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PATTERNS AND CONSEQUENCES OF DISPERSAL
IN COLUMBIA SPOTTED FROGS (*RANA LUTEIVENTRIS*)

by

William Christopher Funk

B.A. Wesleyan University, 1994

presented in partial fulfillment of the requirements

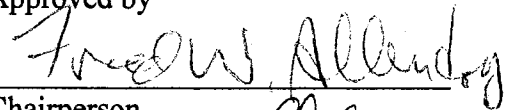
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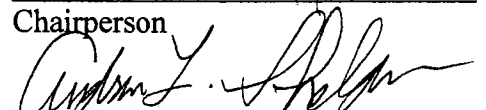
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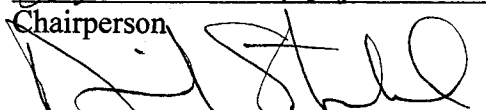
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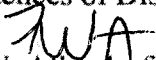
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Patterns and Consequences of Dispersal in Columbia Spotted Frogs (*Rana luteiventris*)

Co-Advisors: Fred W. Allendorf and Andrew L. Sheldon



Ecological and evolutionary theory predicts that dispersal can have important effects on population dynamics and evolutionary trajectories. The objective of my dissertation was to estimate rates and patterns of dispersal and gene flow in Columbia spotted frogs (*Rana luteiventris*) in order to explore the ecological and evolutionary consequences of dispersal. I used multistate capture-recapture analysis of site-specific capture histories and allele frequency data for six microsatellite loci to characterize dispersal and gene flow among populations in western Montana and Idaho. I collected site-specific capture histories for 10,443 uniquely marked frogs from two focal low elevation basins in northwest Montana over four consecutive years of fieldwork. Although amphibians are generally considered to have low dispersal capabilities, I found exceptionally high juvenile dispersal rates of up to 53% annually. Moreover, juveniles dispersed over long distances (> 5 km), large elevation gains (> 750 m), and steep inclines (36° mean incline over 2 km). In contrast, adult dispersal rates and distances were very low. Microsatellite estimates of gene flow were also high for these two basins, suggesting that juvenile dispersers successfully reproduce in the populations to which they immigrate.

I collected microsatellite data from 28 sites from throughout western Montana and Idaho that provide additional insights into movement patterns among Columbia spotted frog populations. In particular, although gene flow is very high among low elevation sites, it is often low among high elevation sites and restricted between low and high elevation sites. Additionally, I observed a strong negative relationship between within population genetic variation and elevation, suggesting that historic effective population sizes are much smaller at high elevations than low elevations. High elevation populations may therefore be more susceptible to stochastic population extinction than low elevation populations. Low elevation populations may also serve as important sources of immigrants and colonists for high elevation populations. Moreover, although there is dispersal and gene flow between low and high elevation populations, gene flow does not appear to constrain local adaptation in egg size.

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The collection of capture-recapture data for over ten thousand frogs was a heroic effort and represents the sweat and blood of many people. Most notably, I am indebted to Allison Greene who worked for me for three years, starting out as a recent high school graduate and ending up as a collaborator. Other froggers include Sherry Adams, Ryan Benson, Britta Bentz, Greg Brownworth, Corissa Crowder, Robin Greene, Nat Johnson, Pat Lizon, Chris Richey, and several volunteers. I also thank Jill Davies and Rodd Gallaway for providing field accommodations. Kathy Knudsen also facilitated lab work and Steve Amish helped run microsatellite gels.

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CHAPTER 1 – Introduction

BACKGROUND

Dispersal is the movement of individuals from one breeding population to another with the potential for reproduction in the new population. Theory predicts that dispersal can have important ecological and evolutionary consequences. Ecological effects include synchronization of population dynamics (Hanski 2001), the rescue of populations from extinction (Brown and Kodric-Brown 1977), and the coexistence of predators and prey (van Baalen and Hochberg 2001). Evolutionary effects include the maintenance of genetic variation and the inhibition of local adaptation through gene flow (Wright 1969; Storfer and Sih 1998). Moreover, the ecological effects of dispersal may have important indirect evolutionary effects, and vice versa. For example, dispersal directly affects gene flow and within population genetic variation which in turn may indirectly affect vital rates and population dynamics (Newman and Tallmon 2001). Dispersal also has important implications for conservation by influencing the geographic distribution of populations, inbreeding depression, the distribution of adaptive genetic variation, population persistence, and patterns of species diversity.

Although the importance of dispersal has been recognized for several decades (Grinnell 1922; Wright 1931), there has been a recent surge in interest in dispersal exemplified by new books dedicated to the subject (Clobert et al. 2001; Bullock et al. 2002). Nevertheless, dispersal theory greatly outpaces the accumulation of dispersal data, so our understanding of dispersal remains limited. In the forward to Clobert et al. (2001), Peter Waser writes in reference to dispersal, “we are almost as limited as was Grinnell [in 1922] with regard to data.” Dozens of modeling papers are published

annually that investigate the population dynamic consequences of dispersal using simulation approaches, while only a handful of studies have estimated dispersal for natural populations (e.g., Roland et al. 2000; Peacock and Ray 2001; Trenham et al. 2001; Lowe 2003; Blums et al. 2003) because dispersal is notoriously difficult to estimate (Koenig et al. 1996). Although modeling serves the useful purpose of refining predictions, improving our understanding of dispersal ultimately will require estimating dispersal patterns for natural populations.

Fortunately, new methods and analyses have recently been developed that have great potential for estimating dispersal and gene flow for natural populations. In particular, multistate capture-mark-recapture (CMR) analysis was developed to allow estimation of movement probabilities from one geographic, life history, or physiological 'state' to another (Nichols and Kendall 1995). Additionally, highly variable molecular markers such as microsatellite loci can be used to estimate genetic differentiation and by inference, gene flow, over small geographic scales. Used in combination, multistate CMR analysis and microsatellite markers provide a powerful approach for characterizing movement patterns.

An understanding of dispersal patterns is particularly relevant to the conservation of amphibians. Amphibian populations are declining on a global scale and habitat fragmentation has been cited as one of the most important factors responsible for their declines (Wake 1991; Bradford et al. 1993; Blaustein et al. 1994). One negative effect of fragmentation is the isolation of populations historically connected by dispersal. Isolation of naturally connected populations may make them more vulnerable to extinction by preventing the rescue of populations by immigrants (Brown and Kodric-

Brown 1977). However, there is little information on how much dispersal there is among amphibian populations, so it is difficult to predict whether the isolating effects of habitat fragmentation are likely to negatively impact amphibians. A better understanding of amphibian dispersal patterns will therefore improve understanding of the effects of fragmentation on amphibian persistence.

RESEARCH OBJECTIVES AND FINDINGS

The main goal of my dissertation was to estimate dispersal and gene flow among populations of Columbia spotted frogs (*Rana luteiventris*) and investigate their ecological, evolutionary, and conservation consequences. The specific questions I address in chapters two, three, and four, respectively, are:

1. How much dispersal is there among populations and what are the implications for spatial population dynamics?
2. How do landscape features affect patterns of gene flow and genetic variation?
3. What causes elevational divergence in egg size and does gene flow constrain divergence?

Columbia spotted frogs are an excellent species for investigating dispersal because they are abundant, can be caught, and can be uniquely marked using toe clips. Moreover, many *Rana* species in the western U.S. have undergone precipitous population declines in the last few decades (Drost and Fellers 1996; Davidson et al. 2001), so the genus is of conservation concern. Columbia spotted frogs populations appear stable in the northern Rocky Mountains which provides the unique opportunity to understand dispersal patterns among healthy populations of a *Rana* species.

In chapter two, I investigated dispersal patterns in Columbia spotted frogs and discuss the implications for spatial population dynamics. Amphibian dispersal patterns are poorly known, but amphibians are generally considered to have limited dispersal abilities (Gill 1978; Daugherty and Sheldon 1982). I used multistate CMR analysis to estimate dispersal patterns and rates over four years in two replicate basins. I also collected allele frequency data at six microsatellite loci from these same two basins to infer patterns of gene flow. Analysis of over ten thousand uniquely marked frogs shows that juvenile dispersal rates are exceptionally high in some years, but that adults disperse little. Gene flow was also high, in line with CMR results. Moreover, juveniles often dispersed over long distances (> 5 km), large elevation gains (> 750 m), and steep inclines (mean incline of 36° over 2 km).

These results show that at least some Columbia spotted frog populations are highly connected by dispersal, suggesting that dispersal may play an important role in their population dynamics. This suggests that some amphibians may be vulnerable to the isolating effects of habitat fragmentation. This is the first study I am aware of that rigorously estimates dispersal in an amphibian over large spatial scales (> 7 km) in replicate basins with CMR analysis. It is likely that other amphibians have similarly high dispersal rates that remain undocumented because studies have not previously been designed to detect long distance dispersal.

In chapter three, I used the same six microsatellite loci as I used in my two focal basins to estimate patterns of genetic variation within and among populations and infer patterns of gene flow for 28 breeding ponds throughout western Montana and Idaho. Landscape features such as mountain ridges, rivers, and ecological gradients likely affect

dispersal patterns which in turn should affect patterns of gene flow and genetic variation (Manel et al. 2003). The observation of high dispersal rates in Columbia spotted frogs in chapter two suggests that gene flow may also be high among some populations. I found that the landscape strongly influences patterns of genetic variation. In particular, I found a strong negative relationship between within population genetic variation and elevation, low differentiation among low elevation sites, high differentiation among high elevation sites, and moderately high differentiation between low and high elevation sites.

I developed a model to explain patterns of genetic variation in Columbia spotted frogs that I term the 'valley mainland-mountain island' model. This model has three basic features: (1) low elevation populations with large historic effective population sizes and high levels of gene flow; (2) high elevation populations with small historic effective population sizes and little to no gene flow; and (3) gene flow is restricted, but not absent, between low and high elevation populations. An important conservation implication of this model is that high elevation populations may be more susceptible to extinction than low elevation populations. Moreover, low elevation populations may also serve as important sources of immigrants for high elevation populations.

In my fourth chapter, I investigated the causes of elevational divergence in Columbia spotted frog egg size and whether gene flow constrains divergence. Gene flow is predicted to constrain local adaptation. Although gene flow appears to be restricted across elevation in Columbia spotted frogs, gene flow may still be high enough to constrain local adaptation in the very different environments found in low elevation valleys versus high elevation mountains. Egg size is positively related to elevation for many taxa and this pattern may represent local adaptation to harsher high elevation

environments. I examined the relationship between egg size and elevation in Columbia spotted frogs and tested whether the pattern is due to divergent natural selection or genetic drift. I also tested whether gene flow constrains egg size divergence. I found that egg size is strongly positively related to elevation along two independent elevational transects in western Montana. All lines of evidence also supported the hypothesis that elevational divergence in egg size is caused by divergent natural selection. Moreover, gene flow does not appear to constrain egg size divergence.

These results are in line with previous work suggesting that elevational gradients may be important sources of adaptive genetic variation and therefore merit high conservation priority (McKay et al. 2001). Also, the observation that gene flow does not appear to constrain egg size divergence suggests that selection for larger egg size at high elevations is strong. This is in agreement with previous work demonstrating that natural selection is often strong enough to prevent gene flow from constraining divergence (Danley et al. 2000; Saint-Laurent et al. 2003).

SYNTHESIS

Theory predicts that dispersal can play an important role in population dynamics and evolutionary trajectories. However, dispersal data for wild populations are extremely limited. My capture-recapture data showing high dispersal in Columbia spotted frogs demonstrate the potential for dispersal to play an important role in the population dynamics of amphibians. Moreover, because theory predicts that dispersal is important for population persistence, these data suggest that fragmentation of Columbia spotted frog populations and other *Rana* frog populations may increase local extinction rates.

This prediction is in agreement with the fact that many low elevation populations of *Rana* frogs in highly modified regions such as the Central Valley of California, Willamette Valley of Oregon, and Puget Trough of Washington have already gone extinct (Green et al. 1996, 1997; Davidson et al. 2001).

Microsatellite data add to the capture-recapture data by demonstrating that landscape features strongly influence patterns of gene flow. In particular, although gene flow is high among low elevation sites, it is often low among high elevation sites and restricted between low and high elevation sites. Additionally, the strong negative relationship observed between within population genetic variation and elevation suggests that historic effective population sizes are much smaller at high elevations than low elevations. High elevation populations may therefore be more susceptible to stochastic population extinction than low elevation populations. Low elevation populations may also serve as important sources of immigrants and colonists for high elevation populations. Moreover, although there is dispersal and gene flow between low and high elevation populations, gene flow does not appear to constrain local adaptation in egg size.

This study represents the first step in trying to understand the role of dispersal in amphibian population dynamics. The next step is to use ecological sensitivity analysis to investigate the relative importance of dispersal for population growth and persistence relative to other within population vital rates (Biek et al. 2002). I have already collected the necessary vital rate data to model Columbia spotted frog population dynamics and plan on pursuing this work in the future. More work is also needed to test the effects of landscape modifications such as roads, agriculture, and wetland loss on amphibian

movement patterns in order to assess whether habitat fragmentation actually isolates amphibian populations.

TWEB INTERNSHIP

During my dissertation, I was supported by an NSF Graduate Research Traineeship called the Training Within Environmental Biology (TWEB) program. A major component of the TWEB program was an internship that allowed TWEB students the opportunity to work with a government agency, a non-governmental organization (NGO), or other academics on an applied conservation project. For my internship, I worked with Ecuadorian biologists from the Universidad Católica del Ecuador to develop an amphibian monitoring program in Ecuador. Our monitoring program was also supported by an NGO called the Conservation, Food and Health Foundation. The main goal of my internship was to start an effective amphibian monitoring program to assess changes in population density that could be transferred over to my Ecuadorian colleagues. The first year of the program we focused on testing alternative methods for monitoring *Eleutherodactylus* frogs which resulted in a publication included here as chapter five. Closed population CMR analysis proved to be the most precise and unbiased method and had the highest power to detect major population declines. I was also able to secure funding for two more years of monitoring. Most importantly, I have transferred the monitoring project over to my Ecuadorian colleagues and am helping them apply for additional funding to continue the project.

CHAPTER 2 – High Dispersal in a Frog Species Suggests Amphibians Vulnerable to Habitat Fragmentation

Abstract.—Global losses of amphibian populations are a major conservation concern and have generated substantial scientific debate concerning their causes (Wake 1991; Houlahan et al. 2000). Habitat fragmentation has been cited as one important potential cause of amphibian population declines (Wake 1991; Bradford et al. 1993; Blaustein et al. 1994). Fragmentation of populations naturally connected by dispersal isolates populations, making them more vulnerable to extinction (Brown & Kodric-Brown 1977; Saccheri et al. 1998). However, there is little information on how much dispersal there is among amphibian populations, so it is difficult to predict whether the isolating effects of habitat fragmentation are likely to negatively impact amphibians. We examined dispersal rates in a frog species using a combination of capture-recapture analysis of 10,443 uniquely marked frogs followed over four years and genetic analysis of six microsatellite loci in replicate basins. Here we show exceptionally high juvenile dispersal rates (up to 53% annually) over long distances (> 5 km), large elevation gains (> 750 m), and steep inclines (36° mean incline over 2 km) in Columbia spotted frogs (*Rana luteiventris*) that are corroborated by the genetic data showing high gene flow. These findings show that dispersal can be an important life history feature of amphibians and suggest that isolation of populations from habitat fragmentation may pose a serious threat to amphibians.

Key words.—dispersal, habitat fragmentation, connectivity, Columbia spotted frog, *Rana luteiventris*, capture-recapture analysis, microsatellite.

Dispersal among populations is expected to increase population persistence through the 'rescue effect' whereby immigrants reduce local extinction rates (Brown and Kodric-Brown 1977). Immigrants may reduce extinction rates directly by reproducing in the populations to which they disperse and indirectly by boosting genetic diversity which can reduce negative inbreeding effects on reproductive and survival rates (Newman and Tallmon 2001). Because rescue effects may be important for population persistence of populations naturally connected by dispersal, isolation of these populations through habitat fragmentation is expected to increase extinction rates.

Amphibians are generally considered to have low dispersal rates (Gill 1978; Daugherty and Sheldon 1982), although this view has recently been challenged (Marsh & Trenham 2001). Despite the recognition of the importance of dispersal in population dynamics (Hanski 2001), few studies have attempted to quantify dispersal in amphibians (Berven and Grudzien 1990; Trenham et al. 2001; Lowe 2003) because of the notorious difficulty of estimating dispersal (Koenig et al. 1996). However, recent advances in capture-recapture analysis and new, highly variable molecular genetic markers greatly improve the potential to understand dispersal patterns. In particular, multistate capture-recapture analysis allows statistically rigorous estimation of current movement rates among populations (Nichols and Kendall 1995). Moreover, microsatellite loci are sufficiently variable to look at patterns of gene flow over small geographic scales in order to make inferences about historic dispersal (Spruell et al. 1999). Used in combination, capture-recapture analysis and genetic analysis provide a powerful approach for investigating dispersal.

We uniquely marked and recaptured juvenile and adult Columbia spotted frogs (*Rana luteiventris*) from 21 ponds in two replicate basins, Keeler Creek (9 ponds) and Marten Creek (12 ponds), in northwest Montana. Site-specific capture histories were then used to estimate annual stage-specific movement probabilities between the lower and upper group of ponds in each basin using multistate capture-recapture analysis (Fig. 2.1). Basins were divided into lower and upper groups of ponds at the elevational midpoint between the lowest and highest pond in each basin. In Keeler Creek, the upper group was pond A and the lower group was ponds B – I and in Marten Creek, the upper group was ponds A – D and the lower group was ponds E – L (Fig. 2.1). Frogs were sampled for four consecutive summers starting in 2000. We also analyzed genetic variation in five ponds from Keeler Creek (ponds A, D, F, H, and I) and six ponds from Marten Creek (ponds B, C, E, G, H, and K) at six microsatellite loci to estimate gene flow (Fig. 2.1). If dispersal has been high historically, then gene flow estimates should also be high, whereas the opposite should be true if dispersal has been low.

MATERIALS AND METHODS

Capture-recapture Analysis

We caught Columbia spotted frogs using dip-nets and marked them with unique toe-clip codes (Heyer et al. 1994) during three- to four-week capture sessions in July and August of each year. Ponds were separated by a maximum straight-line distance of approximately 7 km in each basin. Downstream movements were designated as negative and upstream movements as positive. Movement distributions were compared using Kolmogorov-Smirnov tests (Sokal and Rohlf 1981). Upstream or downstream bias in

movement was examined by testing whether movement distributions were significantly skewed (Zar 1984).

Metamorphic and juvenile frogs were lumped into a single juvenile class for multistate capture-recapture analysis because their movement distributions were not significantly different ($P = 0.39$ in Keeler and $P = 0.29$ in Marten). Multistate capture-recapture analysis assumes that survival between year i and $i + 1$ only depends on the location in year i and not on the location in year $i + 1$ (Nichols and Kendall 1995). This is a reasonable assumption for the current analysis because Columbia spotted frogs primarily move after rains (Pilliod et al. 2002) suggesting that movement is most likely to occur during the rainy spring and early summer months immediately prior to sampling in July and August. Therefore, most frogs will likely spend the majority of the sampling interval from year i to $i + 1$ at the location they are found in year i .

We analyzed capture-recapture models with stage-, annual-, and site-specific variation in survival, capture, and movement probabilities in program MARK (Appendices 1 – 4; White and Burnham 1999). A step-down modeling approach (Lebreton et al. 1992) was used to reduce sources of variation in capture and survival probabilities (Appendices 1 and 3) and then test hypotheses about variation in movement probabilities (Appendices 2 and 4). Akaike's information criterion adjusted for sample size (AIC_c) was used to identify the best models in terms of parsimony and fit to the data (Akaike 1973). Models with ΔAIC_c values ≤ 2 were considered to have strong support. Because no generally agreed upon method exists for independently testing the fit of multistate models, we followed recommendations to increase the variance inflation factor (\hat{c}) from one to assess confidence in the best model (Cooch and White 2001). Increasing

\hat{c} favored models with fewer numbers of parameters as expected but did not qualitatively change our finding that juvenile dispersal rates are high in both Keeler and Marten Creeks.

Microsatellite Analysis

We genotyped a total of 312 adult frogs at six microsatellite loci from five ponds in Keeler Creek and six ponds in Marten Creek (mean sample size of 28) that were sampled during spring breeding seasons (Fig. 2.1). The five ponds sampled in Keeler Creek (ponds A, D, F, H, and I) are equivalent to ponds 1 – 5 in Chapter 3 and the six ponds sampled in Marten Creek (ponds B, C, E, G, H, and K) are equivalent to ponds 7 – 12 in Chapter 3. Primer sequences, DNA extraction methods, microsatellite DNA amplification conditions, and Hardy-Weinberg (HW) proportion and gametic disequilibrium analyses can be found in Chapter 3.

RESULTS

Analysis of marked frogs showed high dispersal rates over long distances in both basins. We made a total of 15,008 captures of 10,443 uniquely marked frogs. Juveniles moved much more than adults ($P < 0.001$; Fig. 2.2). Twenty-five percent of recaptured juveniles moved at least 200 m ($N = 108$), 14% moved at least 1,000 m ($N = 60$), nine percent moved at least 2,000 m ($N = 39$), and two percent moved at least 5,000 m ($N = 7$). In contrast, only four percent of adults moved at least 200 m ($N = 13$), two percent moved at least 1,000 m ($N = 6$), and one percent moved at least 2,000 m ($N = 4$). The maximum distance moved was 5,750 m, the maximum elevation gain was 770 m, and the

greatest incline traversed was 36° (700 m elevation gain over 1930 m horizontal distance), all by juvenile frogs (Fig. 2.3).

Annual juvenile movement probabilities between the lower and upper group of ponds were high, but varied over years from almost 0.00 ± 0.00 (SE) in Keeler Creek and 0.07 ± 0.02 in Marten Creek in 2001 to 0.31 ± 0.14 in 2000 and 0.53 ± 0.21 in 2002 in Keeler Creek and 0.27 ± 0.07 in 2000 in Marten Creek (Tables 2.1 – 2.2). Annual adult movement probabilities between the lower and upper group of ponds approximated zero for all years in both basins. Ninety-five percent of frogs (21 of 22) that were marked, recorded in a new location in a subsequent year, and then caught again in another year remained in the site to which they immigrated, indicating that almost all movement represents permanent dispersal rather than temporary migration. Moreover, juvenile survival rates were fairly high in both basins (0.09 ± 0.02 to 0.83 ± 0.31 ; Tables 2.1 – 2.2), suggesting that juveniles often survive long enough to reproduce in the sites to which they immigrate. There was no difference between basins ($P = 0.59$ for juveniles and $P = 0.29$ for adults) or sexes ($P = 1.00$) in movement distributions, nor any bias towards upstream or downstream movement ($0.10 < P < 0.20$).

F_{st} , a measure of population subdivision, for the six microsatellite loci examined was low in Keeler Creek (0.064 ± 0.011) and in Marten Creek (0.016 ± 0.002) as expected if historical dispersal rates and gene flow are high. This degree of subdivision is expected if there are on average 2.5 and 10.5 dispersers ('migrants' in the genetic sense) entering each population each generation in Keeler and Marten Creeks, respectively, assuming an island model of migration corrected for a finite number of populations (Wright 1969; Slatkin 1995). Moreover, the island model estimate of the

number of dispersers is likely biased low for Keeler Creek because of decreasing gene flow with increasing distance ('isolation by distance') in this basin ($P = 0.01$; Wright 1931). Isolation by distance was not observed in Marten Creek ($P = 0.21$).

Average heterozygosity across all microsatellite loci and populations was 0.62. A few populations had one or two loci out of HW proportions ($P < 0.05$), but there was no consistency as to which locus or for heterozygote excess or deficiency. Two loci, *Rp3* and *SFC139*, were in gametic disequilibrium in six out of 11 populations ($P < 0.05$), consistent with weak linkage between these two loci. Removing *Rp3* or *SFC139* from the analysis does not affect the conclusion that global F_{st} values are low in both Keeler and Marten Creeks.

DISCUSSION

Our capture-recapture and microsatellite analyses demonstrate that current and historic rates of dispersal are exceptionally high in Columbia spotted frogs. Importantly, high gene flow also indicates that juvenile dispersers successfully breed and make demographic contributions in the ponds to which they disperse, suggesting that dispersal may have an important effect on spatial population dynamics (Hanski 2001). Microsatellite analysis of genetic variation throughout western Montana also shows fairly low levels of population divergence (pairwise F_{st} values as low as 0.089) among low elevation populations separated by over 200 kms, indicating that populations separated by much greater distances than analyzed here may also be connected by dispersal (Chapter 3). High dispersal in Columbia spotted frogs may have evolved in response to highly

variable recruitment rates in ponds (Pechmann et al. 1991) and to high rates of pond loss and formation.

Other studies have also shown high dispersal rates in amphibians (Berven and Grudzien 1990; Marsh and Trenham 2001; Trenham et al. 2001), but this is the first study we are aware of to rigorously quantify amphibian dispersal using capture-recapture analysis in replicate basins and to confirm that current dispersal patterns are representative of historic patterns using genetic analysis. Moreover, this is the first study to document high dispersal rates between low and high elevation populations of amphibians, suggesting that populations in these different habitats may be demographically connected. It is likely that other amphibian species have high dispersal rates as well that have not been documented because few studies have been designed to estimate dispersal over large distances.

High dispersal rates in Columbia spotted frogs demonstrate the potential for high dispersal in amphibians, a taxonomic group often thought to have low dispersal. This suggests that at least some amphibians may be vulnerable to the isolating effects of habitat fragmentation because populations naturally connected by dispersal may require dispersal for population persistence. Therefore, maintaining habitat connectivity should be a high priority for amphibian conservation. Future research should also focus on identifying human created landscape features that impede amphibian movement.

TABLE 2.1. Juvenile (j) and adult (a) annual survival (S), capture (p), and transition (Ψ) probability estimates for lower (l) and upper (u) populations of Columbia spotted frogs in Keeler Creek, Montana, from the best-fitting multistate model (Appendix 2). Transitions are both population- (rs) and stage-specific.

Parameter	Estimate	Standard Error	Lower 95% CI	Upper 95% CI
$S_{j_{2000}}$	0.32	0.11	0.15	0.55
$S_{j_{2001}}$	0.83	0.31	0.06	1.00
$S_{j_{2002}}$	0.27	0.13	0.09	0.56
$S_{a_{2000}}$	0.56	0.05	0.46	0.67
$S_{a_{2001}}$	0.77	0.07	0.62	0.88
$S_{a_{2002}}$	1.00	0.15×10^{-4}	1.00	1.00
p_j^l	0.02	0.01	0.01	0.04
p_j^u	0.01	0.01	0	0.05
p_a^l	0.24	0.03	0.19	0.31
p_a^u	0.50	0.04	0.43	0.57
$\Psi_{2000}^{r_j s_j}$	0.31	0.14	0.11	0.61
$\Psi_{2001}^{r_j s_j}$	0.14×10^{-12}	0.11×10^{-6}	-0.22×10^{-6}	0.22×10^{-6}
$\Psi_{2002}^{r_j s_j}$	0.53	0.21	0.18	0.85
$\Psi_{2000}^{r_j r_a}$	0.09	0.05	0.03	0.24

$\Psi_{2001}^{r_j r_a}$	0.04	0.02	0.01	0.11
$\Psi_{2002}^{r_j r_a}$	0.17	0.10	0.05	0.44
$\Psi_{2000}^{r_j s_a}$	0.23×10^{-14}	0.70×10^{-8}	-0.14×10^{-7}	0.14×10^{-7}
$\Psi_{2001}^{r_j s_a}$	0.17×10^{-14}	0.29×10^{-8}	-0.57×10^{-8}	0.57×10^{-8}
$\Psi_{2002}^{r_j s_a}$	0.36×10^{-14}	0.73×10^{-8}	-0.14×10^{-7}	0.14×10^{-7}
$\Psi^{r_a s_a}$	0.20×10^{-15}	0.12×10^{-8}	-0.24×10^{-8}	0.24×10^{-8}
Ψ^{a_j}	0.99×10^{-13}	0.81×10^{-7}	-0.16×10^{-6}	0.16×10^{-6}

TABLE 2.2. Juvenile (j) and adult (a) annual survival (S), capture (p), and transition (Ψ) probability estimates for lower (l) and upper (u) populations of Columbia spotted frogs in Marten Creek, Montana, from the best-fitting multistate model (Appendix 4). Transitions are both population- (rs) and stage-specific.

