Genetic structure and disease prevalence of boreal toads (Bufo boreas) in Glacier National Park

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GENETIC STRUCTURE AND DISEASE PREVALENCE OF BOREAL TOADS (BUFO BOREAS) IN GLACIER NATIONAL PARK

By

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ABSTRACT
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GENETIC STRUCTURE AND DISEASE PREVALENCE OF BOREAL TOADS (BUFO BOREAS) IN GLACIER NATIONAL PARK

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Amphibians are more threatened than any other vertebrate group, with 41% of species experiencing declines. The causes of most declines are not well understood, though many declines have been linked to the emerging infectious disease chytridiomycosis. Additionally, amphibians are physiologically constrained to moist habitats and considered poor dispersers; thus, they may suffer genetic consequences of population isolation. To address threats to the persistence of boreal toads (Bufo boreas) in Glacier National Park, USA, I genotyped 551 individuals at 11 microsatellite loci and used spatially independent (STRUCTURE) and spatially explicit (GENELAND) Bayesian methods to describe population genetic structure and identify barriers to gene flow. I found evidence of hierarchical population structure: individuals were split into high and low elevation groups, and 2 secondary groups were detected within the high elevation group. These results indicate that elevation strongly influences genetic structure. Genetic variation was high, but allelic richness declined with increasing elevation. I tested a subset of the samples for Batrachochytrium dendrobatidis (Bd), the fungal pathogen which causes chytridiomycosis. Thirty-seven of 109 toads tested positive for Bd. Infection prevalence was not correlated with elevation, but—surprisingly—increased with individual heterozygosity. This finding suggests that dispersal may be facilitating the spread of disease because heterozygosity is highest where dispersal and gene flow are greatest.
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Introduction

A central focus of conservation biology is determining why certain species are more at risk than others. Species traits such as poor dispersal ability, ecological specialization to specific habitat types, and restricted geographic range, often lead to isolation of populations, and can facilitate declines (Purvis et al. 2000; Harcourt et al. 2002). Geographically isolated populations are less likely to receive immigrants that help maintain positive population growth (Brown & Kodric-Brown 1977). Loss of genetic variation and inbreeding depression are also common in small, isolated populations (Frankham 2005). Both reduced genetic variation and inbreeding depression elevate extinction risk and weaken the ability of a population to cope with environmental change (Newman & Pilson 1997; Saccheri et al. 1998; Frankham 2005). Therefore, understanding the causes of population isolation and associated genetic effects is paramount for conservation and management, and many recent studies have focused on identifying landscape features (e.g. mountains, rivers) that lead to isolation by restricting movement and gene flow (e.g. McRae et al. 2005; Spear et al. 2005; Epps et al. 2005; Pérez-Espona et al. 2008).

Amphibians are more threatened than any other vertebrate group (Stuart et al. 2004). Forty-one percent of described amphibian species are classified as threatened, compared to only 25% of mammals, 13% of birds, and 22% of reptiles (Hoffmann et al. 2010). Additionally, most amphibian declines have occurred relatively recently: of the 34 amphibian species reported to have gone extinct since the year 1500, 9 extinctions have occurred since 1980, and an additional 113 species can no longer be found (Stuart et al. 2004). This suggests that the situation is worsening; yet the causes of most amphibian declines are still not well understood.

Amphibians may be more vulnerable to extinction than other vertebrate taxa due to life history characteristics that lead to patchy distributions and geographically isolated populations. For example, because amphibians have highly permeable skin that is subject to evaporative water loss (Duellman & Trueb 1994), movements are often restricted to riparian corridors and forested areas where desiccation risks are low (Rothermel & Semlitsch 2002). Additionally, amphibians are generally considered poor dispersers and many species exhibit high breeding site fidelity (Tracy & Dole 1969; Daugherty & Sheldon 1982; Blaustein et al. 1994). Such physiological constraints and dispersal limitation can lead to limited connectivity among populations and related genetic and demographic effects of isolation (Lowe & Allendorf 2010).

Boreal toads (Bufo boreas) are widely distributed across western North America and occupy a variety of habitats, from desert springs to mountain wetlands (Hammerson et al. 2004). Boreal toads are of conservation concern because they are declining in portions of their range, causing the species to be listed as near-threatened by the IUCN (Hammerson et al. 2004). In Colorado, where declines are most severe, only 1 of 44 known breeding populations is considered viable (Jackson 2008). Factors that influence population isolation and patterns of genetic variation are likely important for predicting long-term persistence of this species, particularly for populations in the southern Rocky Mountains where extreme geographic isolation and aridity make immigration an unlikely source of demographic and genetic rescue (Switzer et al. 2009).
Boreal toads are an ideal species to test for landscape effects on population connectivity, particularly the influence of elevation, because they are found at a range of elevations, from sea level up to 3640 m (Hammerson et al. 2004). Breeding immediately follows snowmelt and usually occurs during a 2-4 week period from mid-May to mid-June, but can occur as late as August at higher elevations (Carey et al. 2005). Thus, toads living at different elevations may be reproductively isolated because breeding times may not overlap. In some landscapes, topographic complexity (i.e., mountain ridges) impedes gene flow (Murphy et al. 2010), while in others it does not (Moore et al. 2011), suggesting that factors influencing population connectivity cannot be broadly applied to all landscapes where boreal toads occur.

