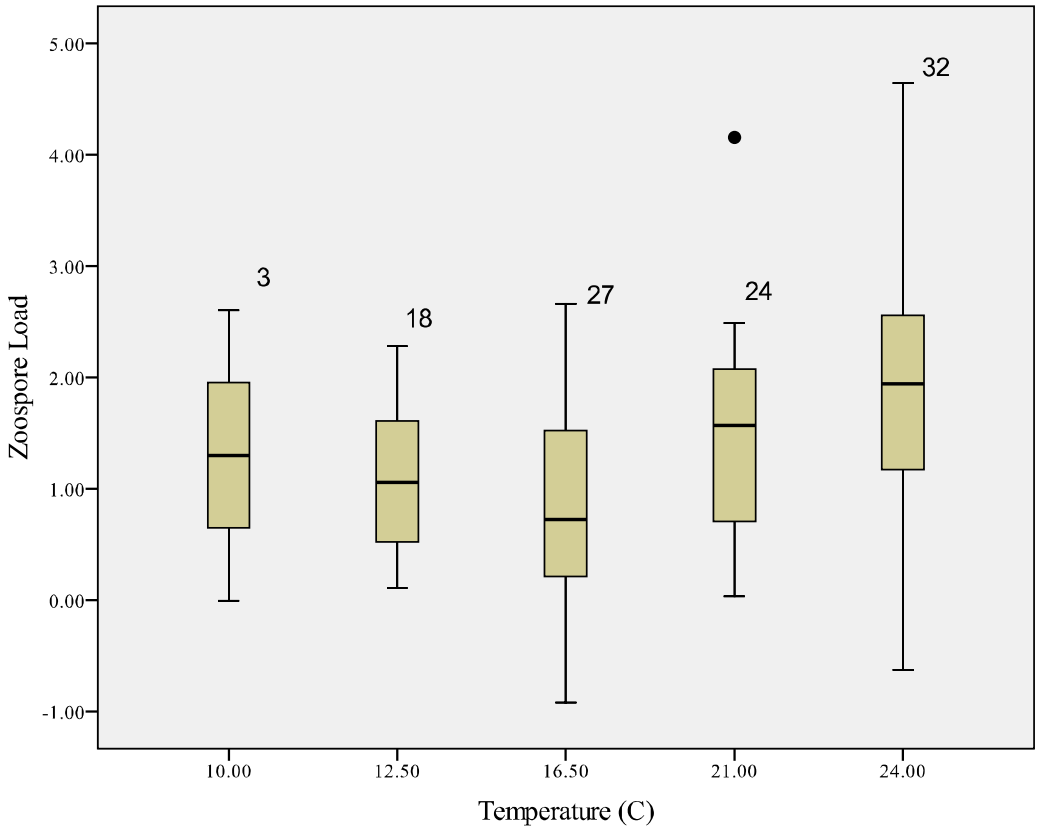
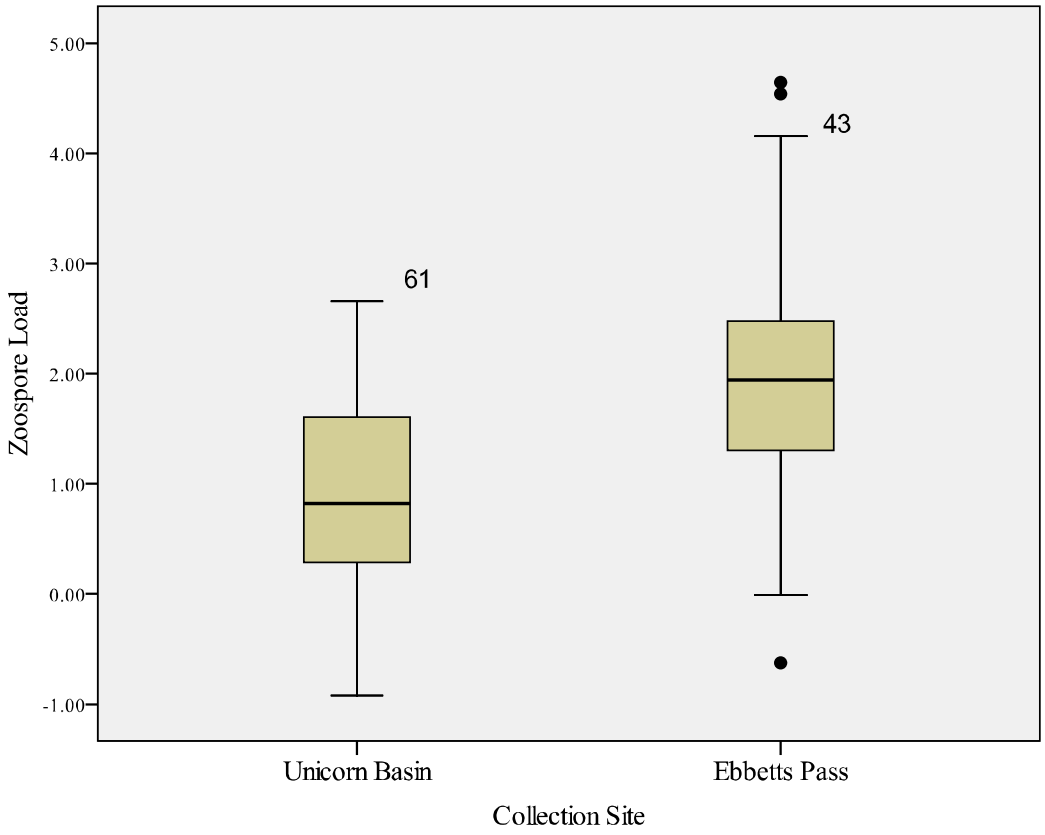

5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Zoospore Load
3
18
27
24
32
10.00
12.50
16.50
21.00
24.00
Temperature (C)

Figure 15.


5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Zoospore Load
61
43
Unicorn Basin
Ebbetts Pass
Collection Site

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