Parameter	Estimate	Standard Error	Lower 95% CI	Upper 95% CI
S_{j2000}^l	0.31	0.06	0.21	0.43
S_{j2001}^l	0.28	0.05	0.20	0.38
S_{j2000}^u	0.13	0.03	0.08	0.21
S_{j2001}^u	0.09	0.02	0.06	0.14
S_{a2000}^l	0.44	0.09	0.29	0.61
S_{a2001}^l	0.50	0.09	0.33	0.66
S_{a2000}^u	0.73	0.25	0.19	0.97
S_{a2001}^u	0.58	0.13	0.32	0.80
p_j	0.22	0.06	0.12	0.36
p_{a2001}	0.16	0.03	0.11	0.24
p_{a2002}	0.25	0.04	0.18	0.33
Ψ_{2000}^{r,s_j}	0.16	0.06	0.08	0.30
Ψ_{2001}^{r,s_j}	0.04	0.02	0.02	0.09
Ψ_{2000}^{r,s_a}	0.36	0.09	0.21	0.55

$\Psi_{2001}^{r_j r_a}$	0.33	0.08	0.20	0.50
$\Psi_{2000}^{r_j s_a}$	0.11	0.04	0.05	0.23
$\Psi_{2001}^{r_j s_a}$	0.03	0.01	0.01	0.08
$\Psi^{r_a s_a}$	0.23×10^{-15}	0.14×10^{-8}	-0.27×10^{-8}	0.27×10^{-8}
Ψ^{aj}	0.15×10^{-14}	0.30×10^{-8}	-0.60×10^{-8}	0.60×10^{-8}

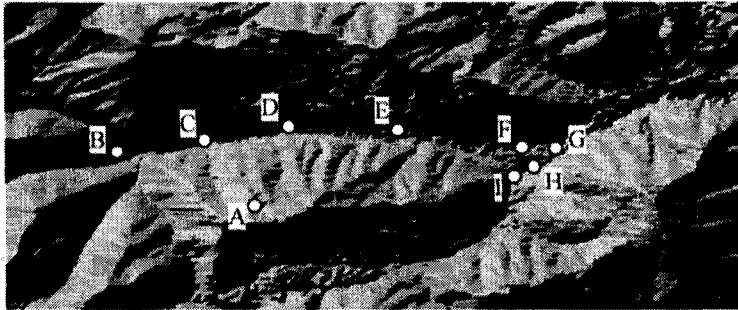
FIGURE LEGENDS

FIG. 2.1. Location of Columbia spotted frog breeding ponds in Keeler and Marten Creeks, Montana, U.S.A., sampled for capture-recapture and genetic analyses.

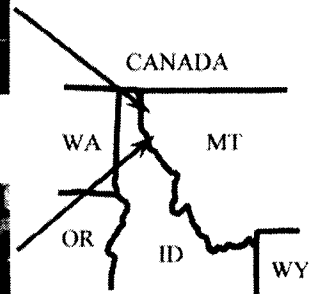
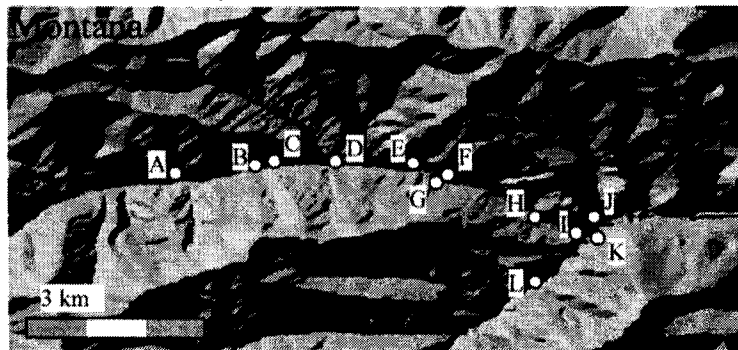
FIG. 2.2. Movement distributions of (a) juvenile and (b) adult Columbia spotted frogs from Keeler and Marten Creeks, Montana, U.S.A. Negative values represent downstream movements and positive values upstream movements.

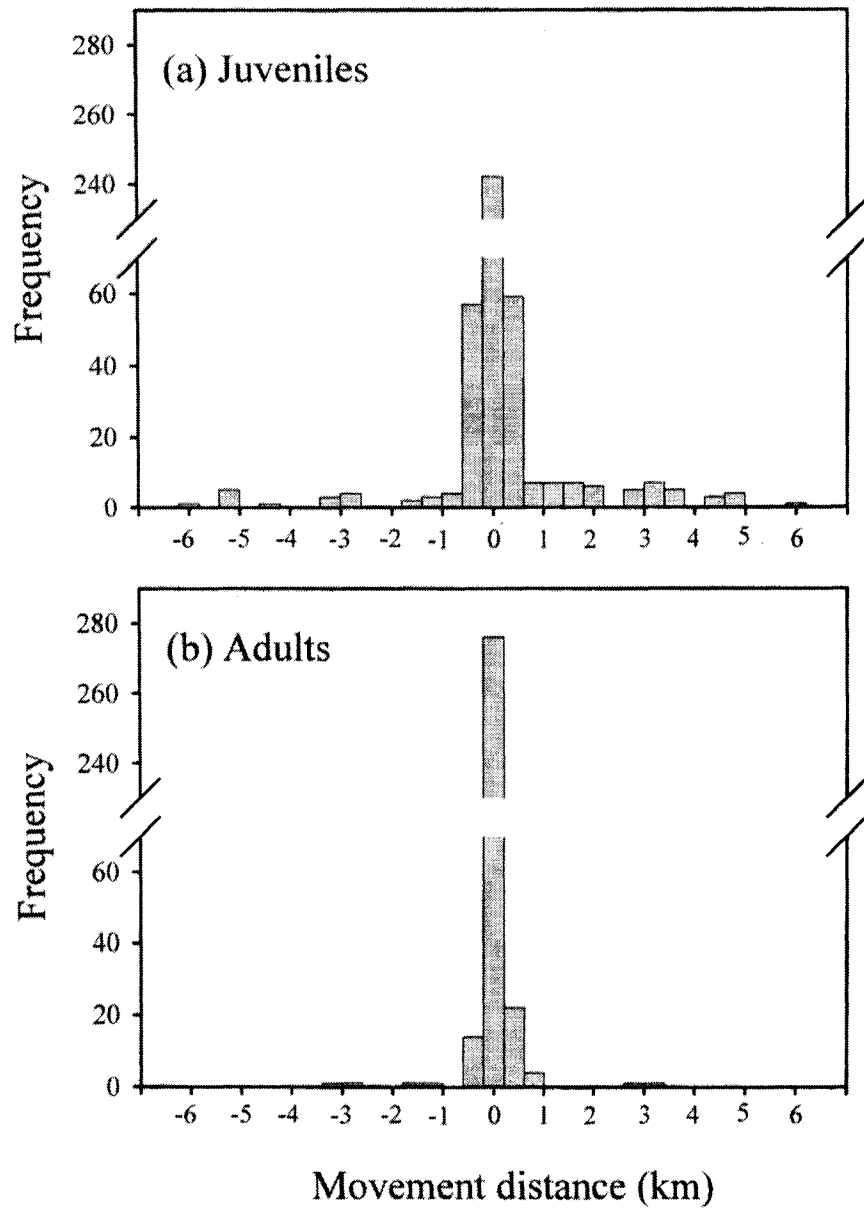
FIG. 2.3. Movements of Columbia spotted frogs from different low elevation ponds to a high elevation lake in Keeler Creek, Montana, U.S.A. The inset shows a juvenile Columbia spotted frog (approximately 25 mm total length), the life history stage responsible for most dispersal in this species. Vector A represents an elevation gain of 770 m over a horizontal distance of 4,240 m (18° mean incline); vector B an elevation gain of 760 m over 4,620 m (16° incline); and vector C an elevation gain of 700 m over 1,930 m (36° incline). The number of frogs observed moving from each low elevation pond to the high elevation lake is indicated in parentheses.

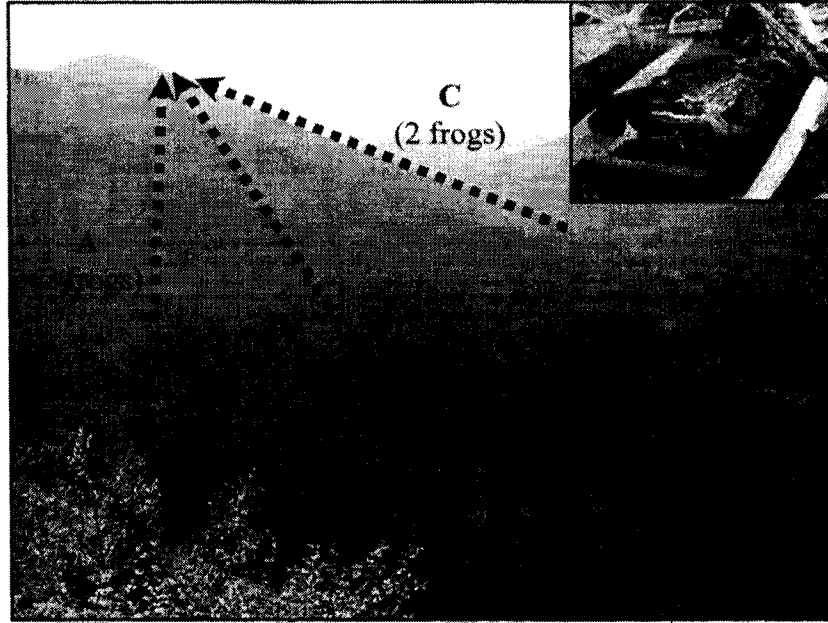
Keeler Creek, Montana



Marten Creek,
Montana







CHAPTER 3 – Population Structure of Columbia Spotted Frogs (*Rana luteiventris*)
is Strongly Affected by the Landscape

Abstract.—Landscape features such as mountains, rivers, and ecological gradients may strongly affect patterns of dispersal and gene flow among populations and thereby shape population dynamics and evolutionary trajectories. The landscape may have a particularly strong effect on patterns of dispersal and gene flow in amphibians because amphibians are thought to have limited dispersal abilities. We examined genetic variation at six microsatellite loci in Columbia spotted frogs (*Rana luteiventris*) from 28 breeding ponds in western Montana and Idaho, USA, in order to investigate the effects of the landscape on patterns of gene flow. We were particularly interested in addressing three questions: (1) Do ridges act as barriers to gene flow? (2) Is gene flow restricted between low and high elevation ponds? (3) Does a pond equal a ‘randomly mating population’ (a deme)? Mountain ridges and elevational differences were associated with increased genetic differentiation among sites, suggesting that gene flow is restricted by ridges and elevation in this species. Populations of Columbia spotted frogs generally include more than a single pond except for very isolated ponds. We also found evidence for surprisingly high levels of gene flow among low elevation sites separated by large distances. Moreover, genetic variation within populations was strongly negatively correlated with elevation, suggesting effective population sizes are much smaller at high elevation than low elevation. Our results show that landscape features have a profound effect on patterns of genetic variation in Columbia spotted frogs. We develop a model of population structure to explain our results, discuss the evolutionary and conservation

implications of the model, and explain how this model may account for conflicting studies on gene flow in amphibians.

Key words.—Columbia spotted frog, *Rana luteiventris*, landscape genetics, microsatellite, gene flow, dispersal, evolution, conservation.

Describing the effects of landscape features on genetic variation is essential for understanding how landscapes shape dispersal, gene flow, population divergence, and speciation (Manel et al. 2003). For example, many models of population divergence and speciation invoke specific landscape features such as rivers, mountains, or habitat gradients as the primary cause of divergence (Wallace 1852; Smith et al. 1997; Lougheed et al. 1999). However, because little is known about the effects of these features on genetic variation, it is difficult to predict their potential for causing population divergence. Understanding the effects of the landscape on genetic variation is also important for identifying the geographic units most suitable for management of populations of different species.

The landscape may have particularly strong effects on genetic variation in amphibians because amphibians are generally thought to have low dispersal abilities. Evidence for low dispersal in amphibians comes from field studies showing high philopatry (Gill 1978; Daugherty and Sheldon 1982; Driscoll 1997) and genetic studies showing low levels of among population gene flow (Larson et al. 1984; Driscoll 1998; García-Parris et al. 2000; Shaffer et al. 2000; Tallmon et al. 2000; Monsen and Blouin 2003). However, other studies on amphibian dispersal suggest that amphibian movement

may not always be so limited (Breden 1987; Berven and Grudzien 1990; Marsh and Trenham 2001; Trenham et al. 2001). Therefore, there may be potential for high gene flow in some species or among some populations (Berry 2001; Newman and Squire 2001; Squire and Newman 2002; Lampert et al. 2003).

Mountain ridges are one landscape feature that may act as important barriers to dispersal and gene flow in amphibians. Because amphibians are subject to high evaporative water loss due to their permeable skin (Duellman and Trueb 1994), amphibians may tend to move along riparian corridors rather than over drier mountain ridges. Or, if ridges are high enough, they may be impassible because they exceed the physiological temperature limits of some species. Loughheed et al. (1999) found that an historic mountain ridge acted as an important barrier to gene flow in a frog, supporting the hypothesis that ridges act as barriers for amphibians. Support for this hypothesis also comes from biogeographic evidence showing that the ranges of some amphibians are bounded by mountains (Lynch and Duellman 1997).

Elevational differences among amphibian populations may also restrict dispersal and gene flow. First, dispersal might be restricted from low to high elevation populations simply because of the energetic costs of moving up steep slopes. Second, even if dispersal is not restricted, pre-mating and post-mating barriers to gene flow may restrict gene flow between low and high elevation populations. Pre-mating barriers to gene flow may include lower survival of dispersers or lower mating success of dispersers due to elevational differences in breeding phenology (Howard and Wallace 1985) or differences in sexually-selected traits such as advertisement calls (Narins and Smith 1986; Lüddecke

and Sánchez 2002). Moreover, if dispersers do successfully mate, post-mating barriers such as hybrid sterility or inviability may reduce reproductive success.

Mountain ridges, elevation, and other landscape features may also influence the distribution of amphibian populations across the landscape. In many ecological and genetic studies of pond and lake breeding amphibians, ponds or lakes are considered to be synonymous with randomly mating populations (Gill 1978; Sjögren 1991; Hecnar and M'Closkey 1996; Tallmon et al. 2000). This is an appealing definition of a population because ponds and lakes are discrete physical units bounded by the shoreline. However, data showing substantial interpond movements in amphibians suggest that populations may sometimes include more than a single pond (Berven and Grudzien 1990; Marsh and Trenham 2001; Trenham et al. 2001). Resolving the spatial extent of amphibian populations is important for determining the most appropriate geographic unit for management.

Columbia spotted frogs (*Rana luteiventris*) are pond breeding frogs distributed from the southern Rocky Mountains northward through southeast Alaska (Green et al. 1996, 1997). They are found in a variety of habitats, ranging from low elevation wetlands to high elevation lakes. Field studies demonstrate that Columbia spotted frogs can move long distances, but the effects of these movements on fine-scale patterns of genetic variation remain unknown (Turner 1960; Reaser 1996; Pilliod et al. 2002; Chapter 2). Columbia spotted frog populations appear stable except for isolated populations in the southern portion of the species' range in Nevada and Utah (Bos and Sites 2001). However, the sister species of the Columbia spotted frog, the Oregon spotted frog (*Rana pretiosa*), has declined dramatically throughout its range in northeast

California, western Oregon and Washington, and southwest British Columbia (Green et al. 1997) and is a candidate for listing under the U.S. Endangered Species Act (ESA). Moreover, other *Rana* species in the western U.S. such as the California red-legged frog (*Rana aurora draytonii*) and the mountain yellow-legged frog (*Rana muscosa*) have suffered dramatic declines as well and are already listed under the ESA in all or parts of their ranges (Drost and Fellers 1996; Davidson et al. 2001). Therefore, the study of genetic variation in Columbia spotted frogs provides the unique opportunity to understand natural patterns of genetic variation in a western *Rana* species in relatively undisturbed habitats.

We investigated patterns of genetic variation at microsatellite loci within and among populations of Columbia spotted frogs to address three primary questions: (1) Do ridges act as barriers to gene flow? (2) Is gene flow restricted between low and high elevation ponds? (3) Does a pond equal a randomly mating population? Our results show that the landscape has strong effects on genetic variation in Columbia spotted frogs. We develop a model to explain the patterns of genetic variation observed, discuss the evolutionary and ecological implications of the model, and explain how this model may account for conflicting studies on gene flow in amphibians.

MATERIALS AND METHODS

Samples

We sampled approximately 30 adult Columbia spotted frogs from each of 28 ponds and lakes (sites) across western Montana and Idaho for a total of 790 individuals using toe-clips (Table 3.1; Fig. 3.1; Heyer et al. 1994). We used a hierarchical sampling

scheme that allowed us to test the effects of mountain ridges and elevational differences on genetic variation. Specifically, sites were sampled in adjacent basins to allow us to test the effects of intervening ridges and at different elevations within basins to allow us to test the effects of elevational differences. Moreover, this same sampling scheme was used in three different regions (Cabinet and Coeur d' Alene Mts., Montana; Bitterroot Mts., Montana; and Bighorn Crags, Idaho) to broaden the geographic scope of inference of the study.

We sampled frogs in the breeding season or shortly thereafter to make sure they were associated with the breeding population from the given pond rather than temporary seasonal migrants. Males and females were considered adults if they were greater than or equal to 45 mm and 50 mm snout-vent-length, respectively, based on the minimum sizes of frogs seen breeding. Males can be distinguished from females based on the presence of nuptial pads on the thumbs (Turner 1960). In site number 17, tadpoles were sampled because no adults were found. In site number 18, juvenile frogs and hatchling tadpoles were sampled in addition to adult frogs to supplement the sample size. In this case, only a single hatchling was taken from each egg mass to avoid disproportionate sampling of a few families.

We sampled most sites in 2000, but some were sampled in 2002 and 2003. We tested whether temporal changes in allele frequencies between 2000 and 2003 could obscure spatial patterns of population divergence by testing for significant differences in allele frequencies between samples from 2000 ($N = 28$) and 2003 ($N = 27$) at a single site, site number 8 (Table 3.1; Fig. 3.1), with exact tests of population differentiation. There was no difference in allele frequencies at this site between 2000 and 2003 ($P =$

0.36). Therefore, we concluded that allele frequencies were likely sufficiently stable at single sites to avoid confounding between temporal and spatial genetic variation.

Microsatellites

DNA was extracted using the Pure Gene® kit (Gentra) following the manufacturer's instructions. We used six microsatellite loci that were developed originally for Oregon spotted frogs (*Rana pretiosa*) (*Rp3*, *Rp15*, *Rp17*, and *Rp23*) and Columbia spotted frogs (*R. luteiventris*) (*SFC134* and *SFC139*; Table 3.2; Blouin, unpubl. data). *Rp15* had odd-sized alleles consistent with variation in both microsatellite repeat number and non-microsatellite insertion-deletions (Table 3.2; Appendix 5). Loci were amplified using the PCR reagents described in Monsen and Blouin (2003) and the annealing temperatures shown in Table 3.2. PCR was conducted in a MJ Research PTC-100 thermocycler with a total reaction volume of 10 μ L.

Amplified alleles were separated on 7% denaturing polyacrylamide gels and visualized using a Hitachi FMBIO-100 fluorescent imager. Allele sizes were determined relative to a standard base pair size ladder (MapMarkerLow, Bioventures). Previously amplified products were included on each gel to ensure consistent scoring of individuals across all gels.

Data analysis

Allele frequencies, exact probabilities for Hardy-Weinberg proportions, exact probabilities for genotypic disequilibrium, *F*-statistics, and exact probabilities of differentiation in allele frequencies were calculated using GENEPOP version 3.3

(Raymond and Rousset 1995). Expected heterozygosities and allelic richness were calculated using FSTAT version 1.2 (Goudet et al. 1996). Linear regression analysis of expected heterozygosity vs. elevation and allelic richness vs. elevation was performed in MINITAB version 13.

We used two methods to examine broad geographic subdivisions across all three regions. First, we conducted analysis of molecular genetic variance with F_{st} (AMOVA; Excoffier et al. 1992) using ARLEQUIN version 2.001 (Schneider et al. 2000). We compared five alternative population groupings with AMOVA to test which grouping explained the greatest proportion of variance (Table 3.3). Secondly, we conducted principle components analysis (PCA) using MINITAB version 13 (Spruell et al. 2003). We computed the principle component (PC) scores based on the covariance among allele frequencies, omitting the largest allele at each locus to account for the non-independence of alleles within each locus. We then plotted PC2 vs. PC1 and PC3 vs. PC1 to estimate genetic divergence as the relative linear distance between points representing each population.

We also used two approaches to investigate the effect of landscape features on population divergence within regions. First, we examined pairwise F_{st} 's to qualitatively assess the effects of mountain ridges and elevational differences on genetic divergence. Second, we used Mantel tests (Mantel 1967) and partial Mantel tests (Smouse et al. 1986) to examine the effect of straight-line distance, river distance, elevational differences, and mountain ridges on F_{st} using FSTAT version 1.2. The natural logarithm of straight-line distances and river distances were used to linearize the relationship between distance and F_{st} . A pair of sites was considered to be separated by a mountain ridge if a straight line

between the two sites intersected one or more ridges. Partial Mantel tests measure the effect of a variable on F_{st} after controlling for another variable, analogous to partial correlation coefficients. We used partial Mantel tests to test two alternative hypotheses concerning movement patterns in Columbia spotted frogs. First, to test the hypothesis that frogs primarily move along riparian corridors, but that elevational differences along rivers impede movement, we estimated the partial correlation between F_{st} and elevation after controlling for river distance. Second, to test the hypothesis that frogs primarily move overland, but that ridges impede overland movement, we estimated the partial correlation between F_{st} and mountain ridges after controlling for straight-line distance. The α value for each test was determined using a sequential Bonferroni adjustment (Rice 1989).

Finally, we used two methods to investigate how many ponds make up a 'randomly mating population,' equivalent to a deme or subpopulation in the population genetics literature (Hartl and Clark 1989). First, we examined exact probabilities of population differentiation to identify sites that had indistinguishable allele frequencies. Secondly, we used a Bayesian clustering approach implemented in STRUCTURE version 2.1 (Pritchard et al. 2000) to estimate the number of populations (K) in a sample and to assign individuals to one or more of these populations (k). This approach assumes Hardy-Weinberg equilibrium within populations and linkage equilibrium between loci within populations. We used the admixture model which assumes gene flow among populations. The admixture model assigns a proportion of each individual's genome to each population (q_k). We assigned sites to populations by calculating the mean q_k for each site (\bar{q}_k) and assigning sites to the population with the largest \bar{q}_k . For each basin or

set of adjacent basins, we calculated the probability that there are from $K = 1$ to the total number of sites sampled in the basin or set of adjacent basins. We ran five independent simulations for each K , used a burn-in length of 50,000 and a run length of 10^6 , and assumed correlated allele frequencies.

RESULTS

Variation Within Populations

Genotypic frequencies generally conformed to the expected Hardy-Weinberg proportions. Fifteen of 151 tests for deviation from Hardy-Weinberg proportions were statistically significant ($P < 0.05$) which is greater than the 7 tests expected to deviate by chance. However, after correcting for multiple tests (Rice 1989), only site 17 deviated significantly from expected Hardy-Weinberg proportions at *Rp3* with $F_{is} = -0.579$ indicating heterozygote excess. Heterozygote excess at site 17 is likely due to sampling tadpoles at this site which, as mentioned above, may only represent the reproductive contribution of a few adults. No loci had an excess of homozygotes as would be expected if there were null alleles.

Tests for linkage disequilibrium did not reveal any strong associations between loci. Twenty-eight out of 352 tests were significant ($P < 0.05$), eleven more than the 17 significant tests expected by chance. Fourteen of the significant tests were between *Rp3* and *SFC139*, consistent with weak linkage between these loci. After correcting for multiple comparisons, four associations were significant: *Rp3* and *Rp17*, *Rp3* and *SFC139*, and *Rp17* and *SFC139* in site 7; and *Rp3* and *SFC139* in site 17. This suggests some degree of population subdivision within site 7.

Overall levels of genetic variation within Columbia spotted frog populations varied substantially among populations and loci (Tables 3.1 – 3.2; Appendix 5). The total number of alleles per site ranged from 11 alleles in site 28 to 36 alleles in sites 7 and 8. Average expected heterozygosity ranged from 0.23 in site 28 to 0.70 in site 11. The number of alleles per locus also varied substantially among loci, ranging from 5 alleles at *SFC134* to 16 at *SFC139*.

Average expected heterozygosity and average allelic richness were strongly negatively correlated with elevation (Table 3.1; Fig. 3.2). The correlation coefficient between expected heterozygosity and elevation was $r = -0.88$ ($P < 0.001$). The correlation coefficient between allelic richness and elevation was $r = -0.85$ ($P < 0.001$). Expected heterozygosity and allelic richness were low at site 17 given the site's elevation (999 m), causing this site to act as an outlier. This site was unique in that only tadpoles were sampled which may only represent the reproductive contribution of a few adults.

Divergence Among Populations

Microsatellite analysis of the entire data set suggests that Columbia spotted frog sites do not form distinct regional groups. Instead, most sites group with other sites in the same basin or adjacent basins. This is reflected in the analysis of molecular genetic variance with basins explaining the most among group variance (17.6%; Table 3.3). The second best grouping is the Snake River (all sites in Bighorn Crags, Idaho) vs. the Clark Fork River and Kootenai River (all Montana sites) (14.7%).

Principle components analysis also suggests that sites tend to group with other sites in the same basin or adjacent basins, with a few interesting exceptions (Figs. 3.1, 3.3). In the plot of PC2 vs. PC1, one notable exception is a low elevation site (17) from the Bitterroot Mts. region which groups more closely with other low elevation sites (7 – 12) in the Cabinet & Coeur d' Alene Mts. region approximately 200 km away than it does with high elevation sites (13 – 16 and 18) only 13 – 15 km away. This grouping is consistent with pairwise F_{st} 's of 0.130 – 0.156 between site 17 and 7 – 12 compared to pairwise F_{st} 's of 0.228 – 0.316 between sites 17 and 13 – 16 and 18. In the same plot, there are also a few examples of isolated high elevation sites (1, 6, and 19) which group with sites from different regions. In the plot of PC3 vs. PC1, another interesting exception to grouping by basin is the grouping of low elevation sites (2 – 5) in Keeler Creek with low elevation sites (7 – 12) in Marten Creek approximately 50 km away rather than with a high elevation site (1) in the same basin only 2 – 5 km away. This grouping is also consistent with pairwise F_{st} 's of 0.071 – 0.149 between sites 2 – 5 and 7 – 12 which are similar to pairwise F_{st} 's of 0.088 – 0.127 between sites 2 – 5 and 1.

Microsatellite analysis within regions reveals that mountain ridges and elevational differences are associated with increased genetic divergence among sites. The isolating effect of mountain ridges can be seen by comparing pairwise F_{st} 's between sites in adjacent basins with pairwise F_{st} 's between sites within basins (Tables 3.4 – 3.6; Fig. 3.1). For example, in the Cabinet Mts., pairwise F_{st} 's between sites 2 – 5 in Keeler Creek and site 6 in Stanley Creek are much higher than pairwise F_{st} 's between sites 2 – 5 within Keeler Creek (Table 3.4). High pairwise F_{st} 's are also seen between sites 19 in Rock Creek and sites 21 – 23 in Little Rock Creek in the Bitterroot Mts. (Table 3.5) and

between sites 24 – 25 in Skyhigh basin and sites 27 – 28 in Tiptop basin in the Bighorn Craggs (Table 3.6). Ridges do not always isolate populations, however, as can be seen from the low pairwise F_{st} 's between sites 13 in South One Horse Creek and sites 14 – 16 in North Fork Sweeney Creek in the Bitterroot Mts. (Table 3.5).

The isolating effects of elevation can also be seen by examining pairwise F_{st} 's between high and low elevation sites within basins (Tables 3.4 – 3.5; Fig. 3.1). For example, pairwise F_{st} 's between a high elevation site (1) and low elevation sites (2 – 5) in Keeler Creek are higher than pairwise F_{st} 's between the low elevation sites (Table 3.4). Similarly, the pairwise F_{st} between a high elevation site (19) and low elevation site (20) in Rock Creek is high (0.176) despite being separated by only 17 km.