Glacier National Park (GNP) in Montana provides an opportunity to examine how landscape effects on genetic variation may influence future boreal toad population trajectories. The Continental Divide runs through the middle of the park, creating high and low elevation toad breeding habitat across a wide range of elevations (960-2190 m). High elevation sites typically have longer winters and more snowfall, constraining breeding to a much narrower window of time than at low elevations.

Given the topographic complexity of GNP, mountain ridges likely impede boreal toad gene flow, as has been shown in other amphibians (Tallmon et al. 2000; Funk et al. 2005; Giordano et al. 2007). In extreme cases, ridges may facilitate allopatric speciation among amphibian populations (Lougheed et al. 1999). This evolutionary process could already be underway in Hanging Gardens, a high elevation site in mountainous central GNP (Fig. 1). Calling toads have been discovered in HG, but toads elsewhere in the park lack vocal slits necessary to produce the call, which is thought to serve as an advertisement to attract mates (Pauly 2008). Thus calling and non-calling toads may be reproductively isolated, reinforcing the potential for divergence at high elevations due to mountain ridges. Understanding how HG toads are genetically differentiated from surrounding populations will provide insight into the role of landscape features, such as mountain ridges, in this pattern of divergence.

Variation in local environmental conditions may also influence population connectivity. Amphibians often show phenotypic variation at microgeographic scales, and this variation is usually related to source pond conditions (Denver et al. 1998; Skelly 2004). For example, breeding ponds in close proximity can differ in light and thermal environments due to differences in shading (Skelly & Freidenburg 2000; Skelly et al. 2002; Halverson et al. 2003). Skelly (2004) collected wood frog embryos from shaded and unshaded wetlands (20-4800 m apart) and conducted a common garden experiment where embryos were exposed to different temperatures that span the range of average in situ temperatures. Individuals from dark wetlands developed faster than individuals from light wetlands, demonstrating a genetic basis for phenotypic variation at microgeographic scales. Similarly, differences in pond hydroperiod may influence the distribution of genotypes by offsetting breeding timing and facilitating reproductive isolation. Four breeding ponds near the Two Medicine Lakes in southeastern GNP (Fig. 1) are densely clustered (ranging from 150-430 m between ponds), but breeding in one pond consistently occurs two weeks earlier than the rest (mid-May v. late-May/early-June; Hossack and Corn, unpublished data). The pond where early breeding occurs is also the only pond to dry completely by the end of the summer. This variation in breeding pond hydroperiod provides an opportunity to test for reproductive isolation at a local, rather than landscape, scale.
Chytridiomycosis, an infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) is gaining attention as a major contributor to amphibian declines worldwide (Berger *et al.* 1998; Lips *et al.* 2006; Skerratt *et al.* 2007), including boreal toad declines in Colorado and Montana (Muths *et al.* 2003; Pilliod *et al.* 2010). The disease is transmitted via contact with infected individuals and by zoospores in water (Nichols *et al.* 2001). Chytridiomycosis has been reported on all 6 continents where amphibians occur (Fisher *et al.* 2009) and in a range of habitats, including lowland rainforests, cold mountaintops, and deserts (Ron 2005). Studies have demonstrated a relationship between Bd infection and temperature (Bosch *et al.* 2007; Olson *et al.* 2013) and Pounds *et al.* (2006) showed that climate warming will likely accelerate the spread of chytridiomycosis by shifting temperatures toward the growth optimum of Bd.

We know that amphibian populations differ in susceptibility to Bd (Crawford *et al.* 2010), and Savage and Zamudio (2011) showed major histocompatibility complex (MHC) heterozygosity was strongly associated with survival in Bd-infected leopard frog populations. More broadly, increases in disease and parasite resistance with increasing heterozygosity has been demonstrated across taxa (Roelke *et al.* 1993; Coltman *et al.* 1999; Meagher 1999; Hedrick *et al.* 2001; Acevedo-Whitehouse *et al.* 2003; Spielman *et al.* 2004; Luikart *et al.* 2008). Further efforts to detect a genetic basis for natural variation in host resistance to chytridiomycosis could be important for predicting local extinction risks and prioritizing conservation efforts for amphibians worldwide.

Boreal toads in GNP have tested positive for chytridiomycosis (Hossack *et al.* 2013b), thus providing an opportunity to test for landscape and genetic factors that lead to patterns of variation in disease prevalence. Based on previous studies, it might be expected that individuals infected with chytridiomycosis will have lower mean heterozygosity than uninfected individuals. Alternatively, individuals in populations experiencing high gene flow, with resulting high levels of heterozygosity, might experience higher exposure to Bd-infected immigrants and have a higher prevalence of Bd infection than individuals from isolated, low-heterozygosity populations. Distinguishing between the two alternatives is important because they have very different management implications (i.e., promoting v. preventing dispersal and gene flow).

Overall, this study had three major goals. The first goal was to describe the population genetic structure of boreal toads in GNP. Second, I tested for landscape effects on genetic population structure. Finally, I used genetics to understand patterns of chytrid transmission and resistance.

**Methods**

*Study site and DNA sampling protocol*

Glacier National Park (GNP), Montana, USA is characterized by steep topography due to extensive glaciation during the Pleistocene. Boreal toads breed at a range of elevations, from 960 m to > 2190 m, and in a variety of habitats, including beaver ponds, forest ponds, and small cirque lakes. They commonly occur in the same wetlands as Columbia spotted frogs (*Rana*...
Columbia spotted frogs are the only other known hosts of Bd in GNP (Muths et al. 2008; Hossack et al. 2013b).