Mantel tests and partial Mantel tests also reveal that the straight-line distances, river distances, mountain ridges, and elevational differences tend to be positively correlated with genetic divergence, although these correlations vary by region. In the Cabinet and Coeur d' Alene Mts., F_{st} is significantly correlated with all four landscape variables and all correlations have large coefficients of determination (Table 3.7; Fig. 3.4). Moreover, the partial correlation of F_{st} and elevation is significant after controlling for the effect of ln river distance and the partial correlation of F_{st} and ridges is significant after controlling for the effect of ln straight-line distance (Table 3.7; Fig. 3.5). The overall coefficient of determination for the model including ln river distance and elevation is 0.87 compared to 0.69 for the model including ln straight-line distance and ridges, lending more support for movement along riparian corridors impeded by elevation than for movement overland impeded by ridges. In the Bitterroot Mts., F_{st} is only

correlated with ln straight-line distance, ln river distance, and ridges (Table 3.7; Figs. 3.4 – 3.5). In the Bighorn Crags, no correlations are significant.

Exact tests of population differentiation and the clustering method implemented in STRUCTURE both show that populations often encompass more than a single pond. However, exact tests (Tables 3.4 – 3.6) tended to split populations more finely than did the clustering method (Tables 3.8 – 3.9; Fig. 3.6). For example, exact tests show significant differences in allele frequencies between site 2 and the other three low elevations sites (3 – 5) in Keeler Creek, splitting the low elevation sites in Keeler into an upper and lower population (Table 3.4). In contrast, the clustering method identifies a total of three populations in Keeler and Stanley Creeks (Table 3.8) and assigns the majority of individuals' genomes from sites 2 – 5 to a single population (Table 3.9; Fig. 3.6). In another example, exact tests reveal significant differences in allele frequencies among all five sites (24 – 28) in the Bighorn Crags, suggesting each site is its own population (Table 3.6). However, the clustering method identifies a total of two populations for these five sites (Table 3.8) and places sites 24 – 26 in one population and sites 27 – 28 in another (Table 3.9; Fig. 3.6).

DISCUSSION

Do Ridges Act as Barriers to Gene Flow?

Our microsatellite data show that in most cases, mountain ridges act as barriers to gene flow in Columbia spotted frogs. This suggests that dispersal rates over ridges are low despite the potential for long distance movements in the species (Turner 1960; Reaser 1996; Pilliod et al. 2002; Chapter 2). Previous work has shown a similar isolating

effect of mountain ridges on gene flow in a different frog species (Lougheed et al. 1999), suggesting that ridges may generally act as barriers to gene flow in amphibians. These results also imply that amphibian populations in mountainous regions should show high levels of population differentiation. This prediction holds for several species of amphibians in mountains (García-Paris et al. 2000; Shaffer et al. 2000; Tallmon et al. 2000; Monsen and Blouin 2003). The observation that ridges impede gene flow also suggests that ridges may facilitate allopatric speciation among amphibian populations (Lougheed et al. 1999).

There was one notable exception to the observation that ridges impede gene flow among Columbia spotted frog populations. In the Bitterroot Mts., pairwise F_{st} 's were low between site (13) in One Horse Creek and sites (14 – 16) on the other side of a ridge in North Fork Sweeney Creek (Table 3.5; Fig. 3.1). The clustering method also identified all of these sites as a single population (Tables 3.8 – 3.9; Fig. 3.6). We suspect that this exception is due to an exceptionally large breeding population of frogs in North Fork Sweeney Creek (Maxell, unpubl. data) which would be expected to result in high levels of gene flow ($N_e m$) even if dispersal rates (m) over the ridge are low. Because population differentiation is inversely proportional to the absolute amount of gene flow, not dispersal rates, high gene flow will lead to low pairwise F_{st} 's (Wright 1969).

Is Gene Flow Restricted between Low and High Elevation Ponds?

Our microsatellite data also demonstrate that gene flow tends to be restricted between low and high elevation ponds in Columbia spotted frogs. Two alternative explanations for restricted gene flow between low and high elevations are that dispersal is

restricted between low and high elevations or that there are pre-mating or post-mating barriers to gene flow between low and high elevations. Capture-recapture analysis in Columbia spotted frogs shows that dispersal rates between low and high elevation populations can be exceptionally high (Chapter 2), suggesting that dispersal is not restricted between low and high elevations. This implies that there may be pre-mating or post-mating barriers to gene flow that have restricted gene flow between low and high elevations. Alternatively, the discrepancy between high dispersal and restricted gene flow between low and high elevations may be due to unusually high dispersal during the capture-recapture study.

No significant relationship was observed between F_{st} and elevational differences in the Bitterroot Mts. using Mantel tests which seems to contradict high pairwise F_{st} 's between low and high elevation sites in this region. The reason for this apparent contradiction is that in the Bitterroot Mts., we primarily sampled high elevation sites. Because many high elevation sites were separated by one or more mountain ridges, pairwise F_{st} 's among high elevation sites tended to be high despite the fact that these sites were at similar elevations. This resulted in many data points in the upper left-hand quadrant (little elevational differences but high pairwise F_{st} 's) of the regression between F_{st} and elevational differences, resulting in a non-significant regression. Nevertheless, high pairwise F_{st} 's between low and high elevation sites in the Bitterroots suggest that gene flow is restricted across elevation in this region as also seen in the Cabinet and Coeur d' Alene Mts.

Does a Pond Equal a Randomly Mating Population?

Finally, our microsatellite data also show that Columbia spotted frog populations usually encompass more than a single breeding pond. In most cases, populations are made up of multiple ponds within a basin (Fig. 3.6). Some basins only contain a single population, whereas other basins contain two. In the cases where ponds or lakes are equivalent to populations, usually the ponds or lakes are very isolated from other ponds by distance, mountain ridges, or elevation (sites 1, 6, 18, and 19). Low elevation sites 17 and 20 in the Bitterroot Mts. region are identified as discrete populations, but this is likely due to the fact that we did not sample adjacent, low elevation sites.

A notable exception to the generalization that most populations are contained within basins is sites 13 – 16 which represent a single population despite being located in two different basins. As explained previously, we suspect this is due to a very large breeding aggregation of frogs in North Fork Sweeney Creek (sites 14 – 16) causing high gene flow from North Fork Sweeney Creek into One Horse Creek (site 13). Nonetheless, the observation that most basins contain one or two populations of Columbia spotted frogs and that most populations are bounded by a single basin suggests that basins in the size range studied here (a few to several kilometers long) may be an appropriate geographic unit for management for this species.

Exact tests often split populations more finely than did the clustering method in STRUCTURE. This is expected because allele frequency differences (tested with exact tests) will likely become manifest sooner than Hardy-Weinberg or linkage disequilibrium (tested by the clustering method) after population subdivision. The question then arises, which method is better for identifying ‘randomly mating populations’? We argue that

neither is better, but that they measure population subdivision in different ways. We therefore suggest that they should be used together to delineate populations. Specifically, we recommend using the clustering method as a lower bound to the number of populations and exact tests as an upper bound.

Negative Relationship between Genetic Variation within Populations and Elevation

A striking result of this study was the strong, negative relationship between genetic variation within populations and elevation (Fig. 3.2). Correlation coefficients between expected heterozygosity and elevation ($r = -0.88$) and between allelic richness and elevation ($r = -0.85$) were both very large and highly significant ($P < 0.001$). This observation suggests that effective population sizes (N_e) are much smaller at high elevations than low elevations in Columbia spotted frogs. Effective population sizes may be smaller at high elevations either because local N_e 's are smaller or because gene flow is restricted at high elevations. Our data suggest that gene flow is restricted by mountain ridges at high elevations, supporting the latter hypothesis. Moreover, some high elevation ponds such as ponds 14 – 16 (Fig. 3.1) support very large breeding aggregations, suggesting that local N_e 's can be large at high elevations (Maxell, unpubl. data).

Valley – Mountain Model of Population Structure

Columbia spotted frogs have a fairly consistent population structure across all three regions analyzed in this study which we refer to as a 'valley – mountain' population structure. This population structure has three distinct characteristics. First, low elevation

populations have large historic effective population sizes and high levels of among population gene flow. Second, high elevation populations have small historic effective population sizes and lower levels of among population gene flow, as has been shown previously in long-toed salamanders (*Ambystoma macrodactylum*) in the Bitterroot Mountains (Funk et al. 1999; Tallmon et al. 2000). Third, gene flow is restricted, but not absent, between low and high elevation populations.

The valley – mountain model of population structure has at least two important evolutionary implications for Columbia spotted frogs. First, restricted gene flow across elevation should facilitate local adaptation to these very different habitats. Restricted gene flow may also indicate that reproductive isolation has already evolved in association with local adaptation. Second, high elevation populations may have largely independent evolutionary trajectories compared to low elevation populations which are much more connected by gene flow.

The valley – mountain model of population structure also has several important implications for conservation of Columbia spotted frogs. First, small effective population sizes and isolation may make high elevation populations particularly susceptible to extinction (Newman and Pilson 1997; Sacchieri et al. 1998). Second, because low elevation populations have been historically connected by dispersal and gene flow, habitat fragmentation of low elevation populations may increase local extinction rates. Next, connectivity between low and high elevation populations by dispersal and gene flow may be important for the persistence (Brown and Kodric-Brown 1977; Newman and Tallmon 2001) and recolonization (Levins 1969; Funk and Dunlap 1999) of high elevation populations. Moreover, if low elevation populations are important sources of

immigrants and genetic variation for high elevation populations, then fragmentation of low elevation populations may have the unexpected consequence of reducing the persistence of mountain populations. In other words, if the 'mainland' is destroyed, eventually there may be nothing left but 'islands.' This is not an unrealistic possibility given that low elevation valleys are often the first to be developed.

A review of previous population genetics studies of amphibians suggests that the valley – mountain model of population structure may explain a substantial portion of the variance among studies in the levels of gene flow reported. Some studies report very high levels of genetic differentiation and low levels of gene flow (Larson et al. 1984; Driscoll 1998; García-Parris et al. 2000; Shaffer et al. 2000; Tallmon et al. 2000; Monsen and Blouin 2003), whereas others report very low levels of genetic differentiation and high gene flow (Berry 2001; Newman and Squire 2001; Squire and Newman 2002; Lampert et al. 2003). A closer examination reveals that most of the studies that report high levels of genetic differentiation are for species or populations from mountainous regions (García-Parris et al. 2000; Shaffer et al. 2000; Tallmon et al. 2000; Monsen and Blouin 2003) and most of the studies that report low levels of divergence are from low and relatively flat regions (Berry 2001; Newman and Squire 2001; Squire and Newman 2002; Lampert et al. 2003). Some of the variation among studies in levels of gene flow is also likely due to differences in species-specific dispersal rates, the loci analyzed, and the geographical scales analyzed. However, the general correspondence between genetic differentiation and landscape topography suggests that other amphibians may be influenced by the landscape in similar ways as the Columbia spotted frog.

TABLE 3.1. Columbia spotted frog sample site information. Map datum NAD27 was used for UTM coordinates. N is the sample size, H_E is expected heterozygosity at the six microsatellite loci examined, and alleles is the total number of alleles observed. Site numbers correspond to the site numbers in Fig. 3.1.

Region	Basin	Site	UTM	Elev. (m)	N	H_E	Alleles	
Cabinet & Coeur d'Alene Mts., MT	Keeler	1	11 575650E 5352125N	1581	28	0.50	24	
		2	11 576062E 5354011N	884	29	0.59	28	
		3	11 579939E 5353638N	785	19	0.63	31	
	Stanley Marten	4	4	11 580150E 5353173N	824	29	0.55	30
			5	11 579822E 5352880N	812	19	0.62	29
		6	11 580370E 5342957N	1485	27	0.40	21	
		7	7	11 587089E 5304715N	833	25	0.65	36
			8	11 587462E 5304851N	819	55	0.66	36
			9	11 589970E 5304808N	769	29	0.62	31
			10	11 590173E 5304507N	839	25	0.65	32
			11	11 592072E 5303550N	733	24	0.70	34
			12	11 593092E 5303102N	769	30	0.64	34
13	11 711404E 5171317N		2251	30	0.44	17		
Bitterroot Mts., MT	One Horse	14	11 710259E 5168988N	2244	30	0.47	22	
		15	11 710202E 5168893N	2241	30	0.46	23	
		16	11 711573E 5168050N	1982	30	0.43	18	
	Valley	17	11 723231E 5162021N	999	30	0.42	18	
		S. Sweeney	18	11 709043E 5165790N	2238	30	0.40	16
	Rock	19	11 700017E 5099542N	2133	30	0.39	15	

		20	11 715702E 5106121N	1250	25	0.50	22
	L. Rock	21	11 702849E 5098795N	2256	21	0.32	17
		22	11 703869E 5098184N	2139	30	0.29	16
		23	11 705725E 5100021N	1995	24	0.34	14
Bighorn	Skyhigh	24	11 688873E 4998211N	2484	28	0.35	19
Crags, ID		25	11 688911E 4996632N	2463	22	0.38	20
	Bob	26	11 690127E 4999150N	2652	30	0.39	19
	Tiptop	27	11 687947E 4995677N	2548	31	0.26	15
		28	11 687637E 4994117N	2560	30	0.23	11

TABLE 3.2. Primer sequences, PCR annealing temperatures, product lengths, and number of alleles (out of 790 Columbia spotted frogs analyzed) for six microsatellite loci from Oregon spotted frogs (*Rana pretiosa*) (*Rp* loci) and *R. luteiventris* (*SFC* loci). Allele size differences between some *Rp15* alleles are not divisible by the repeat length of four because this locus has non-microsatellite insertion-deletions (see Appendix 5).

Primer	Repeat motif	Primer sequence (5' to 3')	Annealing temp (°C)	Product length (bp)	No. alleles
<i>Rp3</i>	GATA	F: 5'-GAAAGCAAACTGGGAAAGTACATA-3'	45	187-223	10
		R: 5'-CCTGAGAGCCATCCAAATAAGTGCCA-3'			
<i>Rp15</i>	GATA	F: 5'-CTTGATACAGTGTGCAAGAGGC-3'	50	188-209	8
		R: 5'-ATACTCGTGATAGGGAGTTT-3'			
<i>Rp17</i>	GATA	F: 5'-GTGTAGACAACAATAAAGTCAG-3'	50	114-210	15
		R: 5'-TCTCTACTTCCATCCAACCATTC-3'			
<i>Rp23</i>	GATA	F: 5'-ACATAGATACAATAGATAGATAGAC-3'	52	183-203	6
		R: 5'-CACAGGAATGTAAAATCTGGCITTC-3'			
<i>SFC134</i>	TACA	F: 5'-TGGGAAAAGACTCTGTGGT-3'	57	213-229	5
		R: 5'-AGGAAATGTGTGGAAGCAT-3'			
<i>SFC139</i>	TACA	F: 5'-GGCATGGTTAAAGTGGAACTC-3'	58	245-305	16

R: 5'-JGCAJGTCTGTAAJGGACCTC-3'

TABLE 3.3. Results from analysis of molecular variance (AMOVA) with sampling sites grouped in different ways. All sites above 1400 m were considered high elevation sites and all sites below 1400 m were considered low elevation sites. Sites in Keeler and Stanley basins are part of the Kootenai River system; sites in Marten basin and the Bitterroot Mountains are part of the Clark Fork River system; and sites in the Bighorn Crags are part of the Snake River system.

Groups	Number of groups	Variance components	Percentage of variation	P-value
(1) Basins	12	Among groups	17.6	< 0.001
		Among sites	3.8	< 0.001
		Within sites	78.6	< 0.001
(2) High vs. low elevation	2	Among groups	4.1	0.005
		Among sites	17.6	< 0.001
		Within sites	78.3	< 0.001
(3) Regions (Cabinet & Coeur d'Alene Mts. vs. Bitterroot Mts. vs. Bighorn Crags)	3	Among groups	10.4	< 0.001
		Among sites	12.5	< 0.001
		Within sites	77.0	< 0.001
(4) Snake R. vs. Clark Fork R. & Kootenai R.	2	Among groups	14.7	< 0.001
		Among sites	13.6	< 0.001
		Within sites	71.7	< 0.001

(5) Snake R. vs. Clark Fork R. vs. Kootenai R.	3	Among groups	12.5	< 0.001
		Among sites	12.0	< 0.001
		Within sites	75.5	< 0.001

TABLE 3.4. Pairwise F_{st} 's (below the diagonal) and probability that allelic distributions are identical between sampling sites when all loci are combined (above the diagonal) for sites in the Cabinet and Coeur d'Alene Mountains, Montana. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, and NS = not significant.

Site	1	2	3	4	5	6	7	8	9	10	11	12
1	-	***	***	***	***	***	***	***	***	***	***	***
2	0.088	-	***	***	***	***	***	***	***	***	***	***
3	0.096	0.050	-	*	NS	***	***	***	***	***	***	***
4	0.106	0.056	0.016	-	NS	***	***	***	***	***	***	***
5	0.127	0.041	0.008	-0.002	-	***	***	***	***	***	***	***
6	0.182	0.195	0.175	0.183	0.198	-	***	***	***	***	***	***
7	0.209	0.116	0.112	0.143	0.114	0.203	-	NS	**	NS	***	**
8	0.191	0.105	0.096	0.118	0.089	0.168	0.002	-	***	***	***	***
9	0.233	0.134	0.130	0.147	0.116	0.196	0.025	0.027	-	NS	NS	NS
10	0.225	0.123	0.125	0.149	0.109	0.231	0.004	0.021	0.012	-	*	NS
11	0.174	0.092	0.080	0.101	0.071	0.185	0.024	0.024	0.009	0.020	-	*
12	0.238	0.138	0.126	0.155	0.118	0.227	0.018	0.023	0.004	-0.003	0.021	-

TABLE 3.5. Pairwise F_{st} 's (below the diagonal) and probability that allelic distributions are identical between sampling sites when all loci are combined (above the diagonal) for sites in the Bitterroot Mountains, Montana. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, and NS = not significant.

Site	Site													
	One Horse	N. Sweeney			Valley	S. Sweeney			Rock			L. Rock		
	13	14	15	16	17	18	19	20	21	22	23			
13	-	*	*	NS	***	***	***	***	***	***	***	***	***	***
14	0.013	-	NS	NS	***	***	***	***	***	***	***	***	***	***
15	0.017	0.022	-	NS	***	***	***	***	***	***	***	***	***	***
16	0.001	0.006	0.009	-	***	***	***	***	***	***	***	***	***	***
17	0.238	0.228	0.248	0.245	-	***	***	***	***	***	***	***	***	***
18	0.109	0.122	0.059	0.094	0.316	-	***	***	***	***	***	***	***	***
19	0.146	0.170	0.125	0.122	0.274	0.204	-	***	***	***	***	***	***	***
20	0.238	0.236	0.213	0.241	0.209	0.247	0.176	-	***	***	***	***	***	***
21	0.294	0.315	0.256	0.300	0.308	0.283	0.197	0.073	-	NS	***	***	***	***
22	0.325	0.342	0.277	0.326	0.329	0.306	0.212	0.110	-0.009	-	***	***	***	***
23	0.276	0.311	0.220	0.293	0.345	0.228	0.233	0.139	0.131	0.154	-	-	-	-

TABLE 3.6. Pairwise F_{st} 's (below the diagonal) and probability that allelic distributions are identical between sampling sites when all loci are combined (above the diagonal) for sites in the Bighorn Crags, Idaho. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, and NS = not significant.

Site	Site				
	Skyhigh		Bob	Tiptop	
	24	25	26	27	28
24	–	***	***	***	***
25	0.069	–	***	***	***
26	0.105	0.037	–	***	***
27	0.236	0.153	0.179	–	***
28	0.242	0.185	0.126	0.156	–

TABLE 3.7. Results of simple and partial Mantel tests to investigate the relationship between F_{st} 's, straight line distance, river distance, elevation, and mountain ridges. Four simple Mantel tests and two partial Mantel tests were performed for each region. The two partial Mantel tests are $(F_{st} \times \text{elev}).\ln(\text{riv dist})$ which tests the partial correlation between F_{st} and elevation after controlling for $\ln(\text{river distance})$ and $(F_{st} \times \text{rid}).\ln(\text{SL dist})$ which tests the partial correlation between F_{st} and ridges after controlling for $\ln(\text{straight line distance})$. The α value for each test was determined by a sequential Bonferroni adjustment. * indicates a significant test and NS indicates a non-significant test. r is the standardized Mantel test statistic which is equivalent to a Pearson product-moment correlation coefficient and r^2 is the coefficient of determination.

Region	Mantel test	<i>P</i> -value	Bonferroni α value	Significance	<i>r</i>	<i>r</i> ²
Cabinet & Coeur	$F_{st} \times \ln(\text{straight line dist})$	0.0005	0.0085	*	0.719	0.517
d'Alene Mts.	$F_{st} \times \ln(\text{river dist})$	0.0005	0.0102	*	0.770	0.593
	$F_{st} \times \text{elevation}$	0.0005	0.0127	*	0.691	0.478
	$F_{st} \times \text{ridge}$	0.0005	0.0170	*	0.832	0.692
	$(F_{st} \times \text{elev}).\ln(\text{riv dist})$	0.0005	0.0253	*	0.528	0.279
	$(F_{st} \times \text{rid}).\ln(\text{SL dist})$	0.001	0.0500	*	0.418	0.175
Bitterroot Mts.	$F_{st} \times \ln(\text{straight line dist})$	0.0005	0.0085	*	0.761	0.580
	$F_{st} \times \ln(\text{river dist})$	0.0005	0.0102	*	0.618	0.382
	$F_{st} \times \text{ridge}$	0.0015	0.0127	*	0.459	0.210
	$F_{st} \times \text{elevation}$	0.047	0.0170	NS	0.267	0.071
	$(F_{st} \times \text{elev}).\ln(\text{riv dist})$	0.073	–	NS	0.238	0.057
	$(F_{st} \times \text{rid}).\ln(\text{SL dist})$	0.784	–	NS	0.039	0.002
Bighorn Crags	$F_{st} \times \ln(\text{river dist})$	0.078	0.0085	NS	0.575	0.331
	$F_{st} \times \text{elevation}$	0.293	–	NS	-0.377	0.142
	$F_{st} \times \ln(\text{straight line dist})$	0.361	–	NS	0.321	0.103
	$F_{st} \times \text{ridge}$	0.435	–	NS	0.285	0.082
	$(F_{st} \times \text{elev}).\ln(\text{riv dist})$	0.620	–	NS	-0.182	0.033

$(F_{st} \times \text{rid}).\ln(\text{SL dist})$	0.690	-	NS	0.144	0.021
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TABLE 3.8. Inference of the number of populations of Columbia spotted frogs in different basins or sets of adjacent basins using the model-based clustering method of Pritchard et al. 2000. K is the number of populations, $\ln P(X|K)$ is the \ln probability of the data given K , and $P(K|X)$ is the estimated posterior probability of K given the data. Five independent runs for each K were used to estimate mean $\ln P(X|K)$. The highest $P(K|X)$ for each basin or set of adjacent basins is shown in bold.

Basins	K	Mean $\ln P(X K)$	$P(K X)$
Keeler & Stanley	1	-2010.1	~0.0
	2	-1978.9	~0.0
	3	-1922.1	~1.0
	4	-1938.5	~0.0
	5	-2008.5	~0.0
	6	-2100.0	~0.0
Marten	1	-2662.2	~1.0
	2	-2959.9	~0.0
	3	-2880.1	~0.0
	4	-3490.5	~0.0
	5	-3180.8	~0.0
	6	-3900.6	~0.0
One Horse, N. Sweeney, Bitterroot, & S. Sweeney	1	-1839.8	~0.0
	2	-1578.4	~0.0
	3	-1548.0	~1.0
	4	-1589.2	~0.0
	5	-1654.3	~0.0

	6	-1714.2	~0.0
Rock & L. Rock	1	-1097.4	~0.0
	2	-1053.4	~0.0
	3	-1007.3	0.20
	4	-1005.9	0.80
	5	-1121.5	~0.0
Skyhigh, Bob, & Tiptop	1	-1057.1	~0.0
	2	-978.6	~ 1.0
	3	-1038.8	~0.0
	4	-1103.7	~0.0
	5	-1061.2	~0.0

TABLE 3.9. Mean proportion of genome from each site estimated to have originated from population k (\overline{q}_k) in given set of adjacent basins using the admixture model of Pritchard et al. 2000. Dashes indicate that the k^{th} population was not inferred for the given set of adjacent basins. Data is not shown for Marten because only one cluster was inferred for Marten (and therefore all genomes originated from $k = 1$). The population to which each site was assigned is indicated in bold.

Basins	Site	k			
		1	2	3	4
Keeler & Stanley	1	0.71	0.10	0.19	–
	2	0.40	0.43	0.17	–
	3	0.21	0.59	0.20	–
	4	0.23	0.57	0.20	–
	5	0.15	0.67	0.18	–
	6	0.14	0.05	0.81	–
One Horse, N. Sweeney, Bitterroot, & S. Sweeney	13	0.64	0.06	0.31	–
	14	0.61	0.03	0.35	–
	15	0.65	0.04	0.31	–
	16	0.59	0.04	0.37	–
	17	0.02	0.97	0.01	–
	18	0.10	0.02	0.88	–
Rock & L. Rock	19	0.82	0.08	0.05	0.05
	20	0.06	0.59	0.17	0.18
	21	0.12	0.24	0.41	0.24
	22	0.13	0.20	0.43	0.24

	23	0.07	0.12	0.25	0.56
Skyhigh, Bob, & Tiptop	24	0.81	0.19	–	–
	25	0.71	0.29	–	–
	26	0.72	0.28	–	–
	27	0.18	0.82	–	–
	28	0.13	0.87	–	–

FIGURE LEGENDS

FIG. 3.1. Location of Columbia spotted frog breeding ponds and lakes in Montana and Idaho, USA, sampled for microsatellite analyses. Site numbers correspond to the site numbers in Table 3.1.

FIG. 3.2. Relationship between (a) mean expected heterozygosity (H_E) and elevation and (b) mean allelic richness and elevation for all 28 sites analyzed.

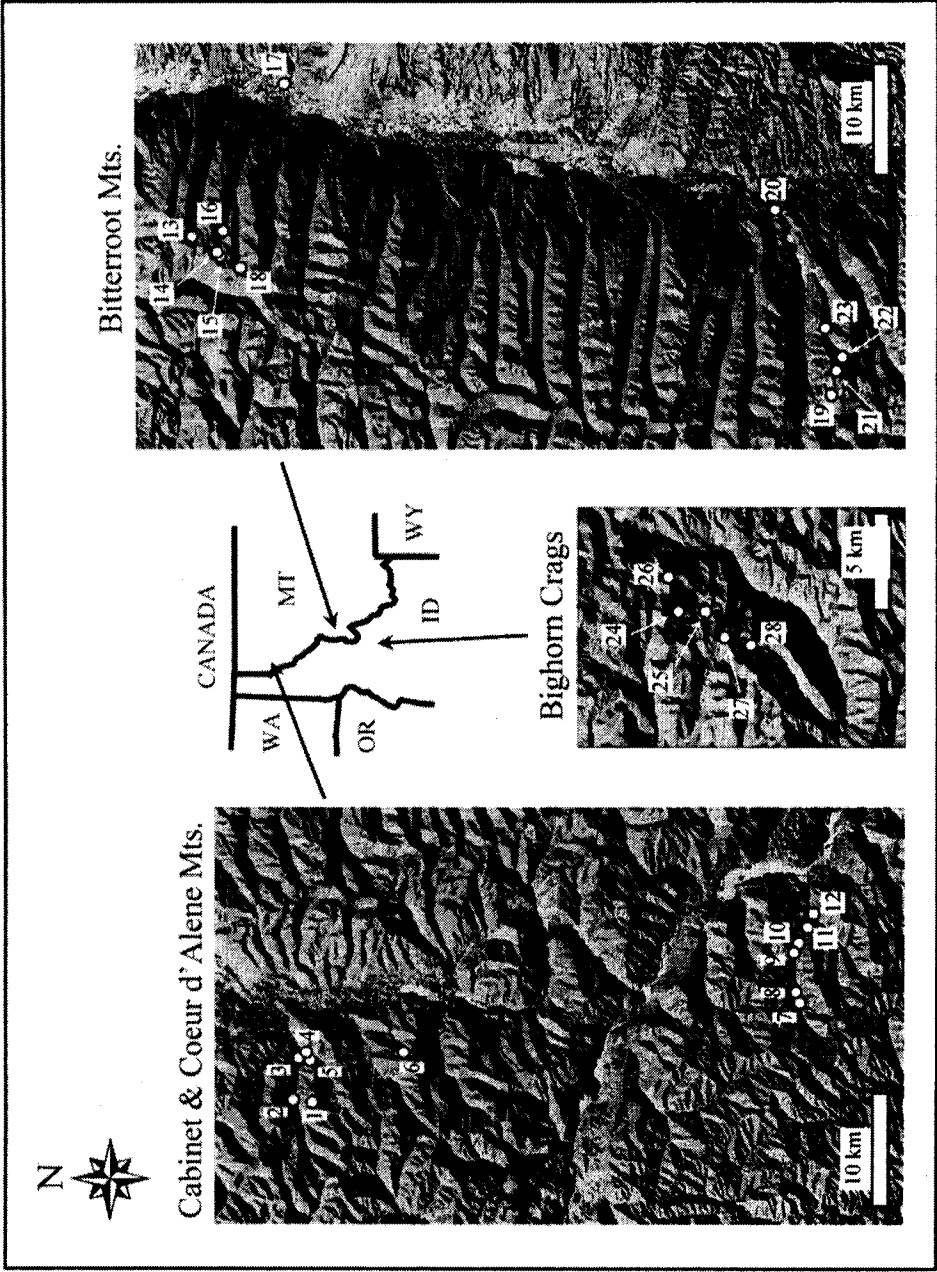
FIG. 3.3. Plots of (a) first two principle component scores derived from allele frequencies for all population samples and (b) first and third principle component scores. Numbers refer to sites (Fig. 3.1) and different symbols represent different basins.