DNA samples were collected from 551 toads older than 1 year in GNP during the summers of 2008-2011 (Fig. 1). Twenty-two toads were sampled in 2008, 246 in 2009, 125 in 2010, and 158 in 2011. Toads were captured at randomly selected wetlands in 17 catchments as a part of an amphibian monitoring program (Corn et al. 2005). Toads were also sampled opportunistically in terrestrial habitats; therefore, not all sampled toads were associated with a wetland. DNA was collected by swabbing the buccal cavity with a foam-tipped or cotton-tipped swab (Pidancier et al. 2003). Buccal swabs were stored in 95% ethanol until DNA extraction.

**DNA isolation and microsatellite amplification**

Tissues were digested using a detergent-based cell lysis buffer followed by ammonium acetate protein precipitation and isopropyl alcohol DNA precipitation. Isolated DNA was resuspended in 100µl TE buffer. DNA was not diluted for polymerase chain reaction (PCR). PCR were carried out in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA) using the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, CA.). All multiplex PCR reactions used a total volume of 10µl. We amplified 11 microsatellite loci that were developed for *Bufo boreas* (BBR29, BBR17, BBR86, BBR87b, BBR36, BBR4, BBR292, BBR281, BBR34-2, BBR16, BBR201; Simandle et al. 2005). PCR conditions followed Murphy et al. (2010). PCR products were visualized on an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Allele sizes were determined using the ABI GS600LIZ ladder (ABI) and called using Genemapper version 3.7 (ABI).

**Genetic analyses**

I did not know *a priori* whether wetlands represented discrete populations, and not all sampled individuals were associated with wetlands. Therefore, I used the Bayesian clustering models in STRUCTURE 2.3.3 (Pritchard et al. 2000) and GENELAND 3.2.4 (Guillot et al. 2005b) to delineate genetic groups (K) in the sample and assign individuals to groups. Both programs group individuals into the most likely number of clusters (K) so that departures from Hardy-Weinberg proportions and gametic disequilibrium are minimized. STRUCTURE bases its inference on genetic data alone, whereas GENELAND incorporates spatial information for the samples.

For the STRUCTURE analysis, the number of clusters (K) is a fixed parameter, and 10 independent runs for K = 1 to 6 were tested assuming an admixture model with correlated allele frequencies. Evanno et al. (2005) showed that STRUCTURE accurately detects the uppermost level of population structure where different layers of population structure exist (i.e., the first round of analysis reveals the major genetic clusters, but further rounds of analyses can reveal substructure within each major genetic cluster). I used a burn-in period of 100,000 with 100,000 Markov Chain Monte Carlo repeats during the first round of analysis to identify the major genetic clusters, and a burn-in length of 300,000 and 300,000 MCMC repeats for subsequent rounds to test for further substructure.
The STRUCTURE run with the highest posterior probability is often used to infer the number of K in the sample; however, this statistic can sometimes be misleading, as once the real K is reached the posterior probability may continue to increase slightly at higher K (Evanno et al. 2005). Evanno et al. (2005) found that the ad-hoc statistic ΔK, which is based on the second order rate of change of the likelihood with respect to K, is a more accurate estimator of the true number of genetic clusters. However, because ΔK is a second order statistic, it cannot differentiate between K = 1 and K = 2, thus the ΔK method was used in conjunction with the highest mean posterior probability to infer the most likely K. STRUCTURE provides the proportion of membership to each cluster (q) for each individual, and individuals were assigned to a population according to their highest q-value.

For the GENELAND analysis, K is not a fixed parameter and 10 independent runs with 100,000 MCMC iterations were performed allowing K to vary from 1 to 9. The uncorrelated allele frequency model was used, as it is known to perform better than the correlated model (Guillot et al. 2005a). The maximum rate of the Poisson process was fixed at 551 (the number of individuals; Guillot et al. 2005a), the maximum number of nuclei in the Poisson-Voronoi tessellation was set at 1653 (3 times the number of individuals; Guillot et al. 2005a), and the uncertainty of spatial coordinates was set at 500 m. The most likely number of clusters was inferred as the modal K with the highest posterior probability. Varying the spatial uncertainty did not alter the most likely number of K.

Genetic structure was also visualized using a principle coordinate analysis (PCA) implemented in GenAlEx version 6.4 (Peakall & Smouse 2005). The PCA was constructed from a pairwise, individual-by-individual genotypic distance matrix. To visualize population patterns, I used MINITAB (Version 16) to conduct a principal component analysis based on the covariance matrix among allele frequencies, omitting the largest allele at each locus to account for the non-independence of allele frequencies within each locus.

I tested for gametic disequilibrium and significant departures from Hardy-Weinberg proportions across loci and identified clusters using exact tests in GENEPOP version 4.0 (Raymond & Rousset 1995). P-values were calculated using Markov Chain permutations (1000 dememorizations, 100 batches, 1000 iterations per batch) according to the algorithm of Guo & Thompson (1992). Genetic variation within populations was calculated as observed heterozygosity (H_0), expected heterozygosity (H_E), and allelic richness. H_0 and H_E were calculated in GenAlEx version 6.41 (Peakall & Smouse 2005) and allelic richness was determined using Fstat 2.9 (Goudet 1995). Departures of observed and expected heterozygosity were quantified using Wright’s (1951) F_{IS} and were calculated in Fstat 2.9 (Goudet 1995). Genetic variation among populations was assessed using pairwise F_{ST} calculated in GENEPOP version 4.0 (Wright 1931; Weir & Cockerham 1984; Raymond & Rousset 1995).