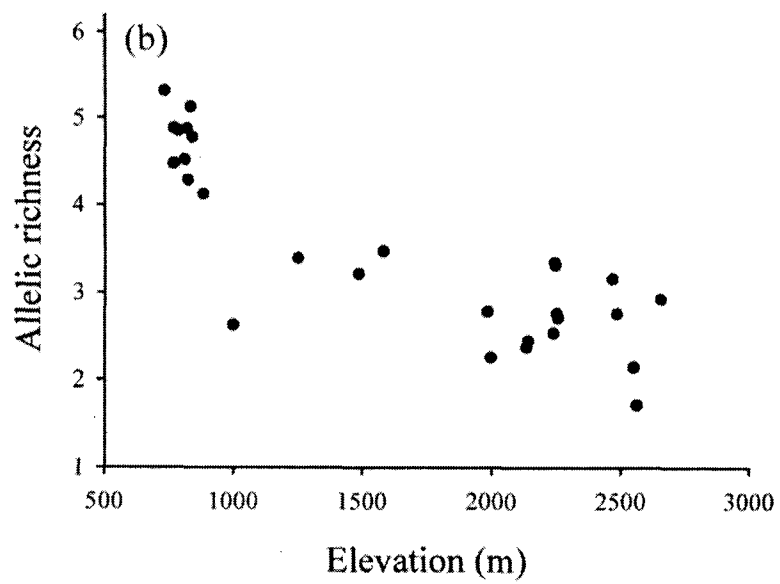
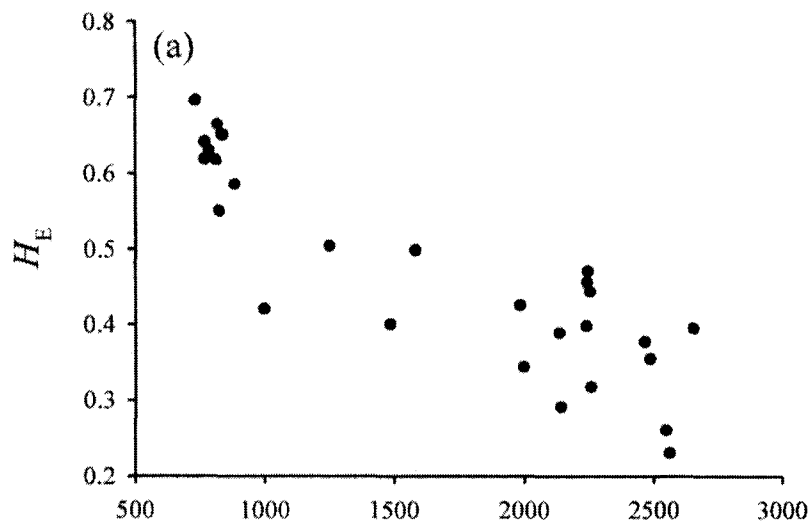
FIG. 3.4. Plots of pairwise F_{st} 's vs. straight-line distance for (a) the Cabinet and Coeur d' Alene Mts., (c) the Bitterroot Mts., and (e) the Bighorn Crags and plots of pairwise F_{st} 's vs. river distances for (b) the Cabinet and Coeur d' Alene Mts., (d) the Bitterroot Mts., and (f) the Bighorn Crags.

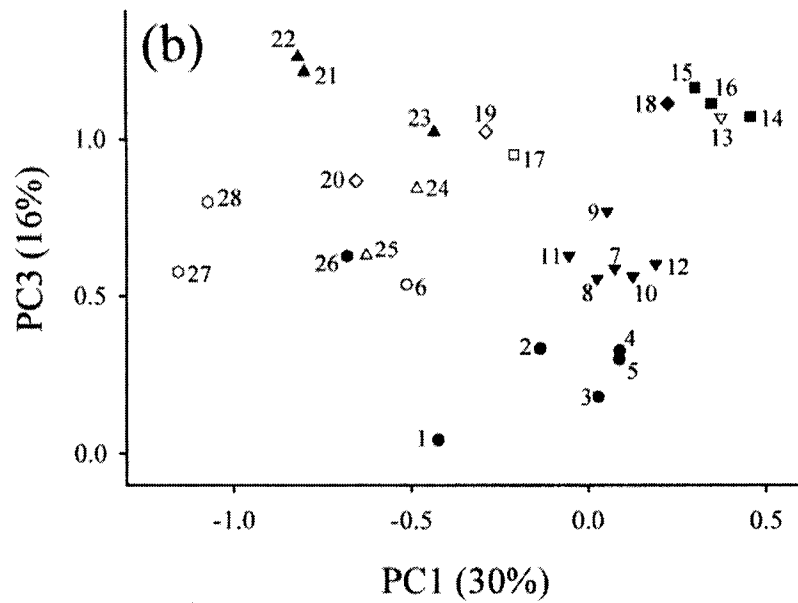
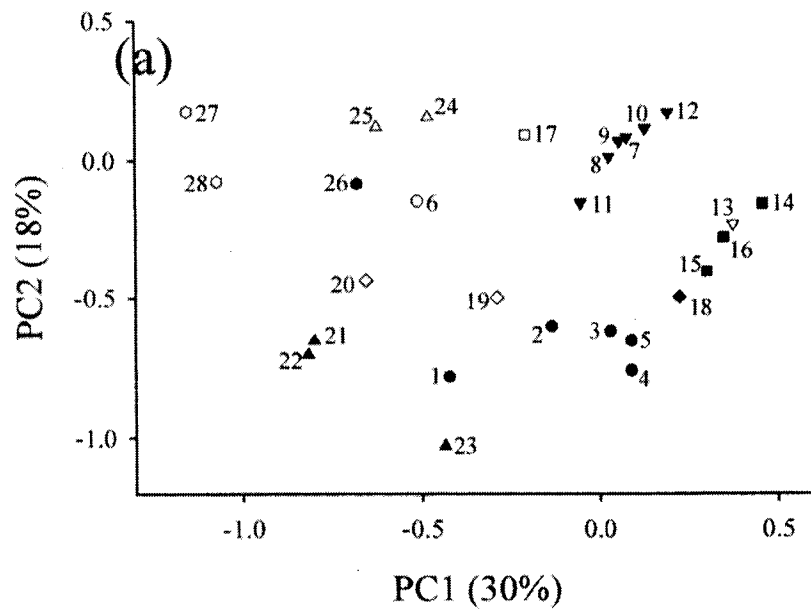
FIG. 3.5. Plots of pairwise F_{st} 's vs. the residuals of elevational difference vs. In river distance for (a) the Cabinet and Coeur d' Alene Mts., (b) the Bitterroot Mts., and (c) the Bighorn Crags.

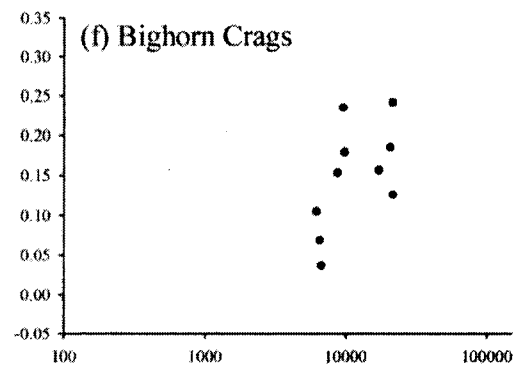
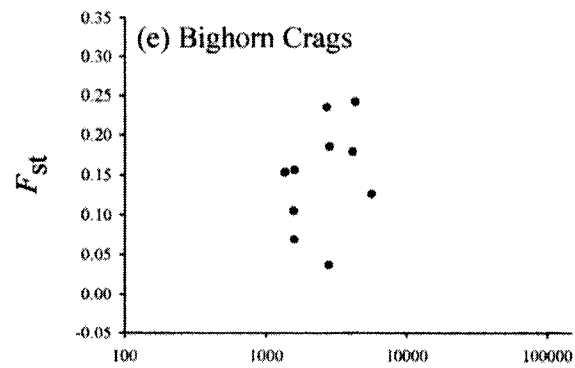
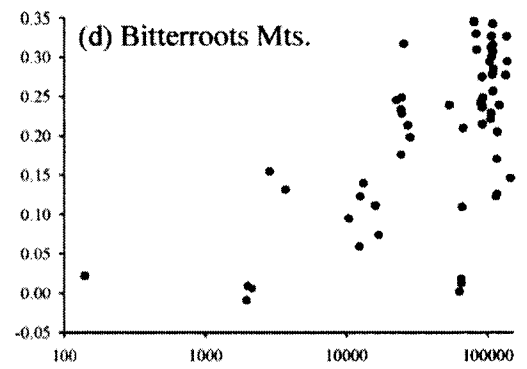
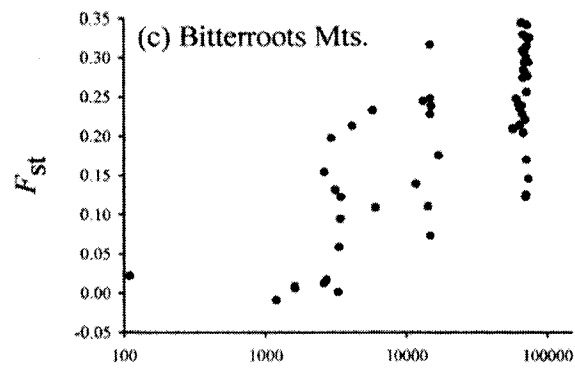
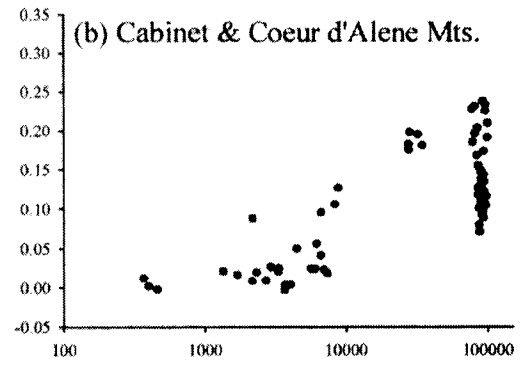
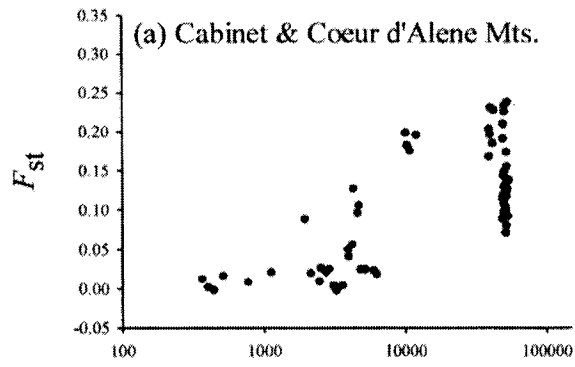
FIG. 3.6. Grouping of sites into populations (enclosed by dashed lines) using Bayesian clustering approach in STRUCTURE.

FIG. 3.7. 'Valley – mountain' model of population structure for Columbia spotted frogs. This population structure has three distinct characteristics: (1) low elevation populations with large historic effective population sizes (large circles) and high levels of among population gene flow (thick arrows); (2) high elevation populations with small historic effective population sizes (small circles) and little (thin and medium arrows) to no among population gene flow; and (3) gene flow is restricted, but not absent, between low and high elevation populations (thin and medium arrows).



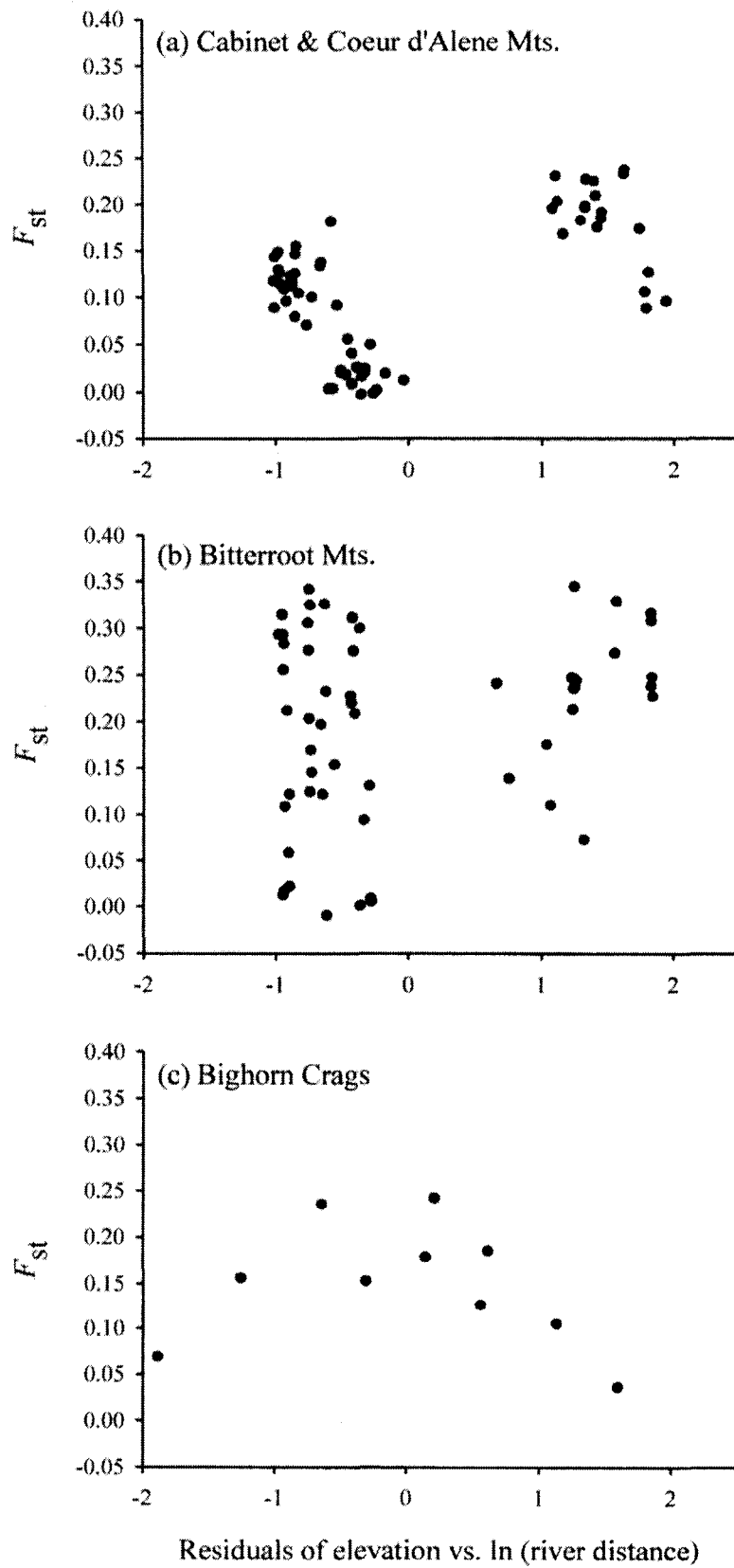


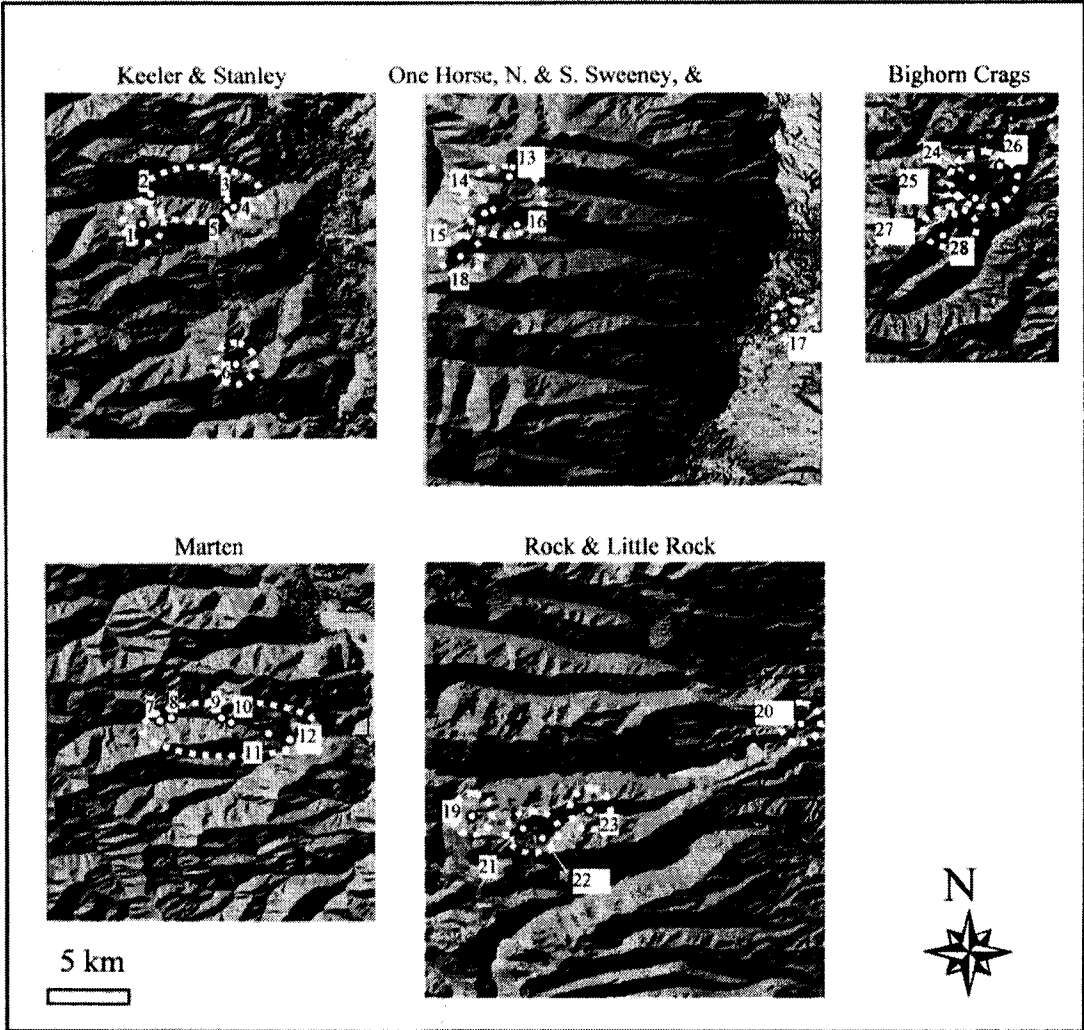


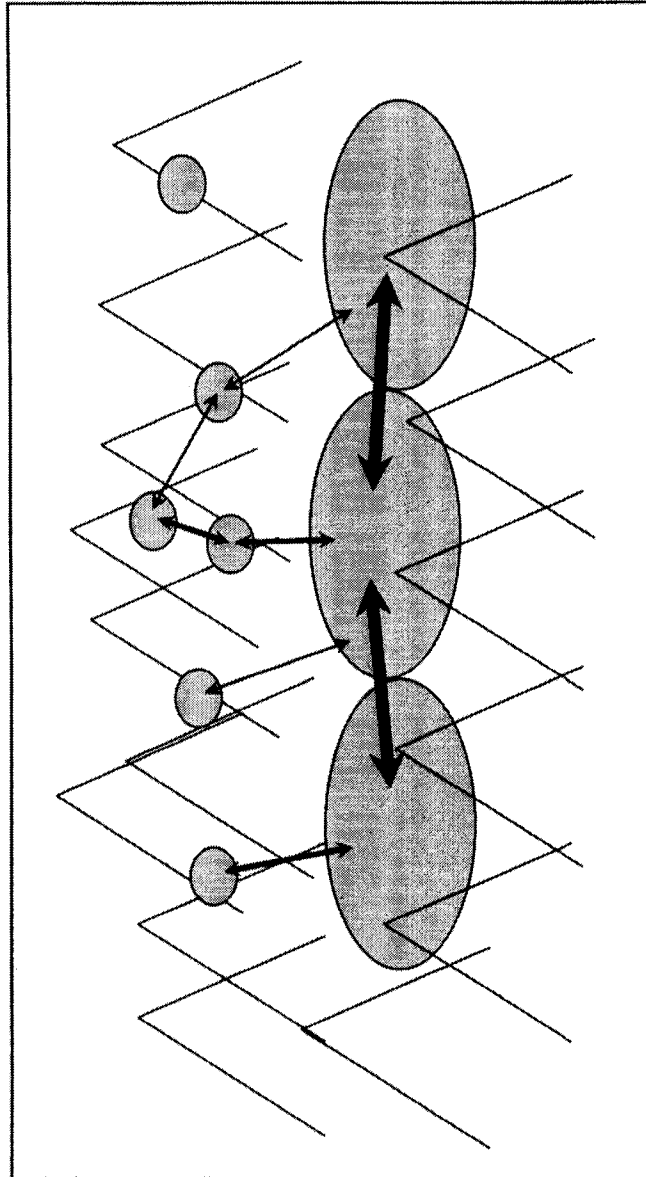


Straight line distance (m)

River distance (m)







CHAPTER 4 – Elevational Divergence in Frog Egg Size: A Test of the Roles of
Divergent Natural Selection, Genetic Drift, and Gene Flow

Abstract.—Larger egg size at high elevations is a pervasive, yet unexplained pattern in many taxa, including amphibians. Elevational divergence in egg size may be caused by divergent natural selection, genetic drift, or phenotypic plasticity, although elevational divergence in egg size is genetically based in all taxa examined, strongly suggesting that plasticity is an unlikely explanation. Gene flow may also influence elevational divergence in egg size by constraining local adaptation. We investigated the relative roles of divergent selection and genetic drift in generating elevational divergence in egg size in Columbia spotted frogs (*Rana luteiventris*) from Montana, USA, by testing: (1) whether egg size is positively related to elevation along two, independent elevational transects (predicted by divergent selection); (2) whether larger egg size is adaptive at high elevations by increasing embryonic developmental rates, embryonic survival at low temperatures, or hatchling size (also predicted by divergent selection); and (3) whether egg size divergence among populations is better predicted by elevational differences (also predicted by divergent selection) or by genetic isolation at microsatellite loci (predicted by genetic drift) using Mantel tests. We also used Mantel tests to test whether gene flow constrains egg size divergence. We found that egg size was strongly positively related to elevation along both transects examined. We also found that hatchling size was strongly positively related to egg size and that egg size divergence was predicted much better by elevational differences than by genetic isolation, all of which suggest that divergent selection drives egg size divergence. Gene flow did not appear to constrain egg size

divergence. We argue that selection for quicker metamorphosis or larger size at metamorphosis at high elevations has selected for larger eggs that result in larger hatchlings that grow and develop faster.

Key words.—Columbia spotted frog, *Rana luteiventris*, Montana, elevation, egg size, life history evolution, divergent natural selection, genetic drift, gene flow, microsatellite.

A fundamental goal of evolutionary biology is to explain the processes that generate phenotypic variation. One ubiquitous phenotypic pattern that remains unexplained is the positive relationship between parental investment per offspring, often manifest as egg size, and elevation. This pattern is observed across a variety of taxa including insects (Blanckenhorn 1997), snails (Baur and Raboud 1988), birds (Badyaev 1997a; Badyaev and Ghalambor 2001), mammals (Wynne-Edwards 1998), reptiles (Rohr 1997), and amphibians (Pettus and Angleton 1967; Berven 1982; Howard and Wallace 1985). Understanding the causes of variation in egg size is of particular interest because egg size is thought to have important fitness consequences for later life history stages (Roff 1992; Stearns 1992). Moreover, understanding the evolutionary processes that generate phenotypic variation is relevant to conservation because a better understanding of these processes can be used to improve conservation of adaptive phenotypic variation (McKay and Latta 2002). Conserving adaptive variation is particularly important now in light of global warming (Root et al. 2003).

There are three alternative hypotheses for elevational divergence in egg size. First, divergent selection pressures at low and high elevations may select for different

optimal egg sizes in these different environments. Life history theory predicts that shorter growing seasons, longer winters, and colder temperatures will select for greater parental investment per offspring at high elevations (Berven 1982; Badyaev 1997*a, b*; Blanckenhorn 1997). Specifically, larger eggs may improve fitness with shorter growing seasons by hatching faster or at a larger size. Larger hatchlings may then develop faster, resulting in quicker metamorphosis for taxa that metamorphose (Kaplan 1980, 1998; Berven and Chadra 1988; Parichy and Kaplan 1992; Loman 2002). Larger hatchlings may also grow faster resulting in larger metamorphs that are better at surviving long winters because of increased energy reserves (Kaplan 1980; Berven and Chadra 1988; Parichy and Kaplan 1992). Increased yolk reserves in larger eggs may also improve embryonic survival at cold temperatures (Heath et al. 2003).

A second hypothesis for elevational divergence in egg size is genetic drift due to finite population size (Wright 1969). This hypothesis seems less likely because of the consistent positive relationship observed between egg size and elevation in most taxa, but at the very least serves as a useful null hypothesis. Finally, elevational divergence in egg size may simply represent phenotypic plasticity in response to environmental variation. However, transplant experiments in flies (Blanckenhorn 1997), snails (Baur and Raboud 1988), and frogs (Berven 1982) all show that elevational variation in egg size has a genetic basis, strongly suggesting that phenotypic plasticity is an unlikely explanation for the pattern. This leaves divergent natural selection and genetic drift as the two most plausible hypotheses.

Elevational divergence in egg size may also be modified by gene flow which may constrain egg size divergence. Theory predicts that gene flow will constrain local

adaptation, but the effect of gene flow depends on the strength of divergent natural selection relative to the level of gene flow. If divergent selection is strong, gene flow may not be able to constrain divergence (Danley et al. 2000; Saint-Laurent et al. 2003). In contrast, if divergent selection is relatively weak, even moderate levels of gene flow may be sufficient to constrain divergence (Storfer and Sih 1998; Lenormand 2002). The effect of gene flow on local adaptation is an important question for conservation because of an emphasis in conservation on maintaining gene flow among populations to prevent the loss of within population genetic variation and negative inbreeding effects (Mills and Allendorf 1996; Newman and Tallmon 2001). However, if gene flow erodes local adaptation at some point, then it will suggest that too much gene flow may have negative fitness consequences.

The divergent selection and genetic drift hypotheses for elevational divergence in egg size have specific predictions. If divergent selection is responsible for the pattern, then: (1) there should be a positive relationship between egg size and elevation along multiple, independent elevational transects; (2) larger egg size should be adaptive at high elevation by increasing developmental rates, embryonic survival at low temperatures, or hatchling size; and (3) egg size should be predicted by elevational differences among populations rather than by genetic isolation as quantified by F_{st} (Fig. 4.1a). Alternatively, if genetic drift causes egg size divergence, then egg size should be predicted by F_{st} rather than elevational differences (Fig. 4.1d). Moreover, if gene flow constrains egg size divergence across elevation, then egg size divergence should be positively related to F_{st} for between elevation comparisons, assuming gene flow is inversely proportional to F_{st} (Fig. 4.1b-c). The steepness of the slope of the relationship between egg size divergence

and F_{st} for between elevation comparisons indicates how strongly gene flow constrains divergence.

We had three general objectives in the current study. The first objective was to determine whether egg size is positively related to elevation in Columbia spotted frogs as it is in other amphibians. Our second objective was to test the relative importance of divergent selection and genetic drift in generating egg size divergence and gene flow in constraining divergence by testing the previously described predictions. Finally, our third objective was to infer the specific selection pressures that cause elevational divergence in egg size if the evidence supports the divergent natural selection hypothesis. Columbia spotted frogs are pond-breeding frogs widely distributed throughout the northwestern U.S., western Canada, and southeast Alaska (Stebbins 1985; Greene et al. 1996, 1997). Columbia spotted frogs are an excellent species for investigating the evolutionary causes of elevational divergence in egg size because: (1) they have a broad elevational range, extending from low elevation wetlands to high elevation subalpine lakes; (2) they are a congener of wood frogs in which elevational divergence in egg size is completely genetically based (Berven 1982); and (3) microsatellite data is available (Chapter 3) which allows testing of alternative hypotheses for egg size divergence.

MATERIALS AND METHODS

Sampling Design

We sampled Columbia spotted frog eggs along two independent elevational transects in Montana, USA, in order to test whether egg size is consistently larger at high elevations (Table 4.1; Fig. 4.2). The northern transect consisted of two low elevation

ponds and one high elevation lake in Keeler Creek and one high elevation lake in Stanley Creek in the Cabinet Mountains of northwest Montana. The southern transect contained three low elevation ponds in Rock Creek and two high elevation lakes in Little Rock Creek in the Bitterroot Mountains of western Montana. The ponds ranged in elevation from 824 – 2256 m. Allele frequency data at six microsatellite data have already been collected for seven of these nine ponds (pond numbers 1, 2, 4, 6, 20, 21, and 22; Chapter 3).

We sampled 13 – 74 clutches from each pond for a total of 275 clutches from all nine ponds. Ten to twenty eggs were sampled from each clutch for a total of 2,843 eggs. Eggs were fixed in 10% formalin in the field for later measurement. Sampling different Columbia spotted frog clutches is straightforward because the clutches are laid as discrete egg masses. In 2002, eggs were sampled from 175 clutches from all nine ponds to test for a positive relationship between egg size and elevation. In 2003, eggs were sampled from 50 clutches from one low elevation pond (pond 4) and 50 clutches from one high elevation pond (pond 1) to test the temporal stability at these ponds between 2002 and 2003 and to test the relationship between egg size and various fitness parameters in the embryo experiment.

Egg Measurement

We measured the frog eggs with a Leica MZ6 microscope attached to a MacIntosh G4 computer using Scion Image 1.62c software. Prior to measurement, the jelly layer was removed from eggs, but the vitelline envelope was left intact. Eggs were positioned with the dorsal, pigmented side (animal pole) facing up. The longest and

shortest diameters of eggs were measured. However, since these dimensions were similar, we only used the longest diameter of each egg as a measurement of egg size for analyses. Embryos were staged using Shumway's (1940) staging table for *Rana pipiens* which is the same as the commonly used Gosner (1960) staging table through stage 25.

Embryo Experiment

We raised embryos in Percival Scientific Series 101 temperature chambers in the laboratory to test the effects of egg size, population origin, and temperature on days to hatching, embryonic survival, and size at hatching. In 2003, 25 embryos were sampled from each of the 100 clutches sampled for egg size measurement, as described previously. Samples were taken from 50 clutches from pond 4 (low elevation) and from 50 clutches from pond 1 (high elevation). Half of the clutch samples (25) from each of these ponds were then randomly assigned to a warm treatment (approximately 20° C) and the other half to a cold treatment (approximately 10° C). Embryos from each clutch were raised in separate 250 ml beakers filled with 200 ml of well water. Only one temperature chamber was available for each temperature treatment, so it was not possible to unambiguously separate chamber effects from temperature effects. However, chambers appeared identical in all respects, so differences in developmental rates, embryonic survival, and hatching size were likely due to temperature effects.

Because the breeding season started on April 6 at the low elevation pond and approximately 7 weeks later on May 28 at the high elevation pond, we had to run the experiment at different times for low and high elevation embryos. Chamber temperatures were therefore monitored daily in order to make sure that the low and high elevation

embryos experienced the same temperatures. Temperature was monitored with three to five thermometers placed in different clutch sample beakers in each chamber. We found that mean temperature was the same for the low and high elevation embryos. The mean temperature in the low temperature treatment was $11.0 \pm 0.2^\circ \text{C}$ (SE) for the low elevation embryos and $10.7 \pm 0.2^\circ \text{C}$ for the high elevation embryos ($N = 77$, $t = 1.31$, $P = 0.20$). The mean temperature in the high temperature treatment was $20.3 \pm 0.4^\circ \text{C}$ for the low elevation embryos and $20.2 \pm 0.3^\circ \text{C}$ for the high elevation embryos ($N = 21$, $t = 0.17$, $P = 0.86$).

We raised embryos under a 12 hour light – 12 hour dark cycle. Embryos were checked daily and the number of dead embryos and hatchlings was recorded. Dead embryos were removed and discarded and hatchlings were fixed in 10% formalin for later measurement. We designated embryos as hatchlings as soon as they left the vitelline envelope. Beaker water was changed twice a week.

Hatchlings were also measured with the Leica MZ6 microscope. Ten hatchlings were measured from each clutch. Total length, head length, tail length, abdomen length, head depth, and tail depth were measured for each hatchling as described in Fig. 4.3. Hatchlings were also staged using Shumway's (1940) staging table.

Data Analysis

We first examined the relationship between egg size and elevation along our northern and southern transects. Egg size was ln-transformed to normalize the data. Because we considered clutch samples to be the sampling units rather than individual eggs, we used mean ln egg size for each clutch sample for analyses. Also because egg

size depended on embryo stage, we used the residuals from the regression of mean ln egg size versus mean stage for each clutch sample for analyses (referred to as residual mean ln egg size throughout the text). Simple linear regressions of residual mean ln egg size versus elevation were used to test for a positive relationship between these two variables for each transect and both transects combined. A general linear model was used to test for an interaction between elevation and transect. T-tests were used to test whether residual mean ln egg size changed between 2002 and 2003 in pond 1 or 4.

We also used simple linear regression analysis to test the effects of residual mean ln egg size on days to hatching, embryonic survival, and hatchling size. T-tests were used to test the effects of population origin (low or high elevation) and temperature treatment (low or high temperature) on these same variables. General linear models were used to test for all two-way interactions between the three predictor variables (residual mean ln egg size, population origin, and temperature treatment). Variation in the six hatchling morphological variables was reduced to orthogonal axes using principal components analysis (e.g., Schneider et al. 1999).

We used Mantel tests to test the effects of elevational differences and F_{st} on egg size divergence (Mantel 1967). Egg size divergence was defined as the absolute difference in average residual mean ln egg size between each pair of sites. A pair of sites was coded as having an elevational difference of one if one of the sites was a low elevation site and the other was a high elevation site and was coded as having an elevational difference of zero if both sites were low or high elevation sites. All sites in the mountains were considered high elevation sites (ponds 1, 6, 21, and 22) and all sites in valley bottoms or in foothills were considered low elevation sites (ponds 2, 4, 20, KP,

and SL). We ran three different Mantel tests. First, we tested whether egg size divergence is positively related to elevational differences as predicted by the divergent selection hypothesis (Fig. 4.1a); second, we tested whether egg size divergence is positively related to F_{st} as predicted by the genetic drift hypothesis (Fig. 4.1d); and third, we tested whether egg size divergence is positively related to F_{st} for between elevation comparisons only as predicted if gene flow constrains divergence (Fig. 4.1b-c). Two thousand randomizations were used for all Mantel tests.

RESULTS

Egg Size Variation Across Elevation

Egg size was strongly positively related to elevation along both transects examined. Residual mean ln egg size was significantly positively related to elevation for the northern transect ($N = 87$, $F = 13.49$, $P < 0.001$), southern transect ($N = 88$, $F = 88.82$, $P < 0.001$), and both transects combined ($N = 175$, $F = 139.68$, $P < 0.001$; Fig. 4.4). Mean egg size varied from a minimum of 2.38 ± 0.03 mm (SE) at site 4 at an elevation of 824 m to a maximum of 2.84 ± 0.04 mm at site 22 at an elevation of 2,139 m (Table 4.1). Residual mean ln egg size was significantly different between 2002 and 2003 in pond 4 ($N = 71$, $t = 3.89$, $P < 0.001$), but not in pond 1 ($N = 74$, $t = 0.48$, $P = 0.631$). Nevertheless, residual mean ln egg size remained higher in the high elevation pond (pond 1) than in the low elevation pond (pond 4) in both 2002 ($N = 45$, $t = 4.20$, $P < 0.001$) and 2003 ($N = 100$, $t = 3.58$, $P = 0.001$).

Embryo Experiment

The only fitness variable examined that was affected by egg size was hatchling size measured with principal components analysis. Most of the variance in hatchling morphology could be explained by two principal components (PC's). PC1 explained 52.3% of the variance and had high negative loadings for total length, tail length, and tail depth (Table 4.2). Therefore, hatchlings with high PC1 scores were short and had small tails. PC2 explained 26.1% of the variance and had high positive loadings for abdomen length, head length, and head depth (Table 4.2). Hatchlings with high PC2 scores thus had relatively large bodies. PC3 only explained 11.4% of the variance.

Principle component 1 (PC1) was not affected by egg size, but PC2 was strongly positively related to egg size. This can be seen from the regression between PC1 and residual mean \ln egg size which was not significant ($N = 99$, $F = 0.01$, $P = 0.941$; Fig. 4.5a) compared to the regression between PC2 and residual mean \ln egg size which was highly significant ($N = 99$, $F = 110.72$, $P < 0.001$; Fig. 4.5b). However, PC1 was larger for hatchlings from the high elevation site than for hatchlings from the low elevation site ($N = 99$, $t = 8.87$, $P < 0.001$). Moreover, PC1 was larger for hatchlings raised in the warm treatment than in the cold treatment for the high elevation site ($N = 50$, $t = 4.89$, $P < 0.001$) and the low elevation site ($N = 49$, $t = 4.01$, $P < 0.001$).

Although PC1 was not related to egg size, it was strongly negatively related to stage at hatching, whereas PC2 was only weakly related to stage at hatching. The regression between PC1 and mean stage at hatching was highly significant ($N = 99$, $F = 410.40$, $P < 0.001$; Fig. 4.6a) and had a steep negative slope of -2.06 . The regression between PC2 and mean stage at hatching was also statistically significant ($N = 99$, $F =$

6.46, $P = 0.013$; Fig. 4.6b), but had a much weaker slope of only -0.40 . These results indicate that PC1 largely reflects morphological changes that occur during development, namely an increase in tail size and total length. In contrast, PC2 represents variation in hatchling body size that is independent of stage, but strongly positively related to egg size.

Although mean days to hatching was not affected by egg size, it was affected by temperature treatment. Embryos hatched after an average of 4.93 ± 0.05 days in the warm treatment and after an average of 21.22 ± 0.46 days in the cold treatment ($N = 100$, $t = 35.36$, $P < 0.001$; Fig. 4.5c). Moreover, in the warm treatment, embryos from the low elevation site hatched slightly more quickly (4.79 ± 0.09 days) than embryos from the high elevation pond (5.07 ± 0.02 days, $N = 50$, $t = 3.17$, $P = 0.004$). In contrast, in the cold treatment, embryos from the high elevation pond hatched much more quickly (19.08 ± 0.45 days) than embryos from the low elevation pond (23.36 ± 0.53 days, $N = 50$, $t = 6.17$, $P < 0.001$). Although embryos from the high elevation pond hatched more quickly in the cold treatment than embryos from the low elevation pond, they also hatched at earlier developmental stages. The high elevation embryos in the cold treatment hatched at an average developmental stage of 19.99 ± 0.12 compared to an average stage of 21.19 ± 0.12 for low elevation embryos ($N = 50$, $t = 7.19$, $P < 0.001$). In fact, the regression of mean stage at hatching versus mean days to hatching reveals that this relationship is similar for the low and high elevation ponds, suggesting that embryonic developmental rate is essentially the same for these ponds (Fig. 4.7). Embryonic survival was not affected by egg size, population origin, or temperature treatment (Fig. 4.5d).

Mantel Tests

Mantel tests showed that egg size divergence was predicted by elevational differences, but not by F_{st} (Fig. 4.8). The standardized Mantel test statistic was 0.19 for the correlation between egg size divergence and F_{st} ($P = 0.416$), but 0.54 for the correlation between egg size divergence and elevational difference ($P = 0.015$). Moreover, egg size divergence was not predicted by F_{st} when only considering between elevation comparisons. In this case, the Mantel test statistic was only 0.29 ($P = 0.347$).

DISCUSSION

Positive Relationship between Egg Size and Elevation

We found that egg size was much larger at high elevations than low elevations in Columbia spotted frogs, especially when considering differences in egg volume. The maximum difference in mean egg diameter was observed between pond 4 with a mean egg diameter of 2.38 mm at an elevation of 824 m and pond 22 with a mean egg diameter of 2.84 mm at an elevation of 2,139 m (Table 4.1). This translates to a 19% larger egg diameter and a 70% larger egg volume over an elevation gain of 1,315 m. Even larger increases in egg volume at high elevations are seen in other amphibians. Egg volume increases 93% over 1,057 m in wood frogs (*Rana sylvatica*; Berven 1982), 104% over 1,330 m in long-toed salamanders (*Ambystoma macrodactylum*; Howard and Wallace 1985), and 197% over 1,311 m in boreal chorus frogs (*Pseudacris maculata*; Pettus and Angleton 1967). Among population differences in egg volume of 70 – 197% represent exceptionally high levels of within species phenotypic variation.

Divergent Natural Selection with Gene Flow

Our evidence supports the hypothesis that divergent selection drives elevational divergence in egg size in Columbia spotted frogs. Three lines of evidence support this hypothesis. First, egg size is strongly positively related to elevation along both transects examined (Fig. 4.4). Second, large eggs appear adaptive at high elevations by producing larger hatchlings that likely metamorphose faster or larger (Fig. 4.5). Last, egg size divergence among populations is predicted by elevational differences, but not by genetic isolation (Fig. 4.8). It is important to note, however, that we cannot eliminate the possibility that egg size divergence is caused by phenotypic plasticity in Columbia spotted frogs because we do not know whether elevational divergence in egg size is genetically based. Nonetheless, we argue that it is highly unlikely that there is no genetic basis to the observed pattern given the fact that elevational divergence in egg size is genetically based in all three taxa examined including wood frogs, a congener of Columbia spotted frogs, over a similar elevational range (Berven 1982; Baur and Raboud 1988; Blanckenhorn 1997).

There was one outlying data point in the relationship between egg size divergence and F_{st} that represents a between elevation comparison between ponds 1 (high elevation) and 2 (low elevation; Fig. 4.8). This point is an outlier in that egg size divergence was very low between these two ponds compared to other between elevation comparisons. One explanation for low egg size divergence between these two sites is that the short hydroperiod of pond 2 has selected for large eggs in this low elevation pond relative to other low elevation ponds. Pond 2 dried completely or mostly during all four years of observation which is predicted to select for large eggs (Loman 2001; Doughty 2002).

Another explanation for the low egg size divergence between these sites is that the elevational difference between these two ponds is not as large as many of the other between elevation comparisons.

Our embryo experiment demonstrated that large eggs produce large hatchlings, suggesting that selection for large hatchlings may drive selection for large eggs at high elevation (Fig. 4.5b). Large hatchlings may be advantageous at high elevations because large hatchlings often develop or grow faster. Evidence from previous studies has shown that larger amphibian eggs often produce larger hatchlings that in turn have higher larval developmental rates and growth rates (Kaplan 1980, 1998; Berven and Chadra 1988; Parichy and Kaplan 1992; Loman 2002). Faster developmental rates would allow larger hatchlings to metamorphose earlier. A reduced time to metamorphosis may increase the probability of metamorphosing by the end of the short growing season at high elevation. Because Columbia spotted frogs tadpoles cannot overwinter, tadpoles must metamorphose or die before ponds freeze over, so selection must be strong to metamorphose before freezing. Faster growth rates would also allow hatchlings to metamorphose at a larger size. Metamorphosing at a large size may have an important positive effect on overwinter survival at high elevations because winters are much longer at high elevations. Larger metamorphs may have more energy reserves for surviving long winters.

The lack of a relationship between egg size divergence and F_{st} for between elevation comparisons also suggests that gene flow does not constrain egg size divergence across elevation (Fig. 4.8). The lack of a relationship between egg size divergence and F_{st} for between elevation comparisons may be partly due to low power

given that there were only 12 between elevation comparisons. Nonetheless, if gene flow does constrain egg size divergence, it does so only weakly. It is somewhat surprising that gene flow does not constrain divergence given the short geographic distances separating some of the low and high elevation ponds in this study and the high dispersal rates seen between some low and high elevation ponds in previous work (Chapter 2). The distances between low and high elevation ponds in this study are well within the dispersal distances traversed by Columbia spotted frogs. We have previously documented Columbia spotted frog juveniles moving over 5 km, while the low elevation ponds in Keeler Creek (ponds 2 and 4) are only 2 – 4.5 km from the high elevation lake in the same basin (pond 1; Chapter 2). The large divergence in egg size despite moderately high levels of gene flow between some low and high elevation ponds suggests that divergent selection for larger eggs at high elevations is strong.

Although egg size did not affect days to hatching, embryos from the high elevation pond hatched an average of four days earlier in the cold treatment than embryos from the low elevation pond (Fig. 4.5c). However, earlier hatching did not translate to quicker embryonic development for the high elevation pond because the relationship between hatching stage and days to hatching was similar for both the low and high elevation ponds (Fig. 4.7). It is unclear what the advantage of hatching sooner at an earlier stage would be for high elevation embryos. Moreover, we cannot determine from our experimental design whether this is a phenomenon common to high elevation populations because we only sampled one high and one low elevation population. If embryos do typically hatch earlier at high elevations, we can think of two possible explanations. First, early hatching may allow earlier feeding which in turn may increase

early larval developmental and growth rates. Second, early hatching may also allow earlier mobility to escape mortality from freezing at the surface where clutches are typically laid. Alternatively, early hatching may be unique to pond 1, the high elevation pond sampled for our experiment. The water level in pond 1 dropped approximately one meter within a couple of weeks after breeding every year between 2000 and 2003. Early hatching could therefore also serve as an adaptation to escape desiccation when the water level drops in this pond.

Conservation Implications

This study and previous work suggest that elevational gradients may be important sources of adaptive genetic variation (McKay et al. 2001). We therefore argue that it is important to preserve populations across elevational gradients in order to maintain adaptive genetic variation within species with wide elevational ranges. This recommendation contrasts with the fact that most areas protected in U.S. National Parks and Wilderness Areas primarily consist of high elevation habitats. Low elevation habitats will likely be more difficult to protect because these areas are also preferred by people and have already undergone serious habitat degradation. Nevertheless, protecting these areas may be crucial for species persistence in the face of global warming which may occur too rapidly for high elevation populations to adapt (Root et al. 2003). If high elevation populations are unable to adapt to rapid global warming, low elevation populations may serve as critical sources of colonists.

TABLE 4.1. UTM coordinates (map datum NAD27), elevation, and Columbia spotted frog egg size means and standard errors for sampling sites (Fig. 4.2). Site codes are the same as in Chapter 3. All sites were sampled in 2002 and sites 1 and 4 were also sampled in 2003. Ten to twenty embryos were measured from each clutch.

Basin	Site code	UTM coordinates	Elevation (m)	Egg size		No. clutches sampled
				Mean	SE	
Keeler Creek	1	11 575650E 5352125N	1581	2.69 (2002)	0.05 (2002)	24 (2002)
				2.56 (2003)	0.03 (2003)	50 (2003)
	2	11 576062E 5354011N	884	2.40	0.04	17
Stanley Creek	4	11 580150E 5353173N	824	2.38 (2002)	0.03 (2002)	21 (2002)
				2.47 (2003)	0.01 (2003)	50 (2003)
	6	11 580370E 5342957N	1485	2.64	0.03	25
Rock Creek	20	11 715702E 5106121N	1250	2.42	0.04	13
	KP	11 713132E 5104827N	1311	2.47	0.05	19
	SL	11 714153E 5103820N	1387	2.49	0.04	17
Little Rock Creek	21	11 702849E 5098795N	2256	2.83	0.04	20
	22	11 703869E 5098184N	2139	2.84	0.04	19



TABLE 4.2. Factor scores from principle components analysis of ln-transformed morphological variables of Columbia spotted frog hatchlings (Fig. 4.3). Clutch means were used in the analysis.

Variable	PC1	PC2
Total length	-0.556	0.021
Abdomen length	0.189	0.518
Tail length	-0.553	-0.110
Head length	-0.228	0.557
Head depth	-0.023	0.639
Tail depth	-0.545	0.010
Eigenvalue	3.14	1.57
Percent of variance	52.3	26.1

FIGURE LEGENDS

FIG. 4.1. Predicted relationship between egg size divergence and F_{st} for population comparisons at the same elevation or different elevations with different evolutionary processes involved in divergence: (a) divergent selection, gene flow does not constrain divergence; (b) divergent selection, gene flow weakly constrains divergence; (c) divergent selection, gene flow strongly constrains divergence; and (d) genetic drift.

FIG. 4.2. Map of sites from which Columbia spotted frog eggs were sampled in Montana, USA. Site details are given in Table 4.1. Microsatellite allele frequency data was collected from sites with numbers. Numbers correspond to the numbers in Chapter 3. Keeler and Stanley Creeks are referred to as the northern elevational transect and Rock and Little Rock Creeks as the southern transect.

FIG. 4.3. Morphological variables measured on Columbia spotted frog hatchlings for principal components analysis. Total length = distance from the tip of the snout to the end of the tail; tail length = distance from the cloaca to the end of the tail; abdomen length = distance from the posterior end of the salivary glands to the cloaca; head depth = distance from the posterior end of the salivary glands to the point on the top of the head forming the shortest straight-line distance; tail depth = distance from the cloaca to the top of the body forming the shortest straight-line distance; and head length = distance from the tip of the snout to point on the head depth line that forms a 90° angle with the head depth line.

FIG. 4.4. Regressions of residual mean ln egg size versus elevation for northern (filled circles) and southern transects (open circles).

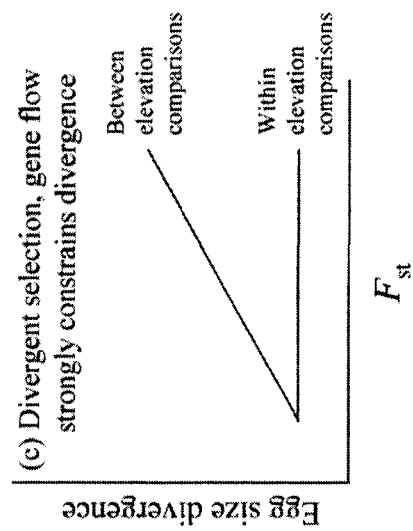
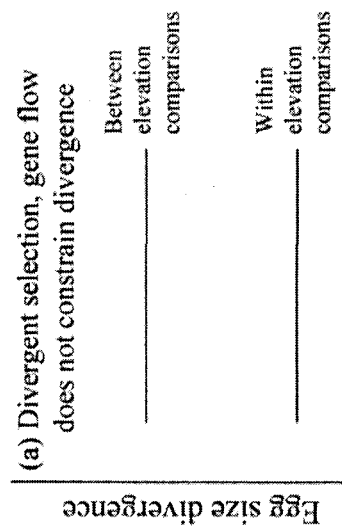
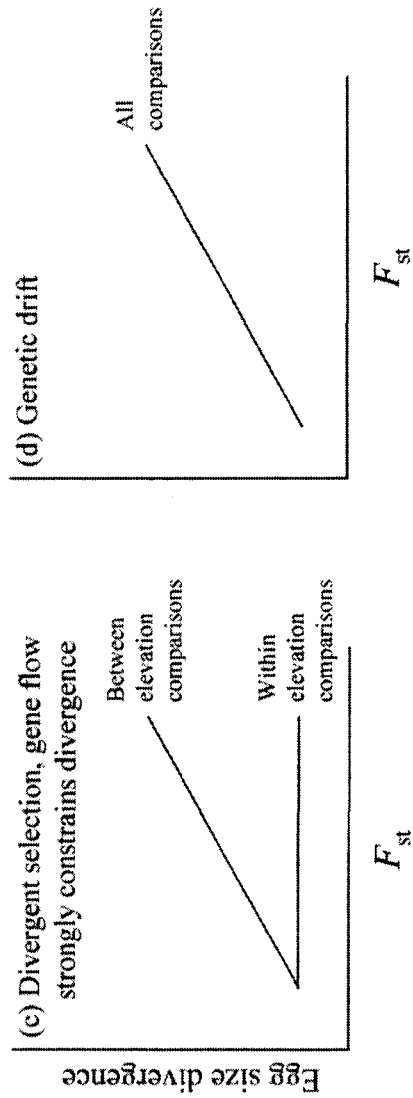
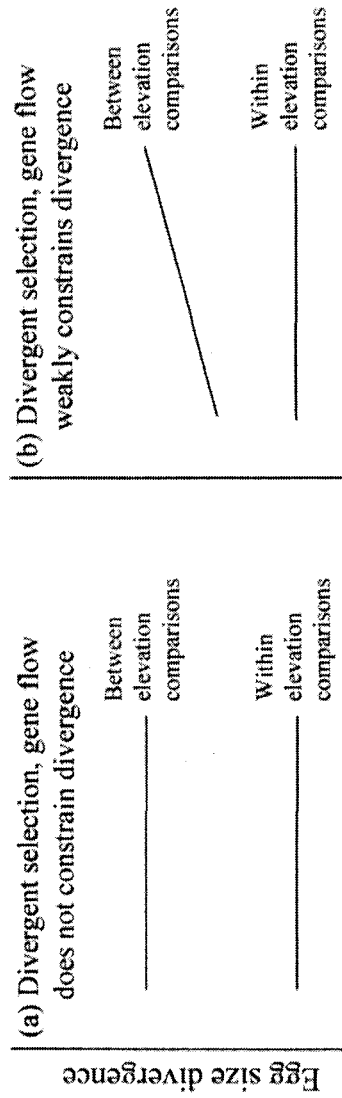
FIG. 4.5. PC1 (a), PC2 (b), mean days to hatching (c), and embryonic survival (d) versus residual mean ln egg size from embryo experiment. Each point represents the mean value of a clutch sample. Filled circles = low elevation site, cold treatment; filled triangles = low elevation site, warm treatment; open circles = high elevation site, cold treatment; open triangles = high elevation site, warm treatment. Sites or temperature treatments with significantly different slopes or means are indicated with different regression lines.

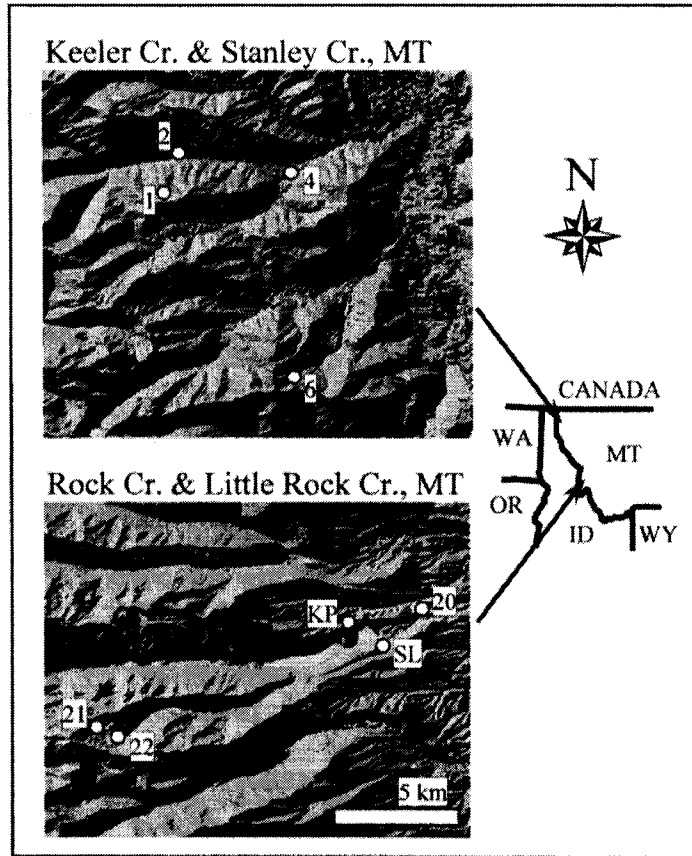
FIG. 4.6. PC1 (a) and PC2 (b) versus mean stage at hatching from embryo experiment. Each point represents the mean value of a clutch sample. Filled circles = low elevation site, cold treatment; filled triangles = low elevation site, warm treatment; open circles = high elevation site, cold treatment; open triangles = high elevation site, warm treatment.