To assess genetic variation among individuals, I used individual multi-locus heterozygosities. I used the R package GENHET (Coulon 2009) to calculate standardized heterozygosity as the proportion of heterozygous typed loci / mean heterozygosity of typed loci (Coltman et al. 1999) because not all loci could be scored in all individuals. I used a Mantel test (Mantel 1967), implemented in GenAlEx version 6.41, to estimate the correlation between individual genetic distances and geographical distances (Peakall & Smouse 2005).
Hanging Gardens and Two Medicine breeding ponds

To test for restricted gene flow due to mountain ridges at high elevations, I used STRUCTURE to determine whether calling Hanging Garden toads are reproductively isolated from toads in two neighboring basins (Heavy Runner and Hidden Lake; Fig. 1). I also used STRUCTURE to determine whether early breeding toads near the Two Medicine Lakes are reproductively isolated from late breeding toads to investigate how differences in breeding phenology may influence genetic structure at a microgeographic scale.

Disease sampling and analyses

Some toads that were sampled for DNA were also sampled for Bd in 2008-2011. To avoid any year effects on infection prevalence, only samples from 2009 were included in further analyses because it was the year with the broadest distribution of samples (n = 109, Fig. 2). Toads were sampled for Bd by swabbing the pelvic patch and undersides of legs and feet with a sterile cotton swab. Swabs were stored in 95% ethanol until analysis for Bd presence.

Bd presence was assessed using a quantitative real-time PCR assay (Hyatt et al. 2007) carried out at the Amphibian Disease Diagnostic Service Center at Washington State University, Pullman, Washington. Samples were categorized as either infected (Bd = 1) or uninfected (Bd = 0). To determine if landscape factors explain variation in Bd prevalence, I tested for an association between Bd infection and elevation using logistic regression. To understand how genetic variation influenced susceptibility to Bd infection, I also tested for an association between individual multi-locus heterozygosity and Bd infection using logistic regression.

Results

Population structure

The first round of STRUCTURE analysis including all 551 individuals revealed the most likely number of clusters to be 2. ΔK was highest at K = 2, but the mean estimated logarithm probability of the data [ln Pr (X|K)] continued increasing at K = 3 through K = 6, a phenomenon which may occur after the true K is reached (Appendix A, Evanno et al. 2005). Because the difference in mean ln Pr (X|K) between K = 2 and K = 6 is much less than the difference between K = 1 and K = 2 (1707 v. 3544, respectively), I concluded the most likely K to be 2.

The mean capture elevations of the two genetic groups (± 1 SD) were significantly different (1763 m ± 232 v. 1484m ± 228, T = 14.173, df = 550, P < 0.001), suggesting that elevation strongly influences genetic structure. Not all individuals assigned to the high elevation group were found at higher elevations than individuals in the low elevation group; there are several instances where individuals assigned to the low elevation group cluster geographically with the high elevation group, and vice versa. In many of these cases, these outlier individuals have low q-values of 0.5 – 0.7, indicating weak assignment to either major group. There is a group of 76 individuals that strongly assign to the high elevation group but were captured at low elevations (Appendix B). These individuals are spatially clustered in the high elevation group.
(close to the Continental Divide, approximately 4 km south of Hanging Gardens; Fig. 1) but are located in the Gunsight valley directly adjacent to other high elevation toads. Thus, the division of individuals into two elevation-based groups is rough; nonetheless, there is a clear pattern indicating that elevation is important in structuring genetic groups.

STRUCTURE analysis was continued in a hierarchical fashion until no further substructure was detected (when \( K = 1 \)). Within the high elevation group, \( \Delta K \) was highest at \( K = 3 \), but the \( \Delta K \) values for \( K = 2 \) and \( K = 3 \) were very similar (Appendix A). The mean \( \ln Pr(X|K) \) values were again highest for \( K = 6 \), but the difference between \( K = 1 \) and \( K = 2 \) is greater than between \( K = 2 \) and \( K = 3 \) (865 v. 417). Pritchard et al. (2000) recommend choosing the smallest value of \( K \) that captures the major structure in the data, thus \( K = 2 \) within the high elevation group. These two groups are roughly arranged along a north-south gradient (Fig. 1), dividing the high elevation group into a northern group and a southern group. Toads from the Hanging Gardens, Hidden Lake, and Heavy Runner basins are included within the southern high elevation group, and STRUCTURE analysis including only these three basins revealed the presence of 3 distinct genetic groups, indicating there are several local populations within the southern high elevation group (Fig. 4).

Within the low elevation group, \( \Delta K \) was highest at \( K = 2 \) (Appendix A). However, the height of the modal value of the distribution of \( \Delta K \) was low, indicating the strength of the signal is fairly weak. Additionally, because \( \Delta K \) is based on the second order rate of change with respect to the likelihood function, it cannot distinguish between \( K = 1 \) and \( K = 2 \). For these reasons, Evanno et al. (2005) warn that the \( \Delta K \) method may not always be the most appropriate criterion for determining the true \( K \). The plotted mean \( \ln Pr(X|K) \) values indicate no clear break in slope and are instead more representative of a single population exhibiting isolation by distance (Schwartz & McKelvey 2009). Additionally, Mantel tests revealed a significant pattern of isolation by distance parkwide (Mantel \( r = 0.30, P < 0.01 \)), suggesting no evidence of further substructure within the low elevation group.