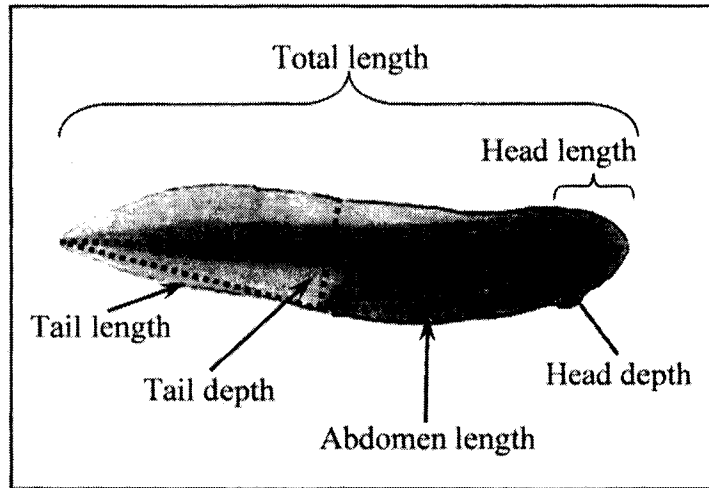
FIG. 4.7. Mean stage at hatching versus mean days to hatching from embryo experiment. Each point represents the mean value of a clutch sample. Filled circles = low elevation site, cold treatment; filled triangles = low elevation site, warm treatment; open circles = high elevation site, cold treatment; open triangles = high elevation site, warm treatment.

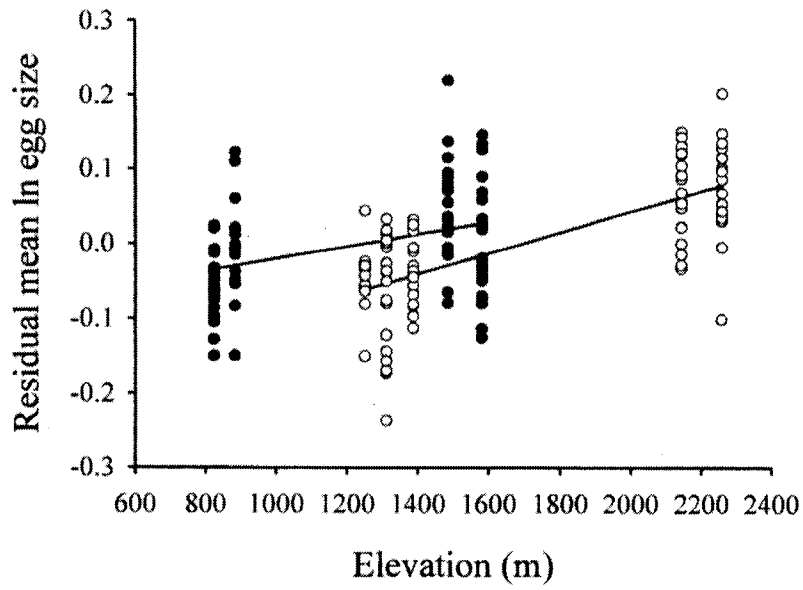
FIG. 4.8. Egg size divergence among sites versus F_{st} . Egg size divergence was measured as the absolute difference in average residual mean ln egg size between each pair of sites.

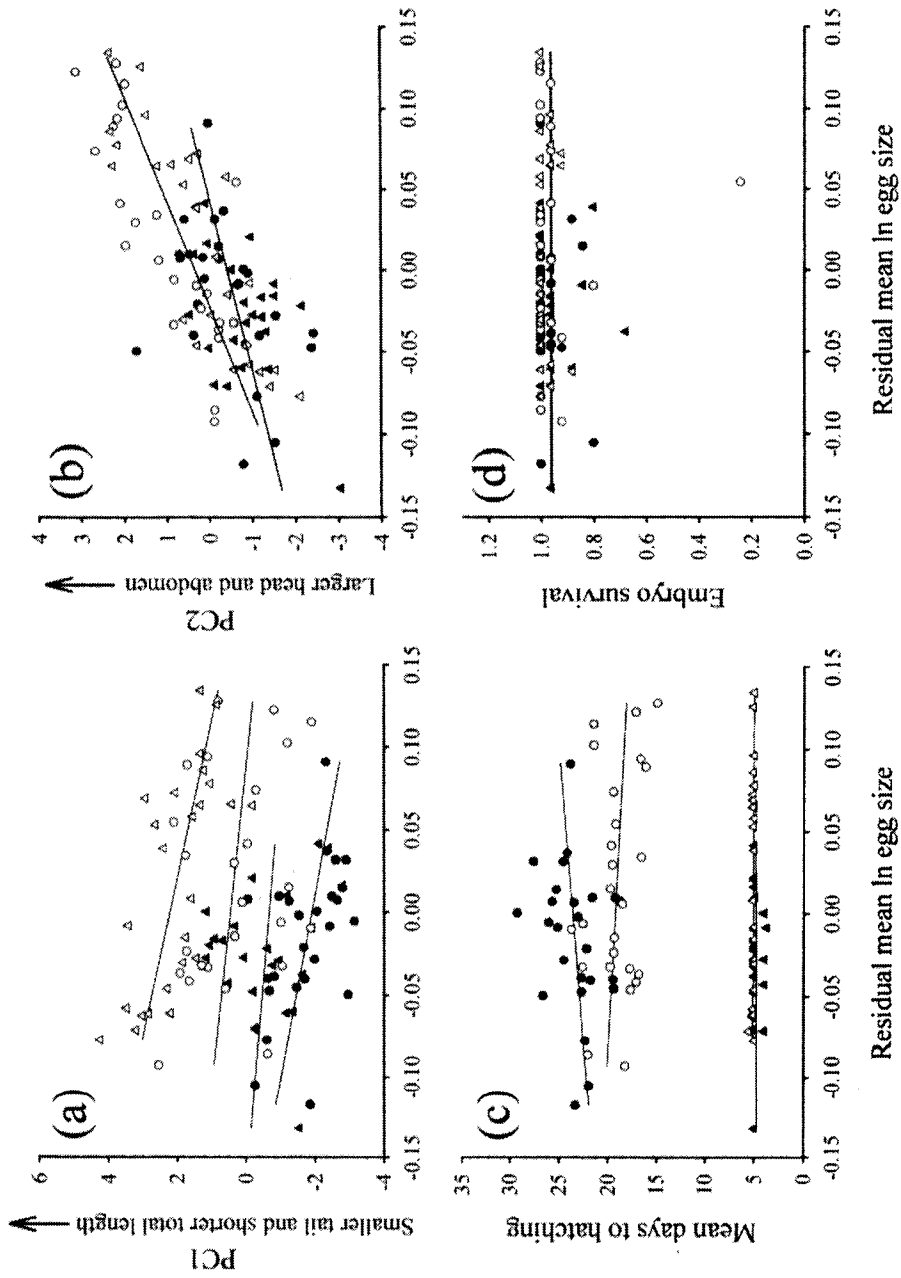
Pairwise F_{st} estimates were calculated from allele frequencies at six microsatellite loci (Chapter 3). Filled circles represent between elevation comparisons (low versus high elevation sites) and open circles are within elevation comparisons (low versus low or high versus high elevation sites).

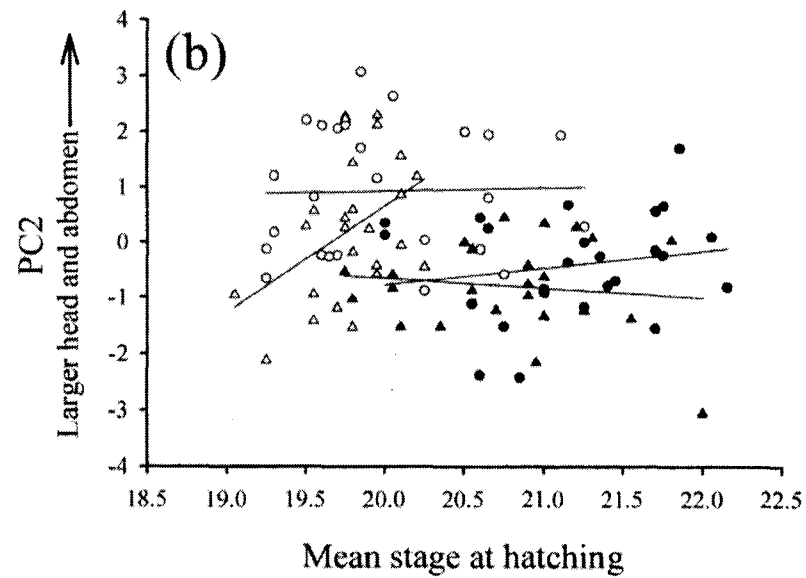
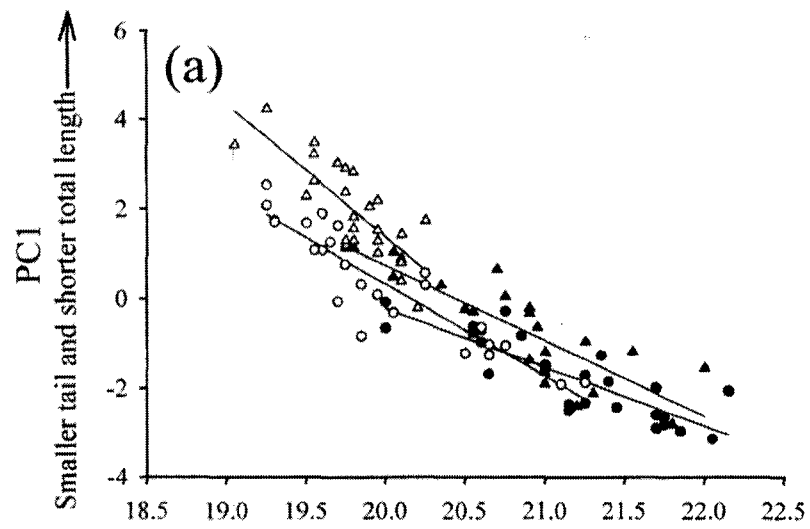


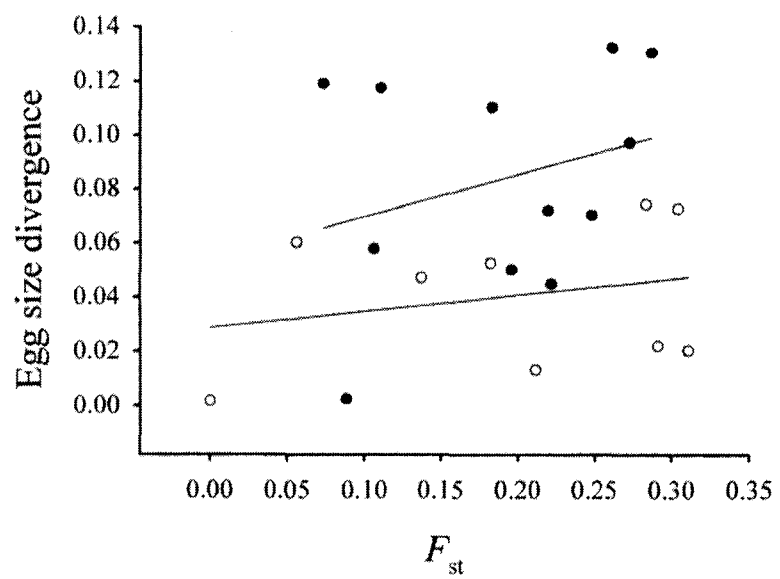












Monitoring Population Trends of *Eleutherodactylus* Frogs

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ABSTRACT.—Like many Neotropical frogs, a number in the genus *Eleutherodactylus* have declined or gone extinct in the past two decades. However, the extent of *Eleutherodactylus* population declines is unknown. Our objective was to identify a good method for monitoring the density of *Eleutherodactylus* populations to assess the extent of declines. We did this in two ways. First, we compared two methods of directly estimating density, closed population capture-recapture analysis and distance sampling, and one method of indirectly estimating density, visual encounter surveys, for multiple *Eleutherodactylus* species at three sites in Ecuador. We then conducted a power analysis to estimate the power of our current sampling design to detect declines. Distance sampling estimates of density were biased low compared to capture-recapture estimates. When we corrected this bias, distance sampling estimates became imprecise. Estimates of density from visual encounter surveys were also imprecise. In contrast, capture-recapture estimates were fairly precise and most likely unbiased. Moreover, capture-recapture analysis had the most power to detect declines, although even with capture-recapture analysis, power was low with only five years of sampling. We conclude that capture-recapture analysis is a good method for monitoring *Eleutherodactylus* density over time, but the sampling area and/or the number of sampling occasions should be increased from the area and number of occasions used here in order to increase sample sizes and therefore power.

RESUMEN.—Como muchas especies de ranas y sapos neotropicales, varias ranas en el género *Eleutherodactylus* han disminuido en número o se han extinguido en las últimas dos décadas, pero no se sabe en que magnitud han disminuido. Nuestro objetivo fue identificar un buen método para monitorear la densidad de poblaciones de *Eleutherodactylus* y de esa forma evaluar la magnitud de sus disminuciones. Esto lo hicimos de dos maneras. Primero, comparamos dos métodos para estimar la densidad directamente, captura-recaptura para poblaciones cerradas y el muestreo de distancia, y un método para estimar densidad indirectamente, registro de encuentros visuales en transectos, en varias especies de *Eleutherodactylus* en tres sitios en Ecuador. Luego hicimos un análisis de poder para estimar el poder estadístico de nuestro diseño de muestreo actualizado para percibir disminuciones. Los cálculos del muestreo de distancia tenían un sesgo a la baja comparados a los cálculos de captura-recaptura. Cuando corregimos este sesgo, los cálculos de muestreo de distancia se volvieron imprecisos. También, los cálculos de registro de encuentros visuales en transectos fueron imprecisos. Los cálculos de captura-recaptura fueron medianamente precisos y probablemente no tuvieron sesgos a la baja o a la alta. Además, el análisis de captura-recaptura tenía el poder estadístico más alto para percibir disminuciones, aunque el poder fue bajo después de cinco años de muestreo. Concluimos que el análisis de captura-recaptura es un buen método para monitorear la densidad de *Eleutherodactylus* a través del tiempo, pero el área de muestreo y/o el número de ocasiones de muestreo deben ser incrementados en relación al área y el número de ocasiones que usamos para aumentar tamaños de muestras y poder.

Perhaps the greatest obstacle to preventing and reversing amphibian declines is that there are few long-term data on population trends for most amphibians (Blaustein, 1994; Pechmann and Wilbur, 1994). As a result, most amphibian declines are not detected until populations have declined precipitously or gone extinct, by which time it may be too late to infer causes of declines, prevent future declines, or restore pop-

ulations. Before it is possible to determine the causes of declines and develop management strategies to prevent and reverse declines, researchers and managers first need to know: (1) which species are declining; (2) where they are declining; and (3) the rate at which they are declining. Moreover, it is critical that this information is gathered quickly.

The only reliable way to gather this information is through well-designed amphibian population monitoring programs. Population mon-

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itoring involves estimating population parameters of interest over time and then using regression analysis to test for significant declines or increases in the parameters (Thompson et al., 1998). The parameters of interest in population monitoring programs are usually abundance (the absolute number of animals) or density (the number of animals per unit area) although parameters of interest may also include population growth rates or vital rates (birth and death rates). Monitoring programs should be designed so that biologically significant changes in the parameter of interest can be detected with a desired level of statistical power, the probability of detecting an actual decline or increase in the parameter of interest (Gerrodette, 1987). In turn, it is important to choose a good method for estimating the parameter of interest because the method used will directly affect the power to detect changes.

For most species of amphibians, little is known about which methods are best for estimating abundance or density. The best estimates are those that are both precise and unbiased. Precision is the degree of spread in estimates generated from repeated samples. Bias is the difference between the expected value of a parameter estimate and the true value of the parameter (Thompson et al., 1998). A lack of precision, manifest as high sampling variance, standard error, and coefficients of variation, reduces power. An estimate that is consistently biased high or low will not reduce power but will simply be an overestimate or underestimate of the true parameter, respectively.

There are two classes of methods for estimating abundance and density: direct estimators and indices. Direct estimators are designed to estimate true abundance or density by first estimating the number or proportion of individuals not encountered. In contrast, indices are count statistics that are assumed to be correlated with abundance or density by some functional relationship (Thompson et al., 1998) but do not directly estimate these parameters. Examples of direct estimators are closed population capture-recapture analysis, distance sampling, and removal sampling (White et al., 1982; Seber, 1982; Buckland et al., 1993). Indices that have been applied to amphibian populations include visual encounter surveys, audio strip transects, and breeding site surveys (Heyer et al., 1994).

There are two problems with indices (Thompson et al., 1998). First, use of an index assumes that there is a functional relationship between the index and the parameter of interest, but often this relationship is unknown. Moreover, even if the function relating the index and parameter is known in a particular case, it is likely not constant over time, space, species, or ob-

servers. Second, indices often have high sampling variance. Because of these problems, direct estimators are expected to give better estimates than indices, both in terms of precision and bias, as long as their assumptions are met. However, direct estimators generally require more effort at a greater cost. Because indices are relatively easy and cheap, they are much more commonly used for studies of amphibian populations than are direct estimators.

The objective of the present study was to identify a good method for monitoring population density of *Eleutherodactylus* frogs and, in particular, to identify a method that has a high probability of quickly detecting rapid declines because many tropical amphibian declines have occurred rapidly (Lips, 1999; Young et al., 2001). *Eleutherodactylus* are direct developing frogs found throughout the Neotropics, some of which have experienced declines and that are in immediate need of population monitoring. The genus is represented by over 600 described species and dozens of undescribed species, making it the most speciose vertebrate genus in the world (Lynch, 1999). At least three *Eleutherodactylus* species have declined or gone extinct in Costa Rica and Panama (Lips, 1999), nine species in Puerto Rico (Hedges, 1993; Joglar and Burrowes, 1996), and several others from other Latin American countries (Hedges, 1993; Young et al., 2001). Because of the extreme species richness of the genus, continued *Eleutherodactylus* population declines could result in a major loss of Neotropical and global amphibian diversity.

We used two direct estimators and one index to estimate the density of multiple *Eleutherodactylus* species from Ecuador and evaluated the relative performance of each method in terms of the precision and bias of its estimates. We then estimated the power to detect *Eleutherodactylus* population declines using these three methods. The two direct estimators we tested were closed population capture-recapture analysis and distance sampling. The index we tested was visual encounter surveys. Capture-recapture analysis uses capture histories of individually marked animals to estimate capture probabilities and from these probabilities, the number of individuals not found. Distance sampling uses the distribution of distances of animals from transect centerlines to estimate a detection function, which is then used to estimate the proportion of animals not encountered. Finally, visual encounter surveys involve systematically searching an area and estimating the number of animals found per person-hour of searching. Capture-recapture analysis is the most labor-intensive of these three methods and was therefore expected to provide the most power to detect declines. However, this is the first study to

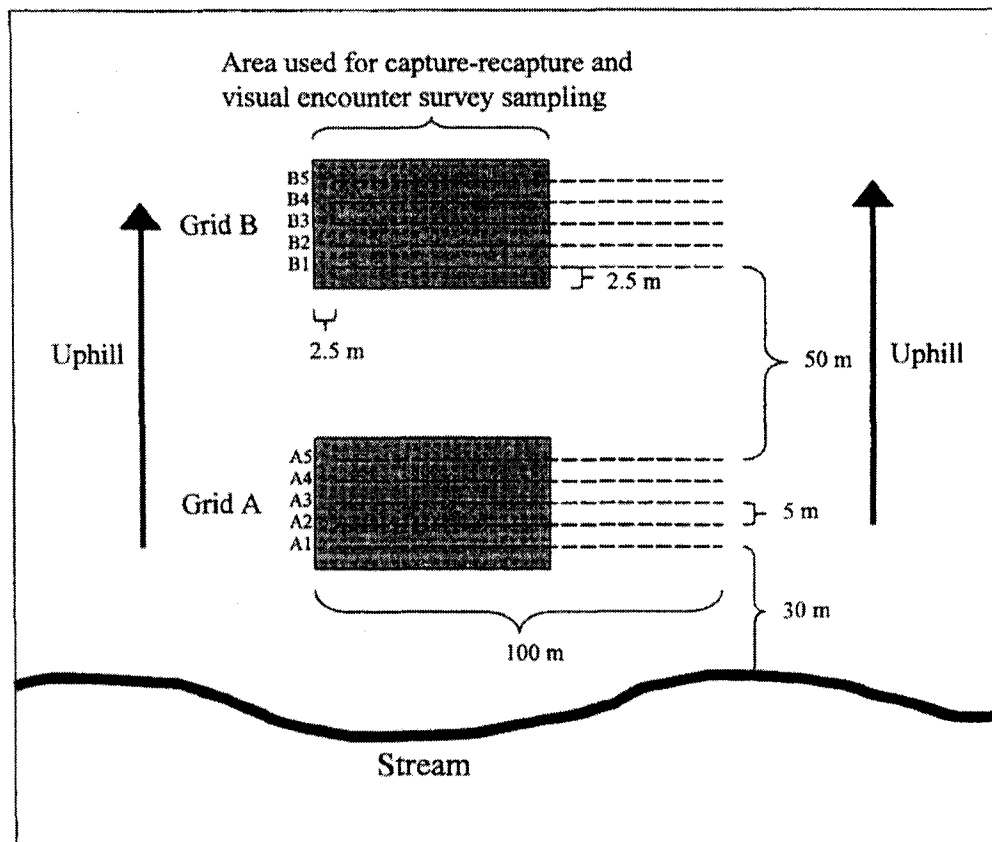


FIG. 1. Grid (A and B) and transect (A1-5 and B1-5) layout used for estimating the density of *Eleutherodactylus* in cloud forest (Cashca Totoras and Yanayacu) and lowland rain forest (Sacha Lodge) using capture-recapture analysis, distance sampling, and visual encounter surveys. The entire length of transects (100 m) was used for distance sampling, but only the area encompassing the first half of transects (0-50 m) was used for capture-recapture and visual encounter survey sampling. Five transects were used in each grid at Cashca Totoras (total transect length (L) = 1000 m), but only four were used in each grid at Yanayacu and Sacha Lodge (L = 800 m). The area used for capture-recapture and visual encounter survey sampling was $55 \text{ m} \times 25 \text{ m} = 1375 \text{ m}^2$ of each grid at Cashca Totoras and $55 \text{ m} \times 20 \text{ m} = 1100 \text{ m}^2$ at Yanayacu and Sacha Lodge.

quantify the power of these methods for tropical frogs to assess whether capture-recapture analysis is sufficiently more powerful than the other two methods to warrant its higher cost.

MATERIALS AND METHODS

Study Sites and Sampling Design.—We estimated the population density of multiple *Eleutherodactylus* species in different forest types at three sites in Ecuador to test the relative performance of closed population capture-recapture analysis, distance sampling, and visual encounter surveys. The three sites we used were the Bosque Protector Cashca Totoras, Yanayacu Biological Station, and Sacha Lodge Biological Station. The Bosque Protector Cashca Totoras is located at approximately 3200 m on the west side of the

Cordillera Occidental of the Andes in Provincia Bolívar at $01^{\circ}43'S$, $78^{\circ}58'W$. The reserve consists of a mixture of pasture and secondary and primary montane cloud forest with a 15–20 m canopy. Yanayacu Biological Station is located at approximately 2100 m on the east side of the Cordillera Oriental of the Andes in Provincia Napo at $00^{\circ}35'S$, $77^{\circ}53'W$. Yanayacu is surrounded by pasture and primary cloud forest with a 20–25 m high canopy. Sacha Lodge Biological Station is located at 250 m in lowland Amazonia in Provincia Sucumbíos at $00^{\circ}26'S$, $76^{\circ}27'W$. The forest at Sacha Lodge is a mixture of secondary and primary lowland rain forest and has a 25–30 m canopy.

At each site, we set up two grids in forest (Fig. 1). Grids were at least 100 m from forest edge

at Yanayacu and Sacha Lodge and 50 m from edge at Cashca Totoras. Each grid consisted of 100 m parallel transects spaced 5 m apart. At Cashca Totoras, each grid had five transects and at Yanayacu and Sacha Lodge each grid had four. We set up the first grid (designated grid A) approximately 30 m uphill from a stream and the second grid (designated grid B) 50 m further uphill of grid A to allow us to test for differences in density at different distances from streams. We used distance sampling along the entire length of transects. For capture-recapture and visual encounter surveys, we only sampled the area encompassing the first 50 m of transects because capture-recapture sampling took more time than distance sampling. The area sampled for capture-recapture analysis and visual encounter surveys was $55 \times 25 \text{ m} = 1375 \text{ m}^2$ of each grid at Cashca Totoras and $55 \times 20 \text{ m} = 1100 \text{ m}^2$ of each grid at Yanayacu and Sacha Lodge.

At each site, we sampled grids using all three methods for six to seven consecutive nights. Grids were searched at night because most *Eleutherodactylus* are nocturnal. We first sampled grids using capture-recapture and visual encounter surveys for five nights at Cashca Totoras and six nights at Yanayacu and Sacha Lodge. Each night was considered one sampling occasion. After capture-recapture and visual encounter surveys, we sampled grids for one night using distance sampling. We started sampling at nightfall (1900–2000 h) and continued until we finished which took approximately 2–7 h depending on the number of frogs that were found and processed. After estimating the density of *Eleutherodactylus* species using these three methods, we compared each method in terms of bias and precision. We considered a method to be "good" if it gave unbiased, precise estimates of density and "poor" if it gave biased and/or imprecise estimates.

A potential problem with using capture-recapture analysis, distance sampling, and visual encounter surveys to estimate frog density on the same grid is that frogs may hop away, hide, and/or become more difficult to catch over time because of the added handling time required for capture-recapture sampling, thereby biasing estimates low. To assess whether this was a problem in our study, we tested whether the number of *Eleutherodactylus* caught per person-hour decreased over time within nights and/or across nights. In the first analysis, we divided each night into two equal time periods, calculated the mean number of *Eleutherodactylus* caught during the first half of the night and the second half of the night for the entire sampling period at each site, and tested whether there was a significant decrease in the mean number of *Eleutherodactylus*

caught in the second half of nights at each site. In the second analysis, we tested whether there was a significant decrease in the number of *Eleutherodactylus* caught per person-hour over nights at each site. In neither analysis did we find a reduction in the number of frogs caught per person-hour over time at any of our three sites. Therefore, the added time of handling frogs during capture-recapture sampling does not appear to bias estimates low.

Voucher specimens of each species sampled were stored at the Museo de Zoología of the Pontificia Universidad Católica del Ecuador in Quito, Ecuador.

Closed Population Capture-Recapture Analysis.—Grids were searched by walking along transects and searching the entire area within 2.5 m of transect centerlines, moving off of transects when this area was not visible from centerlines. Because transects were separated by 5 m, this method assured that the entire area within grids was searched. When frogs were found, we captured them by hand, recorded their locations, and marked animals larger than or equal to 10 mm snout-vent length. Frogs were marked by clipping 3–5 toes in unique combinations similar to those used by Waichman (1992) except that we did not clip thumbs (Finger I) from the forefeet or the longest digits (Toe IV) from the hind feet. We sterilized cut toes with Bactine® and released frogs where they were found. If a frog had already been marked, we recorded its code and location and released it where it was caught.

We used our capture-recapture data to estimate the abundance and density of the four *Eleutherodactylus* species with the largest sample sizes and numbers of recaptures. One species was from Cashca Totoras, one was from Yanayacu, and two were from Sacha Lodge. Initially, we analyzed our capture-recapture data using program MARK (White and Burnham, 1999) rather than the Lincoln-Petersen estimator (Lincoln, 1930) or program CAPTURE (White et al., 1982) for a number of reasons. First, the Lincoln-Petersen estimator requires an assumption which program MARK and program CAPTURE do not. Although all three methods assume that populations are closed (no births, deaths, immigration, or emigration) during capture sessions and that marks are not lost, the Lincoln-Petersen estimator also assumes that all animals have the same probability of being caught during sampling occasions (Thompson et al., 1998). Second, MARK allows the development of more user-defined models than program CAPTURE (White and Burnham, 1999), including models with group covariates, which permits testing alternative hypotheses for differences among groups such as sex or habitat type. Finally, pro-

gram MARK has more advanced model selection features than CAPTURE. Specifically, program MARK uses Akaike's Information Criterion values adjusted for sample size (AICc; Akaike, 1973) to identify the best models in terms of parsimony and fit to the data.

However, for all species analyzed, the best models selected by program MARK gave estimates of abundance and standard error that were very different from each other despite the fact that the models had similar AICc values and therefore similar levels of support. This suggested that some of the abundance estimates and standard error estimates were poor in that the abundance estimates were biased low or high, and the standard error estimates were either unreasonably large or unrealistically small. The likely reason for the poor estimates is that program MARK has little power to select the best model(s) with small sample sizes, such as we had, as has been demonstrated for program CAPTURE (Menkens and Anderson, 1988).

As a result of the poor estimates obtained using program MARK, we decided to use Chapman's unbiased version of the Lincoln-Petersen estimator to estimate abundance and its associated variance (Seber, 1982). Chapman's estimator has been shown to perform well with small sample sizes except when there is extreme individual heterogeneity in capture probabilities and/or extreme behavioral responses in capture probabilities (Menkens and Anderson, 1988). Potential sources of individual heterogeneity in capture probabilities for frogs are heterogeneity among males and females and/or among adults and juveniles. Males may have higher capture probabilities than females because they advertise their locations with calls. Likewise, adults may have higher capture probabilities than juveniles because adults are larger and potentially easier to see. Moreover, there could be a behavioral response in frogs if frogs become more wary and more difficult to catch over time (termed "trap shy" in small mammal trap studies) or if researchers become better at locating and/or capturing animals through time (termed "trap happy" in trap studies).

As recommended by Menkens and Anderson (1988), we tested for evidence of individual heterogeneity and/or behavioral responses in capture probabilities using chi-square tests in program CAPTURE and found no evidence for heterogeneity or behavioral responses for any of the *Eleutherodactylus* species we analyzed. Lack of evidence for heterogeneity and/or behavioral responses may be caused by low power of the chi-square tests, but it does suggest that any existing heterogeneity or behavioral responses or both were not extreme. Therefore, we proceeded to estimate abundance with Chapman's esti-

mator. At each site, the first half of the capture-recapture sampling period was designated as the capture and marking period (three days at all sites) and the second half was designated as the recapture period (two days at Cashca Totoras and three days at Yanayacu and Sacha Lodge).

To convert our estimates of abundance into estimates of density, we calculated the effective capture area for each species analyzed using the mean maximum distance moved procedure as described by Wilson and Anderson (1985). We also used the procedures they described for estimating the variance associated with density estimates.

Distance Sampling.—Prior to sampling, we laid out nylon string along the centerlines of transects to facilitate accurate measurement of distances of frogs from centerlines. During sampling, we walked along transect centerlines and searched on both sides of transects. In contrast to capture-recapture and visual encounter surveys, we remained on centerlines while searching for frogs during distance sampling. Because we rarely observed frogs at a distance of greater than 2 m from centerlines, the probability of observing the same frog twice from different transects was minimal. When a frog was observed, we caught the frog to identify it and then used a metal tape measure to measure the distance of the frog from the centerline to the nearest centimeter. Measurements were likely accurate because frogs did not move away from their original positions when approached. Only frogs equal to or larger than 10 mm snout-vent length were included in the distance sampling analysis.

We used program DISTANCE version 3.5 (Buckland et al., 1993) to analyze our distance data for the two *Eleutherodactylus* species that had total sample sizes of at least 30 (*Eleutherodactylus simonbolivari* from Cascha Totoras and *Eleutherodactylus* sp. 3 from Yanayacu). Program DISTANCE fits distance data to various detection functions and evaluates the detection functions using Akaike's Information Criterion (AIC). The detection function with the lowest AIC value is considered the best function based on the criteria of parsimony and fit to the data (Akaike, 1973). This function is then used to estimate density. We fit our data to all nine of the detection functions available in program DISTANCE, each of which is defined by a key function (uniform, half-normal, or hazard-rate) and series expansion (cosine, simple polynomial, or hermite polynomial). Prior to analyzing our data, we examined them using histograms to make sure that there was no heaping of observations at zero (defined as the disproportionate accumulation of observations near zero distance

from the centerline) and to identify outlying observations because both heaping and outliers can result in poor density estimates (Buckland et al., 1993). We did not find heaping for either of the species we analyzed. We removed observations identified as outliers (Buckland et al., 1993) by truncating the distance data at 1.6 m (four of 54 observations) for *E. simonbolivari* and 1.8 m (four of 35 observations) for *Eleutherodactylus* sp. 3.

Distance sampling requires three main assumptions (Buckland et al., 1993). The first assumption is that objects on the transect are detected with certainty so that the probability of detection on the centerline ($g(0)$) is one. When $g(0)$ is less than one, density estimates (\hat{D}) are biased low by the factor $g(0)$. The second assumption is that objects are detected at their initial location. The third assumption is that measurements are exact. We were confident that the last two assumptions were met for our analysis, but were skeptical that the first assumption was met because some frogs may not be active every night, and others may simply be overlooked. We therefore estimated $g(0)$ for *E. simonbolivari* from the ratio of \hat{D} obtained from distance sampling to \hat{D} obtained from capture-recapture analysis under the assumption that \hat{D} obtained from capture-recapture analysis was an unbiased estimate. We only used data from the first 50 m of distance sampling transects for estimating $g(0)$ because this is the portion of grids that were sampled using capture-recapture (Fig. 1). We used the method described by Mood et al. (1974) to estimate the variance associated with this ratio, assuming that covariance among the two estimates was zero.

Visual Encounter Surveys.—We conducted visual encounter surveys concurrently with capture-recapture sampling. However, the only data we collected for visual encounter surveys was the number of each *Eleutherodactylus* species equal to or larger than 10 mm snout-vent length found during sampling occasions and the time spent searching. These data were then used to calculate an index: the number of frogs seen per person-hour for each sampling occasion (each night). The main assumptions of visual encounter surveys are that (1) all individuals of all species have the same probability of being observed; (2) the probability of being observed is constant over time and space; and (3) there are no differences in the ability of observers to detect animals (Heyer et al., 1994). If all of these assumptions hold, then the functional relationship between the index and density will be constant, and the index can theoretically be used as a surrogate for direct estimates of density.

We used linear regression analysis in program SPSS version 7.0 to test whether there was

a significant positive linear relationship between \hat{D} obtained from capture-recapture analysis and the mean number of frogs caught per person-hour per night (\hat{I}) averaged over five consecutive nights of sampling. We then used the linear regression model relating these two variables to predict \hat{D} and its associated variance from \hat{I} after one, three, or five nights of sampling.

Power Analysis.—We conducted a power analysis using program TRENDS (Gerrodette, 1993) to estimate the power to detect declines in density using capture-recapture, distance sampling, and visual encounter survey estimates of *Eleutherodactylus* density. Power is defined as the probability of detecting an actual decline ($1 - \beta$) where β is the probability of concluding no decline when a decline actually exists (a Type II error). We estimated the power to detect a major decline (20% exponential decline per year) and a less severe, but still substantial, decline (10% exponential decline per year) after five or 10 years of annual sampling given the coefficients of variation obtained from capture-recapture analysis, distance sampling, and visual encounter surveys. We were particularly interested in estimating the power to detect major declines over a short time interval given that many declines of tropical amphibian populations have occurred rapidly (Lips, 1999; Young et al., 2001). We set $\alpha = 0.05$ and used a directional test with 20% of alpha allocated for detecting a positive trend (Rice and Gaines, 1994). Estimates of power obtained from program TRENDS are maximum estimates because program TRENDS does not consider temporal or spatial process variation, which will decrease power to detect population trends (Thompson et al., 1998).

RESULTS

Closed Population Capture-Recapture Analysis.—Sample sizes and numbers of frogs recaptured at least twice were small for all of the *Eleutherodactylus* species encountered during capture-recapture sessions (Table 1). We only analyzed capture-recapture data for *E. simonbolivari* from Cashca Totoras, *Eleutherodactylus eriphus* from Yanayacu, and *Eleutherodactylus lanthanites* and *Eleutherodactylus martiae* from Sacha Lodge because numbers of recaptures and/or sample sizes were very small for the other species.

Capture-recapture estimates of density varied substantially among the four species analyzed, but coefficients of variation were not as variable (Fig. 2). *Eleutherodactylus simonbolivari* had the highest density with $\hat{D} \pm S\hat{E}(\hat{D}) = 564 \pm 112$ frogs/ha. The other three species, *E. eriphus*, *E. lanthanites*, and *E. martiae* had much lower densities of $\hat{D} = 154 \pm 42$ frogs/ha, $\hat{D} = 129 \pm 27$ frogs/ha, and $\hat{D} = 99 \pm 30$ frogs/ha, respec-

TABLE 1. *Eleutherodactylus* species sampled during capture-recapture sessions at three sites in Ecuador. A = total area (m^2) sampled at each site, M_{t-1} = number of individuals caught, recaptures = number of individuals caught on at least two different nights.

Site	A	Species	Grid	M_{t-1}	Recaptures		
Cashca Totoras	2750	<i>E. phoxocephalus</i>	A	2	0		
			B	0	0		
			Total	2	0		
		<i>E. simonbolivari</i>	A	53	13		
			B	39	7		
			Total	92	20		
		<i>E. truebae</i>	A	3	1		
			B	0	0		
			Total	3	1		
Yanayacu	2200	<i>E. eriphus</i>	A	8	1		
			B	14	7		
			Total	22	8		
		<i>Eleutherodactylus</i> sp. 2	A	16	1		
			B	11	1		
			Total	27	2		
		<i>Eleutherodactylus</i> sp. 3	A	29	1		
			B	8	0		
			Total	37	1		
		Sacha Lodge	2200	<i>E. altamazonicus</i>	A	5	1
					B	0	0
					Total	5	1
<i>E. lanthanites</i>	A			12	2		
	B			15	8		
	Total			27	10		
<i>E. martiae</i>	A			12	5		
	B			13	2		
	Total			25	7		
<i>E. ockendeni</i>	A			4	4		
	B			3	1		
	Total			7	5		
<i>E. variabilis</i>	A			2	1		
	B			2	0		
	Total			4	1		

tively. The corresponding coefficients of variation for *E. simonbolivari*, *E. eriphus*, *E. lanthanites*, and *E. martiae* were $CV(\hat{D}) = 0.20, 0.27, 0.21$, and 0.31 , respectively. There were no differences in density among grids A and B for any species that could not be attributed to sampling error.

Capture probabilities during the capture and marking session (the first three nights of sampling at all sites) and recapture session (the last two nights of sampling at Cascha Totoras and last three nights at the other two sites) were fairly low for the species analyzed. Estimated capture probabilities for the capture and marking session (\hat{p}_c) were $0.28, 0.29, 0.41$, and 0.34 for *E. simonbolivari*, *E. eriphus*, *E. lanthanites*, and *E. martiae*, respectively. Estimated capture probabilities for the recapture session (\hat{p}_r) were $0.24, 0.40, 0.46$, and 0.39 for *E. simonbolivari*, *E. eriphus*, *E. lanthanites*, and *E. martiae*, respectively.

Distance Sampling.—Sample sizes from distance sampling were also small (Table 2). We only analyzed distance sampling data for *E. simonbolivari* from Cashca Totoras and *Eleuthero-*

dactylus sp. 3 from Yanayacu because sample sizes were very small for the other species.

Unadjusted ($g(0) = 1$) distance sampling estimates of density for *E. simonbolivari* and *Eleutherodactylus* sp. 3 were substantially different from each other, but their coefficients of variation were the same (Fig. 2). *Eleutherodactylus simonbolivari* had a higher density with $\hat{D} = 260 \pm 49$ frogs/ha compared to $\hat{D} = 162 \pm 31$ frogs/ha for *Eleutherodactylus* sp. 3. The coefficient of variation was $CV(\hat{D}) = 0.19$ for both species.

The density estimate obtained for *E. simonbolivari* from distance sampling was much lower than the estimate obtained from capture-recapture analysis (Fig. 2), suggesting that the assumption that $g(0) = 1$ was violated and that $g(0) < 1$. Based on the ratio of distance sampling and capture-recapture estimates of density for *E. simonbolivari*, $\hat{g} = 0.35 \pm 0.12$ [$CV[\hat{g}(0)] = 0.34$]. When we adjusted density estimates for *E. simonbolivari* and *Eleutherodactylus* sp. 3 using this estimate of $g(0)$, the density estimates and

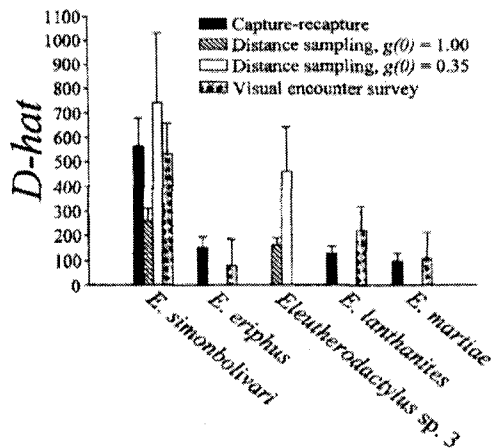


FIG. 2. Density estimates (\hat{D}) for *Eleutherodactylus* obtained from capture-recapture analysis, distance sampling, and visual encounter surveys. Density is expressed as individuals/hectare and error bars represent standard error estimates associated with each density estimate ($SE[\hat{D}]$). Distance sampling estimates are shown with the probability of detection on transect centerlines ($g(0)$) not adjusted ($g(0) = 1.00$; $SE[g(0)] = 0.00$) and adjusted ($g(0) = 0.35$; $SE[g(0)] = 0.12$) for incomplete detection. Visual encounter survey estimates were derived from the mean number of frogs caught per person-hour per night (\bar{l}) averaged over five consecutive nights of sampling using the regression equation $\hat{D} = 298 \cdot \bar{l} - 125$.

their associated variance increased substantially for both species, as expected (Fig. 2). With the adjusted $g(0)$, $\hat{D} = 742 \pm 289$ frogs/ha ($CV[\hat{D}] = 0.39$) for *E. simonbolivari* and $\hat{D} = 461 \pm 180$ frogs/hectare ($CV[\hat{D}] = 0.39$) for *Eleutherodactylus* sp. 3.

Visual Encounter Surveys.—The linear regression model relating the mean number of frogs caught per person-hour per night (\bar{l}) averaged over five nights of sampling to \hat{D} estimated from capture-recapture analysis ($\hat{D} = 298 \cdot \bar{l} - 125$) was marginally significant ($F = 16.841$, $r = 0.945$, $P = 0.055$). However, the regression model was very poor at predicting \hat{D} from \bar{l} , giving density estimates that tended to be very imprecise (Fig. 2). The regression model gave particularly poor estimates of density when \bar{l} was low, even predicting negative densities for *E. eriphus* and *E. martiae* after one night of sampling. All of the 95% prediction intervals for \hat{D} included zero except for the prediction interval for *E. simonbolivari* after five nights of sampling, indicating that in most cases, estimates were indistinguishable from zero. Coefficients of variation for density estimates were also extremely large, ranging from 0.23–1.99 and were negative in two cases based on the negative density esti-

mates for *E. eriphus* and *E. martiae* after one night of sampling.

Power Analysis.—The predicted power to detect declines in density for the *Eleutherodactylus* species analyzed depended strongly on the method used to estimate density, the number of years of sampling, the rate of decline, and the coefficient of variation of density estimates (Fig. 3). As expected, power was higher to detect a 20% decline per year (Fig. 3A–B) than a 10% decline (Fig. 3C–D) and was higher after 10 years of annual sampling (Fig. 3B, D) than after five years (Fig. 3A, C). Moreover, power tended to be highest with capture-recapture estimates of density because of the relatively small coefficients of variation associated with these estimates. In particular, capture-recapture analysis had the highest power to detect a 20% decline per year after 5 years of sampling for all species analyzed (Fig. 3A). Under this scenario, power ranged from 0.35–0.61 for capture-recapture analysis and 0.10–0.52 for visual encounter surveys and was 0.27 for both species analyzed using distance sampling.

DISCUSSION

Density Estimation.—Distance sampling and visual encounter survey estimates of *Eleutherodactylus* density were imprecise and biased relative to capture-recapture estimates, pointing to capture-recapture analysis as the best method for estimating *Eleutherodactylus* density. The main problem with distance sampling estimates was that they were biased very low, most likely caused by violation of the assumption of complete detection on transect centerlines. When we corrected this bias using an estimate of the probability of detection on transect centerlines, the bias was removed, but variance was greatly increased (Fig. 2) giving coefficients of variation of approximately 0.39.

Visual encounter survey estimates of density were also imprecise (Fig. 2). In some cases, imprecision reached astronomical levels, yielding coefficients of variation greater than one. The imprecision of visual encounter survey estimates stem from problems associated with the regression model used to predict density (\hat{D}) from the index (\bar{l}). These problems include small sample size (only four datapoints), among species variation in the functional relationship between \bar{l} and \hat{D} , sampling error of \bar{l} and \hat{D} , and a nonzero y-intercept. It may be possible to partially improve the regression model by increasing sample size, developing different regression models for each species and obtaining more precise estimates of the index and density. However, this would likely require at least as much work as directly estimating density using cap-

TABLE 2. *Eleutherodactylus* species sampled during distance sampling sessions at three sites in Ecuador. L = total length (m) of transects, N = sample size.

Site	L	Species	Grid	N
Cashca Totoras	1000	<i>E. simonbolivari</i>	A	26
			B	28
			Total	54
		<i>E. truebae</i>	A	0
			B	1
			Total	1
Yanayacu	800	<i>E. eriphus</i>	A	2
			B	10
			Total	12
		<i>Eleutherodactylus</i> sp. 1	A	1
			B	0
			Total	1
		<i>Eleutherodactylus</i> sp. 2	A	2
			B	4
			Total	6
		<i>Eleutherodactylus</i> sp. 3	A	23
			B	12
			Total	35
Sacha Lodge	800	<i>E. lanthanites</i>	A	4
			B	8
			Total	12
		<i>E. martiae</i>	A	10
			B	1
			Total	100
		<i>E. ockendeni</i>	A	2
			B	3
			Total	5
		<i>E. variabilis</i>	A	1
			B	2
			Total	3

ture-recapture analysis, defeating the one advantage of indices: lower effort and cost.

The conclusion that capture-recapture analysis gives better estimates of density than distance sampling or visual encounter surveys rests upon the assumption that our capture-recapture estimates were not biased. We believe that this is a good assumption given that the capture-recapture estimator we used, Chapman's estimator, has been shown to give unbiased estimates, even with small sample sizes, as long as there is not extreme heterogeneity or extreme behavioral responses or both in capture probabilities (Menkens and Anderson, 1988). We did not find evidence of heterogeneity or behavioral responses, so we concluded that our estimates should not be biased.

We recommend designing sampling grids so that sample sizes are large enough to allow analysis of capture-recapture data with programs MARK and CAPTURE. These programs are more flexible than Chapman's estimator because they provide estimators of abundance when capture probabilities vary among individuals within sampling occasions. The minimum sample size necessary for model selection pro-

cedures in both programs to function properly depends on capture probabilities. With low capture probabilities, as observed in this study, White et al. (1982) recommend sample sizes of 200 or more animals.

One way of increasing sample sizes is to increase the size of the area sampled. For example, we found 92 *E. simonbolivari* on both of our grids, which together encompassed an area of 2750 m² (Table 1). To increase the sample size to 200 individuals, the area sampled would need to be increased 2.17-fold (= 200 individuals desired/92 individuals observed) to approximately 6000 m² (= 2.17 × 2750 m² = 5968 m²). Alternatively, sample size could be increased by increasing the number of sampling occasions or a combination of increasing sampling area and sampling occasions. However, the number of sampling occasions should not be increased beyond 1–2 weeks, because after this time frame, frogs may begin to emigrate out of grids or immigrate into grids, which will violate the assumption of closure necessary for closed population capture-recapture analysis.

Power Analysis.—The power to detect declines in *Eleutherodactylus* density was generally high-

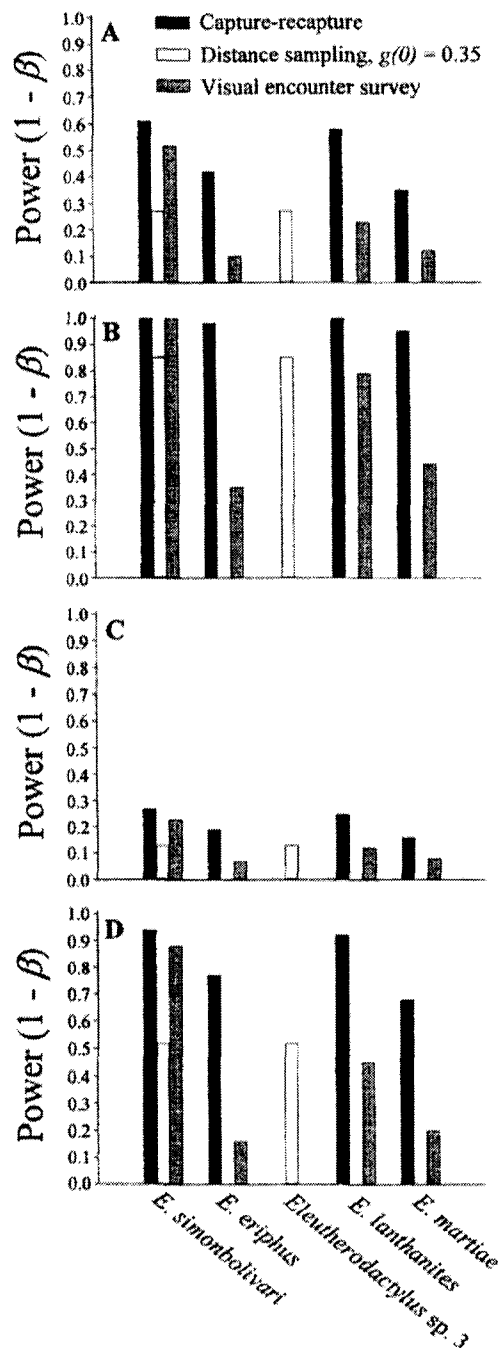


FIG. 3. Predicted power ($1 - \beta$) to detect exponential declines in the density of *Eleutherodactylus* using capture-recapture analysis, distance sampling corrected for incomplete detection on transect centerlines [$g(0) = 0.35$; $SE[g(0)] = 0.12$], or visual encounter surveys. (A) 20% decline per year, five years of annual

est with capture-recapture estimates because these estimates had the smallest coefficients of variation (Fig. 3). In particular, capture-recapture analysis had much more power to detect a rapid decline of 20% per year after five years of annual sampling than did distance sampling or visual encounter surveys (Fig. 3A). An exponential decline of 20% per year translates into a 67% decline after five years (calculated from $100\% \times [1 - (1 - 0.20)^5]$) and is similar in magnitude to the rapid declines observed in many tropical frogs (Lips, 1999; Young et al., 2001). A decline of this magnitude is certainly of conservation concern and monitoring programs should be designed so that they have high power to detect such declines. Because distance sampling and visual encounter surveys will generally not have the power to detect these declines, capture-recapture analysis will usually be the most appropriate method for monitoring *Eleutherodactylus* density.

Nonetheless, even with capture-recapture sampling, power was low to detect an annual decline of 20% in *Eleutherodactylus* density after five years of sampling with the current sampling design (Fig. 3A). Moreover, our estimates of power are maximum estimates because we did not account for temporal or spatial process variation in density. When process variation is included, CVs will increase (Thompson et al., 1998), thereby reducing power, so our power estimates are optimistic. Therefore, we emphasize once again that it is important to increase sample sizes by increasing grid size, the number of nights of sampling, or both as previously described to increase the precision of density estimates and the power to detect declines.

Recommendations for Monitoring *Eleutherodactylus* Frogs.—For the *Eleutherodactylus* species analyzed in this study, we recommend monitoring density using capture-recapture analysis with larger sample sizes. Capture-recapture estimates of density were more precise than estimates generated from distance sampling or visual encounter surveys (Fig. 2), which allowed greater power to detect declines using capture-recapture (Fig. 3). In particular, capture-recapture analysis had the most power to quickly detect rapid declines. Because many declines of tropical amphibians have been rapid, we feel that all amphibian monitoring programs in the tropics should be designed so that they have a high probability of detecting these declines. For

←

sampling; (B) 20% decline per year, 10 years of annual sampling; (C) 10% decline per year, five years of annual sampling; (D) 10% decline per year, 10 years of annual sampling.

TABLE 3. Advantages and disadvantages of capture-recapture, distance sampling, and visual encounter surveys for monitoring the density of *Eleutherodactylus* species.

Method	Advantages	Disadvantages
Capture-recapture	<ol style="list-style-type: none"> 1. More precise and less biased estimates 2. Higher power to detect declines, particularly to quickly detect rapid declines 3. Allows estimation of other parameters such as survival probability if study correctly designed 	<ol style="list-style-type: none"> 1. More labor intensive, which may require reducing the number of species and/or sites monitored
Distance sampling	<ol style="list-style-type: none"> 1. Less labor intensive 	<ol style="list-style-type: none"> 1. Biased low 2. Correcting bias causes estimates to become imprecise 3. Lower power so that may not detect rapid declines
Visual encounter surveys	<ol style="list-style-type: none"> 1. Less labor intensive 	<ol style="list-style-type: none"> 1. Imprecise 2. Lower power so that may not detect rapid declines

all of the species that we analyzed except for *Eleutherodactylus* sp. 3 from Yanayacu, capture-recapture analysis is the best method for detecting rapid declines. In the case of *Eleutherodactylus* sp. 3, there were not enough recaptures to permit capture-recapture analysis (Table 1). Therefore, either distance sampling or visual encounter surveys need to be used to monitor the density of *Eleutherodactylus* sp. 3.

We would also generally recommend using capture-recapture analysis for monitoring the density of other species of *Eleutherodactylus* not analyzed here because of the much higher precision and greater power of this method (Table 3). In addition, capture-recapture analysis can be used to estimate other parameters such as survival probability when studies are designed appropriately (Cormack, 1964; Jolly, 1965; Seber, 1965; Pollock et al., 1990; W. C. Funk and L. S. Mills, unpubl. data). Survival estimates and other vital rate (birth and death rate) estimates are useful because they can be used to conduct ecological sensitivity analyses to help identify likely causes of declines and develop management strategies for preventing and reversing declines (Caswell, 2001; Biek et al., 2002). Although capture-recapture studies are more labor intensive than distance sampling or visual encounter surveys, which may limit the number of species or sites included in a monitoring program (Table 3), we believe that it is much more valuable to have high power to detect declines of one or a few species at fewer sites than it is to have low power to detect declines of many species at many sites. However, for some species such as *Eleutherodactylus* sp. 3 from Yanayacu, it may not be possible to use capture-recapture to estimate density. Therefore, we also strongly recommend

conducting pilot studies prior to implementing long-term monitoring programs to determine which method or combination of methods yield the highest power to detect declines for each species.

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APPENDIX 1. Akaike information criterion values (AIC_c), AIC_c differences (ΔAIC_c), AIC_c weight, and number of parameters in models used to examine annual (i) and population (r) variation in survival (S) and capture (p) probabilities of juvenile (j) and adult (a) Columbia spotted frogs in Keeler Creek, Montana, from 2000 to 2003. Movement probabilities among lower and upper populations are year- and population-specific in all models.