Analyses in GENELAND revealed the most likely number of clusters to be 6. However, two of these clusters contained only 1 and 3 individuals, and there was no spatial coherency in the latter cluster, making those groupings biologically uninterpretable. In the run with the highest posterior probability and a different modal value for \( K \), the most likely number of clusters was 5. Population assignments revealed a pattern very similar to that of STRUCTURE, except that GENELAND found 3 groups in the high elevations and 2 groups in the low elevations. Ninety-eight percent of individuals (541) were assigned to the same elevation grouping (high or low) using both clustering methods. Of the 10 individuals that had different assignments, 7 had \( q \)-values in STRUCTURE ranging from 0.5 – 0.7, indicating weak assignment. The remaining 3 outliers had strong assignments in STRUCTURE (\( q \) values = 0.7 – 1.0), but GENELAND appeared to assign these individuals to the other elevation category based on geographical continuity.

Principal coordinate analysis (PCA) based on pairwise genetic distances between individuals supported the STRUCTURE groupings more than the GENELAND groupings. PC1 clearly separates individuals into the same high (positive coordinate 1 values) and low (negative coordinate 1 values) elevation groups detected by both STRUCTURE and GENELAND (Fig.
 Coordinate 2 differentiates between the two groups within the high elevation cluster detected by STRUCTURE (Fig. 3A, 3C), but does not clearly differentiate between the 3 high elevation groups and 2 low elevation groups detected by GENELAND (Fig. 3B). The GENELAND results may, therefore, overestimate population structure. For further analyses, individuals were grouped according to the results of STRUCTURE. I acknowledge that this is likely a conservative estimate of population structure, and these groups are probably not true populations in a biological sense (Waples & Gaggiotti 2006). Group $F_{IS}$ values indicate a deficit of heterozygotes, likely due to the Wahlund effect (Table 1, Allendorf & Luikart 2007).

Variation within groups

All loci were polymorphic and the number of alleles per locus ranged from 4 alleles at BBR29 to 40 alleles at BBR16. Genetic variation was high; mean expected heterozygosity within groups ranged from 0.68 to 0.74, and allelic richness ranged from 9.89 to 13.04 alleles per locus (Table 1).

Twenty-three $F_{IS}$ values were significantly different from zero before correcting for multiple comparisons. After Bonferroni correction, BBR29 in group 1, BBR87b, BBR292, BBR16, and BBR201 in group 2, and BBR86, BBR36, BBR292, BBR281, BBR34-2, BBR16, and BBR201 in group 3 deviated from Hardy-Weinberg expectations. All loci exhibited heterozygote deficiency, with the exception of BBR201, which exhibited heterozygote excess. Because no locus showed consistent deviations from Hardy-Weinberg expectations in all groups, all loci were retained for further analyses.

Significant gametic disequilibrium was detected among 28 pairs of loci after Bonferroni correction, but only 4 pairs of loci were out of equilibrium in 2 groups (BBR17 & BBR86, BBR4 & BBR34-2, BBR86 & BBR281, BBR87b & BBR36) and no locus-pair was out of equilibrium in all groups.

I also tested whether restricted gene flow at high elevations due to mountain ridges resulted in lower genetic variation compared to the low elevation group. Mean expected heterozygosity was not lower in the high elevation group (binomial sign test, $P_{one-tailed} = 0.50$). However, mean allelic richness was significantly lower in the high elevation group than in the low elevation group ($P_{one-tailed} = 0.03$). This difference was also statistically significant using a randomization test (10,000 randomizations, $P < 0.0001$) where individual genotypes were randomized with respect to elevation designation (high or low) and allelic richness was calculated for each randomization.

Variation among groups

All pairwise $F_{ST}$ comparisons between the 3 groups were significant (Table 2). The greatest $F_{ST}$ values were between the high and low elevation groups, indicating that gene flow is most restricted among low and high elevation groups (Table 2). The significant differentiation between the two high elevation groups suggests that mountain ridges represent a barrier to gene flow.
To further assess how mountain ridges influence genetic connectivity, I used STRUCTURE to test whether toads in Hanging Gardens and two neighboring basins (Heavy Runner and Hidden Lake) were genetically distinct. The ΔK method (Evanno et al. 2005) revealed the most likely number of K to be 3 (Fig. 4) and $F_{ST}$ values between the 3 basins were significant (Table 3), indicating that mountain ridges act as barriers to gene flow in this area, and may underlie divergence in calling phenotypes.

I also used STRUCTURE to determine whether early breeding toads were reproductively isolated from late breeding toads near the Two Medicine Lakes. The ΔK method (Evanno et al. 2005) revealed the most likely number of K to be 3; however, the height of the modal value of the distribution of ΔK is extremely low (Appendix C) and the plotted mean ln Pr (X|K) values exhibit a pattern of isolation by distance (Appendix C, Schwartz & McKelvey 2009), indicating little evidence of substructure. Genetic samples collected at the early breeding pond do not cluster together (Appendix C), thus early breeding toads appear not to be reproductively isolated from late breeding toads.