Model	AIC_c	ΔAIC_c	AIC_c weight	K
$S_{ji}S_{ai}P_j^r P_a^r$	1788.68	0.00	0.33	35
$S_{ji}S_{ai}P_j P_a^r$	1788.70	0.02	0.32	34
$S_j S_{ai} P_{ji}^r P_{ai}^r$	1790.27	1.59	0.15	40
$S_j S_{ai} P_j P_a^r$	1791.73	3.05	0.07	32
$S_j S_a^r P_{ji}^r P_{ai}^r$	1792.57	3.89	0.05	40
$S_{ji}^r S_{ai}^r P_j P_a^r$	1792.81	4.13	0.04	40
$S_j^r S_a P_{ji}^r P_{ai}^r$	1794.17	5.49	0.02	40
$S_j^r S_a^r P_{ji}^r P_{ai}^r$	1794.99	6.31	0.01	41
$S_j S_a^r P_j P_{ai}$	1796.59	7.91	0.01	34
$S_j^r S_a^r P_j P_{ai}$	1798.40	9.72	0.00	35
$S_{ji}S_a P_{ji}^r P_{ai}^r$	1799.25	10.57	0.00	40
$S_{ji}^r S_{ai}^r P_j P_{ai}$	1800.69	12.01	0.00	41

$S_j S_a P_{ji}^r P_{ai}^r$	1801.92	13.24	0.00	39
$S_{ji}^r S_{ai}^r P_j^r P_a^r$	1803.65	14.97	0.00	41
$S_{ji}^r S_{ai}^r P_j^r P_a$	1803.75	15.07	0.00	40
$S_j^r S_a^r P_{ji} P_{ai}$	1803.91	15.23	0.00	33
$S_j S_a P_j^r P_a^r$	1806.13	17.45	0.00	31
$S_{ji} S_a P_j P_a^r$	1806.47	17.79	0.00	32
$S_{ji} S_a P_j^r P_a^r$	1806.90	18.22	0.00	33
$S_j^r S_a P_j^r P_a^r$	1808.16	19.47	0.00	32
$S_j S_a^r P_j^r P_a^r$	1808.71	20.03	0.00	32
$S_j^r S_a^r P_j^r P_a^r$	1809.89	21.21	0.00	33
$S_j S_a P_j P_a^r$	1810.61	21.93	0.00	30
$S_j S_a^r P_{ji} P_a$	1812.27	23.59	0.00	32
$S_j^r S_a^r P_j P_a^r$	1813.65	24.97	0.00	32
$S_j S_a^r P_{ji} P_a$	1815.91	27.23	0.00	30
$S_{ji}^r S_{ai}^r P_{ji} P_a$	1816.50	27.82	0.00	40
$S_j^r S_a^r P_{ji} P_a$	1817.89	29.21	0.00	31
$S_j S_a^r P_j^r P_a$	1817.95	29.27	0.00	31
$S_{ji} S_{ai} P_j^r P_a$	1818.10	29.42	0.00	34
$S_{ji} S_{ai} P_{ji} P_a$	1819.42	30.74	0.00	35
$S_j S_{ai} P_{ji} P_{ai}$	1819.44	30.76	0.00	35

$S_{ji}S_{ai}P_{ji}P_a$	1819.81	31.13	0.00	33
$S_{ji}S_aP_{ji}P_{ai}$	1820.28	31.60	0.00	33
$S'_j S'_a P'_j P_a$	1820.73	32.05	0.00	32
$S_j S_{ai}P_{ji}P_a$	1820.87	32.19	0.00	31
$S_{ji}S_{ai}P_{ji}P_{ai}$	1821.11	32.43	0.00	35
$S_{ji}S_aP_{ji}P_{ai}$	1822.19	33.51	0.00	35
$S_j S_a P_{ji} P_{ai}$	1823.79	35.11	0.00	31
$S'_j S_a P_j P'_a$	1825.15	36.47	0.00	31
$S'_{ji} S'_{ai} P_{ji} P_{ai}$	1826.54	37.86	0.00	40
$S_j S'_a P_j P'_a$	1827.86	39.18	0.00	31
$S_j S_a P_{ji} P_a$	1829.95	41.27	0.00	31
$S_{ji}S_a P_{ji} P_a$	1831.10	42.42	0.00	31
$S_j S_a P'_j P_a$	1831.51	42.83	0.00	30
$S'_j S_a P_{ji} P_a$	1831.88	43.20	0.00	32
$S_{ji}S_a P_{ji} P_a$	1833.36	44.68	0.00	33
$S_{ji}S_{ai}P_{ji}P_{ai}$	1834.80	46.12	0.00	37
$S_j S_a P_{ji} P_a$	1835.29	46.61	0.00	29
$S'_j S_a P_{ji} P_a$	1837.11	48.43	0.00	30
$S'_j S_a P_{ji} P_{ai}$	1868.09	79.41	0.00	32

APPENDIX 2. Akaike information criterion values (AIC_c), AIC_c differences (ΔAIC_c), AIC_c weight, and number of parameters in models used to examine annual (i) and population (rs) variation in movement (Ψ) probabilities of juvenile (j) and adult (a) Columbia spotted frogs in Keeler Creek, Montana, from 2000 to 2003. Survival probability is year-specific for juveniles (S_{ji}) and adults (S_{ai}) and capture probability is population-specific for juveniles (p_j^r) and adults (p_a^r) in all models.

Model	AIC_c	ΔAIC_c	AIC_c weight	K
$\Psi_{ji} \Psi_a$	1764.89	0.00	0.61	21
$\Psi_{ji} \Psi_a^{rs}$	1766.93	2.03	0.22	22
$\Psi_{ji} \Psi_{ai}$	1768.96	4.07	0.08	23
$\Psi_j \Psi_a$	1770.08	5.19	0.05	15
$\Psi_j \Psi_a^{rs}$	1772.10	7.21	0.02	16
$\Psi_j^{rs} \Psi_a$	1773.82	8.93	0.01	18
$\Psi_j \Psi_{ai}$	1774.13	9.23	0.01	17
$\Psi_{ji} \Psi_{ai}^{rs}$	1775.07	10.18	0.00	26
$\Psi_j^{rs} \Psi_a^{rs}$	1775.85	10.96	0.00	19
$\Psi_j^{rs} \Psi_{ai}$	1777.88	12.99	0.00	20
$\Psi_j \Psi_{ai}^{rs}$	1780.21	15.32	0.00	20
$\Psi_{ji}^{rs} \Psi_{ai}$	1782.53	17.63	0.00	32

$\Psi_j^{rs} \Psi_{ai}^{rs}$	1783.98	19.09	0.00	23
$\Psi_{ji}^{rs} \Psi_{ai}^{rs}$	1788.68	23.79	0.00	35
$\Psi_{ji}^{rs} \Psi_a^{rs}$	1793.09	28.20	0.00	31

APPENDIX 3. Akaike information criterion values (AIC_c), AIC_c differences (ΔAIC_c), AIC_c weight, and number of parameters in models used to examine annual (i) and population (r) variation in survival (S) and capture (p) probabilities of juvenile (j) and adult (a) Columbia spotted frogs in Marten Creek, Montana, from 2000 to 2003. Movement probabilities among lower and upper populations are year- and population-specific in all models.

Model	AIC_c	ΔAIC_c	AIC_c weight	K
$S_{ji}^r S_{ai}^r p_j p_{ai}$	5397.50	0.00	0.16	40
$S_j^r S_a^r p_{ji}^r p_{ai}^r$	5397.71	0.21	0.15	41
$S_j^r S_a^r p_{ji}^r p_{ai}^r$	5398.08	0.58	0.12	40
$S_j^r S_a^r p_{ji} p_{ai}$	5398.26	0.76	0.11	35
$S_{ji}^r S_{ai}^r p_j p_{ai}$	5398.59	1.10	0.10	41
$S_{ji}^r S_{ai}^r p_j p_a$	5398.73	1.23	0.09	39
$S_j^r S_a^r p_j p_{ai}$	5399.41	1.91	0.06	33
$S_{ji}^r S_{ai}^r p_j^r p_a$	5400.38	2.88	0.04	40
$S_{ji}^r S_{ai}^r p_j p_a^r$	5400.56	3.06	0.04	40
$S_{ji}^r S_{ai}^r p_j p_a$	5400.60	3.10	0.03	40
$S_{ji}^r S_{ai}^r p_j^r p_a^r$	5402.11	4.61	0.02	41
$S_j^r S_a^r p_j p_a$	5402.71	5.21	0.01	31

$S_j S_a P_{ji}^r P_{ai}^r$	5402.75	5.25	0.01	39
$S_j^r S_a^r P_j P_a^r$	5403.58	6.08	0.01	32
$S_j S_a^r P_{ji}^r P_{ai}^r$	5404.04	6.55	0.01	40
$S_j^r S_a^r P_{ji} P_a$	5404.54	7.04	0.00	33
$S_j^r S_a^r P_j^r P_a$	5404.54	7.04	0.00	32
$S_{ji} S_a P_{ji}^r P_{ai}^r$	5404.71	7.21	0.00	40
$S_j S_{ai} P_{ji}^r P_{ai}^r$	5404.76	7.26	0.00	40
$S_j^r S_a^r P_j^r P_a^r$	5405.07	7.58	0.00	33
$S_{ji}^r S_{ai}^r P_{ji}^r P_{ai}^r$	5405.28	7.78	0.00	45
$S_{ji} S_{ai} P_j^r P_a^r$	5405.54	8.05	0.00	35
$S_{ji} S_a P_j^r P_a^r$	5406.06	8.56	0.00	33
$S_j^r S_a P_j P_a^r$	5406.19	8.70	0.00	31
$S_j^r S_a P_j^r P_a^r$	5406.49	9.00	0.00	32
$S_{ji} S_{ai} P_{ji}^r P_{ai}^r$	5406.72	9.22	0.00	41
$S_j S_{ai} P_j^r P_a^r$	5408.40	10.90	0.00	33
$S_j S_a^r P_j^r P_a$	5408.93	11.44	0.00	31
$S_j S_a^r P_j^r P_a^r$	5409.42	11.92	0.00	32
$S_j S_a P_j^r P_a^r$	5409.97	12.47	0.00	31
$S_j^r S_a P_{ji} P_{ai}$	5415.80	18.30	0.00	34

$S_j^r S_a p_j p_{ai}$	5417.66	20.16	0.00	32
$S_{ji} S_{ai} p_j^r p_a$	5421.08	23.58	0.00	34
$S_j S_{ai} p_j^r p_a$	5422.35	24.85	0.00	32
$S_j^r S_a p_j p_a$	5423.11	25.61	0.00	30
$S_j^r S_a p_j^r p_a$	5424.58	27.08	0.00	31
$S_{ji} S_a p_j^r p_a$	5424.99	27.50	0.00	32
$S_j^r S_a p_j p_a$	5425.01	27.52	0.00	32
$S_j S_a p_j^r p_a$	5426.98	29.48	0.00	30
$S_j S_a^r p_j p_{ai}$	5435.98	38.48	0.00	32
$S_j S_a^r p_j p_{ai}$	5439.29	41.79	0.00	34
$S_j S_a^r p_j p_a$	5441.30	43.81	0.00	30
$S_j S_a^r p_j p_a^r$	5442.07	44.57	0.00	31
$S_j S_a^r p_j p_a$	5445.12	47.62	0.00	32
$S_j S_{ai} p_j p_a^r$	5450.60	53.10	0.00	32
$S_j S_a p_j p_{ai}$	5451.25	53.75	0.00	31
$S_j S_a p_j p_a^r$	5452.43	54.93	0.00	30
$S_j S_{ai} p_j p_{ai}$	5454.14	56.64	0.00	33
$S_{ji} S_{ai} p_j p_a^r$	5454.31	56.81	0.00	34
$S_{ji} S_a p_j p_{ai}$	5454.46	56.96	0.00	33
$S_j S_{ai} p_j p_a$	5454.81	57.31	0.00	31

$S_{ji}S_a p_{ji} p_{ai}$	5455.47	57.98	0.00	35
$S_{ji}S_a p_j p_a^r$	5456.04	58.54	0.00	32
$S_j S_{ai} p_{ji} p_{ai}$	5456.65	59.15	0.00	35
$S_{ji} S_{ai} p_{ji} p_{ai}$	5457.47	59.97	0.00	35
$S_{ji} S_{ai} p_{ji} p_a$	5458.69	61.19	0.00	33
$S_j S_{ai} p_{ji} p_a$	5458.77	61.27	0.00	33
$S_j S_a p_j p_a$	5458.80	61.30	0.00	29
$S_{ji} S_{ai} p_{ji} p_{ai}$	5459.29	61.79	0.00	37
$S_{ji} S_{ai} p_{ji} p_a$	5460.94	63.44	0.00	35
$S_{ji} S_a p_j p_a$	5462.61	65.12	0.00	31
$S_j S_a p_{ji} p_a$	5462.68	65.18	0.00	31
$S_{ji} S_a p_{ji} p_a$	5464.65	67.15	0.00	33

APPENDIX 4. Akaike information criterion values (AIC_c), AIC_c differences (ΔAIC_c), AIC_c weight, and number of parameters in models used to examine annual (i) and population (rs) variation in movement (Ψ) probabilities of juvenile (j) and adult (a) Columbia spotted frogs in Marten Creek, Montana, from 2000 to 2003. Survival probability is year- and population-specific for juveniles (S_{ji}^r) and adults (S_{ai}^r) and capture probability is constant for juveniles (p_j) and time-specific for adults (p_{ai}) in all models.

Model	AIC_c	ΔAIC_c	AIC_c weight	K
$\Psi_{ji} \Psi_a$	5386.84	0.00	0.38	26
$\Psi_{ji}^{rs} \Psi_a$	5387.40	0.56	0.29	35
$\Psi_{ji} \Psi_a^{rs}$	5388.86	2.01	0.14	27
$\Psi_{ji}^{rs} \Psi_a^{rs}$	5389.42	2.58	0.10	36
$\Psi_{ji} \Psi_{ai}$	5390.87	4.03	0.05	28
$\Psi_{ji}^{rs} \Psi_{ai}$	5391.44	4.60	0.04	37
$\Psi_{ji} \Psi_{ai}^{rs}$	5396.91	10.07	0.00	31
$\Psi_{ji}^{rs} \Psi_{ai}^{rs}$	5397.50	10.65	0.00	40
$\Psi_j^{rs} \Psi_a$	5398.22	11.37	0.00	24
$\Psi_j^{rs} \Psi_a^{rs}$	5400.23	13.38	0.00	25
$\Psi_j \Psi_a$	5401.34	14.49	0.00	21

$\Psi_j^{rs} \Psi_{ai}$	5402.24	15.40	0.00	26
$\Psi_j \Psi_a^{rs}$	5403.35	16.50	0.00	22
$\Psi_j \Psi_{ai}$	5405.36	18.52	0.00	23
$\Psi_j^{rs} \Psi_{ai}^{rs}$	5408.28	21.44	0.00	29
$\Psi_j \Psi_{ai}^{rs}$	5410.91	24.06	0.00	26

APPENDIX 5. Allele frequencies at *Rana luteiventris* sample sites. Sites correspond to Fig. 3.1 and Table 3.1.

Site	<i>Rp3</i>													
	*187	*191	*195	*199	*203	*207	*211	*215	*219	*223				
1	0.423	-	0.019	-	0.038	0.404	-	0.115	-	-				
2	0.464	-	-	-	0.143	0.250	0.125	0.018	-	-				
3	0.563	-	-	-	0.063	0.281	0.094	-	-	-				
4	0.648	-	-	0.019	0.056	0.167	0.093	0.019	-	-				
5	0.594	-	-	-	0.094	0.125	0.125	0.063	-	-				
6	0.238	-	-	0.024	-	0.429	0.024	0.286	-	-				
7	-	0.020	0.020	0.220	-	0.240	0.280	0.200	0.020	-				
8	-	0.022	0.034	0.174	-	0.239	0.348	0.141	0.022	-				
9	-	-	0.021	0.333	-	0.292	0.146	0.208	-	-				
10	-	-	0.059	0.176	-	0.324	0.176	0.235	0.029	-				
11	-	-	-	0.222	-	0.222	0.111	0.306	0.139	-				
12	-	-	0.037	0.241	-	0.315	0.278	0.074	0.056	-				
13	0.411	-	-	-	0.071	0.071	0.375	0.071	-	-				
14	0.481	-	0.019	-	0.096	0.038	0.346	0.019	-	-				
15	0.375	-	0.018	-	0.232	0.018	0.321	0.036	-	-				
16	0.483	-	-	-	0.117	0.017	0.350	0.033	-	-				
17	-	-	-	0.117	0.033	0.350	-	0.483	-	0.017				
18	0.346	-	0.231	-	-	0.038	0.385	-	-	-				
19	0.350	-	-	-	0.417	-	0.033	0.200	-	-				
20	-	-	-	0.146	-	0.083	0.021	0.750	-	-				
21	0.025	-	-	-	0.125	-	-	0.850	-	-				
22	0.019	-	-	-	0.204	-	-	0.778	-	-				
23	-	-	-	-	0.342	0.079	-	0.579	-	-				
24	-	-	-	-	0.023	0.114	0.477	0.386	-	-				
25	-	-	-	0.038	0.077	0.231	0.423	0.231	-	-				
26	0.019	-	-	0.074	-	0.278	0.241	0.370	0.019	-				
27	-	-	-	0.352	0.019	-	0.056	0.574	-	-				
28	-	-	-	0.389	-	-	0.037	0.574	-	-				

Site	<i>Rp15</i>									
	*188	*189	*197	*199	*203	*204	*205	*209		
1	--	0.021	--	--	--	0.854	0.104	0.021		
2	--	0.021	--	--	--	0.854	--	0.125		
3	--	0.056	--	--	--	0.694	--	0.250		
4	--	0.020	--	--	--	0.760	--	0.220		
5	--	0.036	--	--	--	0.607	--	0.357		
6	--	--	--	--	--	1.000	--	--		
7	0.021	--	--	0.042	0.063	0.583	--	0.292		
8	0.024	--	--	0.024	0.048	0.595	--	0.310		
9	--	--	--	0.138	0.069	0.414	--	0.379		
10	0.023	--	--	0.182	0.045	0.409	--	0.341		
11	--	--	--	0.071	0.214	0.464	--	0.250		
12	--	--	--	0.190	0.048	0.357	--	0.405		
13	--	--	--	0.159	--	0.659	--	0.182		
14	--	--	--	0.259	--	0.407	--	0.333		
15	--	--	--	0.192	--	0.577	--	0.231		
16	--	--	--	0.140	--	0.640	--	0.220		
17	--	--	--	--	--	0.500	--	0.500		
18	--	--	--	0.214	--	0.643	--	0.143		
19	--	--	0.022	--	--	0.978	--	--		
20	--	--	--	--	0.059	0.676	--	0.265		
21	--	--	0.132	--	--	0.868	--	--		
22	--	--	0.192	--	--	0.808	--	--		
23	--	--	0.024	--	--	0.833	--	0.143		
24	--	--	--	--	--	1.000	--	--		
25	--	--	--	--	--	1.000	--	--		
26	--	--	--	--	--	1.000	--	--		
27	--	--	--	--	--	1.000	--	--		
28	--	--	--	--	--	1.000	--	--		

Site	*114	*118	*122	*126	*130	*138	*154	*158	*182	*186	*194	*198	*202	*206	*210
1	-	0.038	0.308	0.635	-	-	-	-	-	0.019	-	-	-	-	-
2	-	-	0.537	0.315	0.056	-	-	0.019	-	0.074	-	-	-	-	-
3	-	0.361	0.278	0.139	0.139	-	-	0.028	-	0.056	-	-	-	-	-
4	-	0.140	0.420	0.320	0.060	-	-	0.060	-	-	-	-	-	-	-
5	-	0.184	0.421	0.237	0.026	-	0.079	-	0.026	0.026	-	-	-	-	-
6	-	0.020	0.880	0.100	-	-	-	-	-	-	-	-	-	-	-
7	0.022	0.065	0.565	0.065	0.022	0.065	-	-	-	-	0.022	0.152	0.022	-	-
8	-	0.056	0.602	0.028	0.056	0.028	-	-	-	-	-	0.176	0.046	-	0.009
9	-	0.063	0.813	0.063	-	-	-	-	-	-	-	0.021	0.021	0.021	-
10	-	0.119	0.643	0.143	-	-	-	-	-	-	0.024	0.024	0.024	-	0.024
11	0.067	0.133	0.533	0.067	0.067	-	-	-	-	-	0.024	0.033	0.033	0.033	0.033
12	-	0.136	0.682	0.045	0.045	-	-	-	-	-	-	0.023	0.045	-	0.023
13	-	0.135	0.865	-	-	-	-	-	-	-	-	-	-	-	-
14	-	0.040	0.960	-	-	-	-	-	-	-	-	-	-	-	-
15	-	0.034	0.966	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-
17	-	0.025	0.950	-	0.025	-	-	-	-	-	-	-	-	-	-
18	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-
20	-	0.063	0.708	0.229	-	-	-	-	-	-	-	-	-	-	-
21	-	0.048	0.905	0.048	-	-	-	-	-	-	-	-	-	-	-
22	-	-	0.979	0.021	-	-	-	-	-	-	-	-	-	-	-
23	-	-	0.591	0.409	-	-	-	-	-	-	-	-	-	-	-
24	-	0.019	0.923	-	-	-	-	-	-	-	-	-	0.058	-	-
25	-	0.024	0.976	-	-	-	-	-	-	-	-	-	-	-	-
26	-	0.138	0.862	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-

Site	Rp23										SFCI34												
	*183	*187	*191	*195	*199	*203	*213	*217	*221	*225	*229	*183	*187	*191	*195	*199	*203	*213	*217	*221	*225	*229	
1	-	0.643	0.232	0.125	-	-	-	0.125	0.286	-	0.589	-	-	-	-	-	-	-	-	-	-	-	-
2	-	0.589	0.411	-	-	-	-	0.321	0.500	-	0.179	-	-	-	-	-	-	-	-	-	-	-	-
3	0.026	0.763	0.184	-	0.026	-	-	0.158	0.395	0.026	0.421	-	-	-	-	-	-	-	-	-	0.026	0.421	-
4	0.036	0.857	0.089	0.018	-	-	-	0.054	0.482	0.036	0.429	-	-	-	-	-	-	-	-	-	0.036	0.429	-
5	-	0.733	0.267	-	-	-	-	0.083	0.500	0.028	0.389	-	-	-	-	-	-	-	-	-	0.028	0.389	-
6	0.214	0.643	0.143	-	-	-	-	-	0.065	-	0.935	-	-	-	-	-	-	-	-	-	-	0.935	-
7	-	0.684	0.053	-	0.237	0.026	-	0.609	0.196	-	0.196	-	-	-	-	-	-	-	-	-	-	0.196	-
8	-	0.590	0.100	-	0.210	0.100	-	0.350	0.350	0.010	0.290	-	-	-	-	-	-	-	-	-	0.010	0.290	-
9	-	0.679	0.143	-	0.036	0.143	-	0.404	0.365	0.019	0.212	-	-	-	-	-	-	-	-	-	0.019	0.212	-
10	-	0.625	0.200	-	0.075	0.100	-	0.600	0.225	-	0.175	-	-	-	-	-	-	-	-	-	-	0.175	-
11	-	0.711	0.184	-	0.026	0.079	-	0.300	0.400	0.075	0.225	-	-	-	-	-	-	-	-	-	0.075	0.225	-
12	-	0.700	0.220	-	0.020	0.060	-	0.464	0.232	0.125	0.179	-	-	-	-	-	-	-	-	-	0.125	0.179	-
13	-	0.962	0.038	-	-	-	-	0.625	0.375	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	0.900	0.100	-	-	-	-	0.654	0.346	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	0.958	0.042	-	-	-	0.023	0.364	0.614	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	0.925	0.075	-	-	-	-	0.580	0.420	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	0.050	0.950	-	-	-	-	-	0.482	-	-	0.518	-	-	-	-	-	-	-	-	-	-	-	-
18	-	0.889	0.111	-	-	-	-	0.167	0.833	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	0.763	0.237	-	-	-	-	0.524	0.476	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	0.690	0.310	-	-	-	-	0.354	0.604	-	0.042	-	-	-	-	-	-	-	-	-	-	0.042	-
21	-	0.900	0.1000	-	-	-	-	0.158	0.684	-	0.158	-	-	-	-	-	-	-	-	-	-	0.158	-
22	-	0.940	0.060	-	-	-	-	0.091	0.705	-	0.205	-	-	-	-	-	-	-	-	-	-	0.205	-
23	-	1.000	-	-	-	-	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	0.646	0.354	-	-	-	-	0.060	0.100	-	0.840	-	-	-	-	-	-	-	-	-	-	0.840	-
25	-	0.500	0.500	-	-	-	-	0.026	0.053	-	0.921	-	-	-	-	-	-	-	-	-	-	0.921	-
26	-	0.667	0.333	-	-	-	-	-	0.100	-	0.900	-	-	-	-	-	-	-	-	-	-	0.900	-
27	-	0.226	0.774	-	-	-	-	0.017	-	-	0.967	-	-	-	-	-	-	-	-	-	0.017	0.967	-
28	-	0.741	0.259	-	-	-	-	-	-	-	0.979	-	-	-	-	-	-	-	-	-	0.021	0.979	-

SFC139

Site	*245	*249	*253	*257	*261	*265	*269	*273	*277	*281	*285	*289	*293	*297	*301	*305
1	-	-	0.021	-	-	0.708	0.063	0.167	-	0.042	-	-	-	-	-	-
2	0.083	-	0.063	0.083	0.083	0.313	-	0.250	0.042	0.021	0.021	-	-	-	-	0.042
3	0.111	0.111	0.028	0.111	-	0.250	0.056	0.111	0.111	0.056	-	-	-	-	-	0.056
4	0.241	0.019	0.037	0.037	-	0.148	0.148	0.296	0.074	-	-	-	-	-	-	-
5	0.306	0.111	0.056	0.028	-	0.111	0.056	0.278	0.056	-	-	-	-	-	-	-
6	-	0.173	0.115	-	0.058	0.250	0.269	0.038	0.096	-	-	-	-	-	-	-
7	0.300	0.080	0.100	0.080	0.020	0.100	0.240	0.080	-	-	-	-	-	-	-	-
8	0.357	0.071	0.092	0.102	-	0.082	0.245	0.041	0.010	-	-	-	-	-	-	-
9	0.190	0.138	0.034	0.069	-	0.017	0.414	0.103	0.034	-	-	-	-	-	-	-
10	0.455	-	0.045	0.045	0.023	0.045	0.273	0.114	-	-	-	-	-	-	-	-
11	0.200	0.050	-	0.025	-	0.125	0.300	0.250	0.025	-	-	0.025	-	-	-	-
12	0.352	0.019	0.037	0.037	0.019	0.037	0.407	0.056	0.037	-	-	-	-	-	-	-
13	-	-	-	-	-	-	0.404	0.231	-	-	-	0.365	-	-	-	-
14	-	-	-	0.096	-	-	0.346	0.173	-	0.038	0.038	0.250	-	-	0.058	-
15	-	-	-	0.093	0.037	-	0.352	0.167	-	-	0.037	0.259	-	-	0.056	-
16	-	-	-	0.095	-	-	0.310	0.310	-	-	0.024	0.262	-	-	-	-
17	-	-	-	-	-	-	0.018	0.357	0.375	-	0.250	-	-	-	-	-
18	-	-	-	-	-	-	0.339	0.375	0.018	0.268	-	-	-	-	-	-
19	-	-	-	-	-	0.381	-	0.238	0.143	-	-	0.238	-	-	-	-
20	-	-	-	-	0.125	0.475	0.050	0.025	0.125	-	0.175	-	-	-	-	0.025
21	-	-	-	-	-	0.643	0.214	0.095	0.048	-	-	-	-	-	-	-
22	-	-	-	-	-	0.729	0.146	0.083	0.042	-	-	-	-	-	-	-
23	-	-	-	-	-	0.348	0.283	0.304	0.065	-	-	-	-	-	-	-
24	-	-	-	-	-	0.042	0.521	0.042	0.354	0.021	-	-	-	0.021	-	-
25	-	-	-	-	-	0.265	0.147	0.059	0.147	0.265	0.088	-	0.029	-	-	-
26	-	-	-	-	-	0.389	0.111	0.093	-	0.093	0.037	-	0.278	-	-	-
27	-	-	-	-	-	0.533	0.083	0.033	0.350	-	-	-	-	-	-	-
28	-	-	-	-	-	0.704	-	-	0.296	-	-	-	-	-	-	-

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