**Disease prevalence**

A subset of toads that were sampled for DNA were also sampled for Bd to test for a relationship between infection and individual heterozygosity. Of the 109 individuals tested for Bd, 37 were positive (Fig. 2). Logistic regression analysis revealed that individuals with higher multiple-locus heterozygosity had significantly higher prevalence of Bd infection ($\chi^2 = 4.71$, df = 1, $P < 0.03$; Fig. 5). Bd infection was unrelated to capture elevation ($\chi^2 = 0.13$, df = 1, $P < 0.72$), and there was no clear spatial pattern of infection (Fig. 2).

**Discussion**

**Elevation influences genetic structure**

My results show that elevation strongly influences genetic structure of toads in GNP. The first line of evidence comes from the initial split detected by STRUCTURE, which roughly divided individuals into a high elevation genetic group and a low elevation genetic group (Fig. 1). GENELAND also grouped individuals into high and low elevation groups, though the program detected greater substructure than STRUCTURE. Principle coordinate analysis based on pairwise genetic distances between individuals also distinguished between low and high elevation primary genetic groups (Fig. 3). Finally, pairwise $F_{ST}$ values showed that gene flow is restricted among high and low elevation groups (Table 2). However, the assignment of individuals to the high or low elevation genetic groups based solely on capture location is not always clear, as some individuals found at low elevations assign to the high elevation group (i.e., Gunsight valley individuals), and vice versa.

There are several reasons gene flow might be restricted among low and high elevation groups. Upslope dispersal may be limited by energetic costs and heightened desiccation risk with increased elevation due to reduced forest cover (Rothermel & Semlitsch 2002; Lowe et al. 2008; Semlitsch et al. 2009). However, Funk et al. (2005) found restricted gene flow between Columbia spotted frogs at low and high elevations despite evidence of long distance upslope
dispersal. Similar to Columbia spotted frogs, boreal toads are vagile compared to other amphibians (Harris 1975; Semlitsch 1981; Kleeberger & Werner 1982; Muths 2003; Adams et al. 2005) and may be capable of dispersing long distances uphill.

Rather than reflecting dispersal constraints, the genetic difference I observed between low and high elevation toads could be due to reproductive isolation based on elevation-related differences in breeding phenology. Some breeding ponds are separated by > 1 km of elevation in GNP, and breeding at low elevation sites can occur up to 60 days earlier than breeding at high elevations sites (B. Hossack, personal communication). Thus, even though dispersal may not be restricted between high and low elevations, toads living at different elevations are likely reproductively isolated. Snowpack throughout the Northern Rockies is expected to decrease under future climate change scenarios (Leung et al. 2004; Mote 2006; McKelvey et al. 2011), potentially reducing these differences in phenology and genetic differentiation related to elevation.

Although elevation is likely an important source of variation in breeding phenology and reproductive isolation throughout GNP, at microgeographic scales it appears that genetic structure is unrelated to variation in breeding phenology. Although some toads near the Two Medicine lakes breed approximately 2 weeks earlier than the rest, STRUCTURE analyses did not reveal evidence of genetic differentiation among Two Medicine toads. The 2-week difference in breeding timing at Two Medicine is striking, but it is important to note that breeding can be offset by more than 2 months at low and high elevation sites in GNP, making genetic differentiation much more likely.

Parkwide genetic structure

My results suggest there are 3 genetic groups of boreal toads in GNP. However, the Bayesian algorithm implemented in STRUCTURE (Pritchard et al. 2000) is not designed for situations where there is evidence of isolation by distance, as I detected in this study (Pritchard et al. 2010). Schwartz & McKelvey (2009) warn that patterns of spatial autocorrelation of allele frequencies due to mating with neighbors should be considered prior to population structure analyses. This is because autocorrelation along with irregular sampling can be misinterpreted as landscape features acting as barriers to gene flow, leading to faulty conclusions about the role of the landscape in shaping population structure. I sampled continuously at a scale much larger than the dispersal capability of boreal toads, so it is unlikely I detected false barriers. Nevertheless, the designation of 3 discrete groups should be viewed as a coarse estimation of population structure within the park, and group $F_{IS}$ values indicate it is likely an underestimate (Table 1). Further, though I could not resolve true biological populations at a parkwide scale, STRUCTURE analysis including Hanging Gardens, Hidden Lake, and Heavy Runner basins indicates that there are likely several local populations within the high elevation group. Therefore, my analysis only describes general patterns of genetic differentiation.

The amount of population differentiation in this study was higher than boreal toad populations elsewhere (Manier & Arnold 2006; Moore et al. 2011), suggesting that there are stronger barriers to gene flow in this system. Overall, boreal toads in GNP have high genetic diversity. Expected heterozygosity ranged from 0.683-0.736 within groups, and allelic richness
ranged from 9.887-13.038 alleles per locus. These values are at the upper range observed for other pond-breeding amphibians (Newman & Squire 2001; Funk et al. 2005; Manier & Arnold 2006; Moore et al. 2011). For example, expected heterozygosity of wood frog populations in the Prairie Pothole Region in North Dakota ranged from 0.44-0.50, and the maximum number of alleles per locus was 5 (Newman & Squire 2001). The authors hypothesized that extinction/colonization dynamics likely contributed to these low levels of genetic variation because breeding ponds in the region are ephemeral and cycle through periodic dry conditions. Frequent wildfires in GNP also lead to high turnover in wetlands, and toad occupancy increases initially post-fire (Hossack et al. 2013a). Such dynamics could increase genetic variation because toads coming from outside the immediate burn area colonize the burned wetlands.

Mountain ridges are barriers to gene flow

Significant differentiation between genetic groups at high elevations \( (F_{ST} = 0.11) \) adds to evidence that mountain ridges act as barriers to gene flow in amphibians (Lougheed et al. 1999; Tallmon et al. 2000; Funk et al. 2005; Giordano et al. 2007). Hanging Gardens, the only known location in the park where calling toads have historically existed (Pauly 2008), represents one of the highest elevations where toads breed in GNP and is surrounded by > 300 m cliffs on 3 sides and drained by a stream that plunges to a valley bottom 520 m below. Such steep topography could prevent gene flow into the Hanging Gardens basin, promoting divergence and, ultimately, allopatric speciation. When HG toads and toads from 2 neighboring basins (Heavy Runner and Hidden Lake) were included in a separate STRUCTURE analysis, the three basins were significantly differentiated. However, \( F_{ST} \) values among the basins were within the range observed parkwide (0.11-0.14, compared to parkwide range of 0.11-0.23). I would expect higher \( F_{ST} \) values if HG toads were completely reproductively isolated, but these results do not preclude an effect of landscape structure on divergence in calling phenotype.

I found that allelic richness decreased with elevation, but found no relationship between heterozygosity and elevation. This result contrasts with other studies showing reductions in both metrics of genetic variation in high elevation amphibian populations (Funk et al. 2005; Giordano et al. 2007; Martínez-Solano & González 2008). Genetic drift acts more quickly on allelic diversity than heterozygosity (Allendorf & Luikart 2007), which may explain these results. The decrease in allelic richness with increasing elevation in this study indicates that there may be long-term genetic consequences of isolation at higher elevations, particularly the ability to respond to selection. The loss of alleles reduces the genotypic diversity in a population that is subject to natural selection (Allendorf 1986; Allendorf & Luikart 2007), and large populations harboring more genetic variation have a greater response to selection than small, isolated populations with reduced genetic variation (Frankham 1996).

The ability to respond to selection may be especially relevant under future climate change scenarios where rising temperatures are predicted to reduce the amount and duration of mountain snowpack (Leung et al. 2004), causing earlier breeding by montane amphibians (Corn 2003). Associated with early breeding are a suite of consequences, such as increased exposure to killing frosts (Inouye et al. 2000), increased duration of larval period, and shortened pond hydroperiod (Corn 2003), which may pose serious threats to populations with reduced adaptive potential. Reduced allelic richness may also have important implications for chytridiomycosis resistance.
MHC loci characteristically have many alleles and are associated with disease resistance in many taxa (Clarke 1979). Savage and Zamudio (2011) showed that heterozygous leopard frogs bearing a specific allele, MHC allele Q, exhibited reduced risk of death from chytridiomycosis. Thus, reductions in allelic diversity could significantly affect disease susceptibility.

**Higher disease prevalence in more heterozygous individuals**

I found that more heterozygous individuals were more likely to be infected with Bd than less heterozygous individuals (Fig. 5). This result is surprising in view of previous studies reporting that more heterozygous individuals within local populations tend to be more resistant to pathogens or parasites (e.g., Roelke et al. 1993; Coltman et al. 1999; Acevedo-Whitehouse et al. 2003; Luikart et al. 2008). In addition, others have reported that local populations with greater mean heterozygosity tend to be more resistant to pathogens or parasites (e.g., Meagher 1999; Hedrick et al. 2001; Spielman et al. 2004; Pearman & Garner 2005). Inter and intra-population differences in heterozygosity result from different genetic processes (Luquet et al. 2011). Random genetic drift leads to differences in heterozygosity among populations (Kimura et al. 1963), while inbreeding depression due to segregating deleterious alleles within populations leads to differences in heterozygosity among individuals within populations (Charlesworth & Charlesworth 1987). It is important to consider whether correlations between heterozygosity and disease resistance result from intra or inter-population differences in heterozygosity because they have very different management implications (i.e., promoting gene flow vs. preventing mating between kin, Luquet et al. 2011).

My results are best explained by my sampling scheme. I was only able to identify major genetic groups and could not resolve local populations, so it is possible that the positive relationship between individual heterozygosity and infection results from an individual- or population-level effect. Nevertheless, the findings of this study likely result from a population-level effect. That is, individuals with higher heterozygosity likely come from local populations with greater immigration. Populations experiencing higher immigration are expected to have greater heterozygosity and greater exposure to chytridiomycosis. This would result in a positive relationship between heterozygosity and Bd infection. More broadly, this would suggest that dispersal may be facilitating the spread of chytridiomycosis throughout GNP. Additionally, this result would provide empirical support for mathematical models predicting that increased contact among populations increases prevalence, incidence and rate of disease spread (Hethcote 1976; Post et al. 1983; Andreason & Christiansen 1989; Hess 1996), and may even allow disease to persist when it would otherwise decline in isolated populations (e.g., Post et al. 1983; Andreason & Christiansen 1989).

**Conclusions**

My study revealed the presence of 3 genetic groups of boreal toads in GNP, with one low elevation group and two high elevation groups. Thus, elevation strongly influences genetic structure, most likely because of differences in breeding phenology at low and high elevations. I found evidence of greater divergence at high elevations, suggesting that mountain ridges restrict gene flow. Genetic variation in boreal toad groups in GNP was high; however, toads at high elevations exhibited reduced allelic diversity, which could limit adaptive potential under future
environmental change. A potentially more important threat to population persistence is chytridiomycosis. I found that more heterozygous individuals had higher infection prevalence, suggesting that dispersal facilitates disease transmission. In this and other systems, knowledge of gene flow among populations may be very valuable for identifying populations likely to be exposed to disease, thus prioritizing conservation effort.

**Literature Cited**


Hossack BR, Lowe WH, Ware JL, Corn PS (2013b) Disease in a dynamic landscape: host behavior and wildfire reduce amphibian chytrid infection. *Biological Conservation*, 157, 293–299.


Fig. 1 Locations of boreal toad genetic samples \((n = 551)\) in Glacier National Park, Montana, USA. Symbols represent genetic groups revealed by STRUCTURE analysis. Open symbols represent the high elevation genetic group, and closed symbols represent the low elevation genetic group. Open squares and circles represent groups 1 and 2, respectively. Closed circles represent group 3. Symbols overlap where multiple toads were sampled. The blue line indicates the Continental Divide.
Fig. 2 Location of boreal toad disease samples \((n = 109)\) in Glacier National Park, Montana, USA. Red symbols indicate individuals infected with chytridiomycosis and black symbols indicate uninfected individuals. The blue line indicates the Continental Divide.
Fig. 3 Principal coordinate analysis based on pairwise genetic distances between individuals (A and B), and principal component analysis based on covariance matrix of group mean allele frequencies (C). Symbols in (A) and (C) correspond to STRUCTURE group assignments. Symbols in (B) correspond to GENELAND group assignments.
Fig. 4 (A) Population assignments from STRUCTURE analysis including toads from Hanging Gardens ($n = 30$), Heavy Runner ($n = 17$), and Hidden Lake ($n = 23$) basins. Each population is indicated by a different symbol, and symbols overlap where multiple toads were sampled. The blue line indicates the Continental Divide. (B) Plot of q values from STRUCTURE simulations when $K = 3$. (C) Plotted mean $\ln P(X|K)$ values from STRUCTURE simulations for $K = 1-6$. (D) Results of STRUCTURE simulations using the delta K method (Evanno et al. 2005) to detect the most likely number of $K$. 
Fig. 5  (A) Prediction curve from logistic regression analysis for probability of Bd infection relative to individual heterozygosity. A Bd infection value of 0 indicates no infection and a value of 1 indicates infection. (B) Proportion of individuals infected with chytridiomycosis at different heterozygosity levels. Numbers above bars indicate sample size for each heterozygosity level.
### Table 1  Sample size, mean capture elevation, and genetic information of 3 groups of boreal toads in Glacier National Park, Montana

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Mean capture elevation (m)</th>
<th>AR</th>
<th>HE</th>
<th>F&lt;sub&gt;IS&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>High elevation group</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>87</td>
<td>1865</td>
<td>9.989</td>
<td>0.728</td>
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<tr>
<td>2</td>
<td>210</td>
<td>1721</td>
<td>9.887</td>
<td>0.736</td>
<td>0.038*</td>
</tr>
<tr>
<td>Low elevation group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>254</td>
<td>1478</td>
<td>13.040</td>
<td>0.683</td>
<td>0.091*</td>
</tr>
</tbody>
</table>

N, population size; AR, allelic richness; HE, expected heterozygosity; F<sub>IS</sub>, Wright’s (1951) index of deviation from expected heterozygosity, averaged across loci: 1- (H<sub>O</sub>/H<sub>E</sub>). Significance: probability * < 0.05.

### Table 2  Pairwise F<sub>ST</sub> values for groups of boreal toads in Glacier National Park, Montana

<table>
<thead>
<tr>
<th>Group</th>
<th>High</th>
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<th></th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>0.233</td>
<td>0.200</td>
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</tr>
</tbody>
</table>

### Table 3  Pairwise F<sub>ST</sub> values between Hanging Gardens (HG), Heavy Runner (HR) and Hidden Lake (HL) basins in Glacier National Park, Montana

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>HL</th>
<th>HG</th>
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</thead>
<tbody>
<tr>
<td>HR</td>
<td>-</td>
<td>-</td>
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<tr>
<td>HL</td>
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<td></td>
</tr>
<tr>
<td>HG</td>
<td>0.137</td>
<td>0.123</td>
<td>-</td>
</tr>
</tbody>
</table>
Appendix A

Mean of estimated $\ln P(X|K)$ and Delta K of STRUCTURE analyses including all individuals (panel A, $n = 551$), individuals assigning to the high elevation group (panel B, $n = 297$), and individuals assigning to the low elevation group (panel C, $n = 254$).
Appendix B

STRUCTURE plot indicating proportion of membership of each toad ($n=552$) in each of K populations for $K=2$. Individuals are arranged according to capture elevation, lowest to highest.
Appendix C

Results of STRUCTURE analysis including early and late-breeding toads from Two Medicine Lakes. (A) Population assignments for individuals sampled at 4 breeding ponds. Intervals along the y-axis indicate the proportion of assignment (q) to each cluster when K = 3. (B) Mean Ln P(X|K) values from STRUCTURE simulations for K = 1-6. (C) Results of STRUCTURE simulations using the delta K method (Evanno et al. 2005) to detect the most likely number of K.