### Landscape Genetics of Foothill Yellow-Legged Frogs (*Rana boylii*) in regulated and unregulated rivers: Assessing connectivity and genetic fragmentation

by

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#### Abstract

The stream breeding foothill yellow-legged frog (Rana boylii) is experiencing range wide population declines. Because this species inhabits rivers in the foothills of California, a suite of anthropogenic impacts, including habitat alteration, river regulation, aerial drift of pesticides, and invasive species, directly and indirectly affects these frogs. Among multiple stressors, hydroelectric projects may have the greatest potential impact on R. boylii because of flow regulation and riverscape alterations such as dams, reservoirs, and powerhouses. River regulation can fragment the landscape and reduce the connectivity within and among *R. boylii* populations, which ultimately may limit gene flow and reduce genetic diversity. Determining gene flow and levels of genetic diversity within and among populations in regulated systems compared with unregulated rivers can provide valuable information about population structure and riverscape connectivity for conservation management. The hypothesis that *R. boylii* populations in watersheds regulated by hydroelectric generation have lower genetic diversity and riverscape connectivity compared with unregulated watersheds (without hydroelectric generation or dams) was tested. Six different rivers in the Sierra Nevada were compared; pairing similar-sized hydroelectric-regulated and unregulated rivers in adjacent watersheds. Genetic structure within and among R. boylii populations in regulated and unregulated watersheds was characterized and compared using mitochondrial DNA (mtDNA) to estimate gene flow and random amplified polymorphic DNA (RAPD) to estimate genetic diversity. Riverscape connectivity was analyzed with a quantitative geo-spatial network analysis using stream networks, tributary confluences, and frog distribution patterns. Results indicate significant differences in population structure between regulated and unregulated streams, with important implications for watershed management. Rana boylii populations were fragmented spatially and genetically in regulated study rivers compared with unregulated rivers. This species has adapted to inhabit a dynamic ecosystem, and flow regulation has altered the pattern of natural hydrologic variation. As a result, R. *boylii* populations are currently becoming isolated at genetic and spatial scales, limiting potential adaptive plasticity required to survive within these regulated watersheds.

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# Background

As indicators of global environmental change, amphibians are unique; the typical life cycle of a frog includes aquatic development of eggs and larvae, and terrestrial activity as adults, which links individuals to multiple habitats and trophic levels (Power and Dietrich 2002, Wake and Vredenburg 2008). Therefore, many amphibians are particularly sensitive to changes in the ecosystem due to their physiology and life histories (Davidson et al. 2002, Beebee and Griffiths 2005, Vredenburg and Wake 2007). However, amphibian species have persisted through the last four mass extinctions (the earliest occurring approximately 364 million years ago), and all three orders of amphibians escaped extinction (Wake and Vredenburg 2008).

Amphibian populations continue to decline on local and global scales (Beebee and Griffiths 2005, Lannoo 2005, Pounds et al. 2006, Hamer and McDonnell 2008, Wake and Vredenburg 2008), and the underlying reasons behind these declines often remain unknown (Moyle and Randall 1998, Beebee and Griffiths 2005, Brito 2008, Wake and Vredenburg 2008). Human activities have been directly linked to all of the key factors in this recent era of amphibian decline, including climate change, invasive species introductions, habitat fragmentation, and habitat destruction (Karr and Chu 2000, Beebee and Griffiths 2005). Therefore, current amphibian declines may not only represent a severe change in the balance of global biodiversity, but also indicate significant and widespread ecological degradation. This degradation may have ramifications not only for amphibians, but all species that rely on the benefits that "ecosystem services" (Daily 1997) provide, such as drinking water, food supply, purification of human and industrial wastes, and habitat for plant and animal life (Wilson and Carpenter 1999).

The river dwelling foothill yellow-legged frog (Rana boylii) is currently a species of special concern in California, and has declined from over 50 percent of its historic range (Davidson et al. 2002). Research on the life history of *R. boylii* has increased our understanding of the habitat requirements (Kupferberg 1996, Yarnell 2005, Lind 2005, Haggarty 2006), development and competition (Kupferberg 1997), basic movement (GANDA 2007, Bourgue 2008), phylogeny and genetic structure (Macey et al. 2001, Lind 2005), and behavior (Van Wagner 1996, Wheeler 2007) of this species. Adult frogs are found primarily in or near rivers and streams and feed primarily prey on aquatic and terrestrial insects (Nussbaum et al. 1983, Haggarty 2005). They require low stream gradients in breeding and rearing areas (Zweifel 1955), but post-metamorphic individuals have been observed in a wide variety of habitats, including those with very steep gradients. Breeding and oviposition occur in the spring and females only deposit a single egg mass per season, consisting of several hundred to over 2,000 eggs (Zweifel 1955, Kupferberg 1996, Lind et al. 1996, Kupferberg et al. 2008). Breeding occurs in hydraulically stable habitat (generally shallow, low water velocity areas) in rocky substrates, often near cobble or gravel bars with sparse vegetation near tributary confluences (Twitty et al. 1967, Kupferberg 1996, Lind et al. 1996, Van Wagner 1996, Lind and Yarnell 2008). Initiation of oviposition is generally associated with the descending limb of the hydrograph (receding flow from spring snow melt), increasing day length, and warming of water temperatures, although additional variables may be

involved (Zweifel 1955, Lind et al. 1996, Kupferberg 1996). Development from hatching through metamorphosis requires approximately three months, depending on water temperature and food availability, and *R. boylii* do not overwinter as tadpoles (Zweifel 1955). Reproductive maturity may not occur until the frog reaches two to three years in age, and it is unknown whether individuals breed every year (particularly females). *Rana boylii* use habitat patches for breeding at or near tributary confluences (Kupferberg 1996), and generally seem to prefer heterogeneous habitat areas, such as braided channels or tributary junctures (Twitty et al. 1967, Van Wagner 1996, Kupferberg 1996, Lind et al. 1996, Lind 2005, Yarnell 2005, Haggarty 2006), however, connectivity of these habitats with other breeding populations within the same watershed is unknown.

Many current *R. boylii* populations occur in rivers with dams, powerhouses, and diversions, yet only recently has research focused on the effects of river regulation on *R. boylii* (see Lind et al. 1996, Lind 2005, Kupferberg et al. 2008). Research has shown large fluctuations in flow can scour egg masses from breeding locations, and the loss of any egg mass can reduce population fecundity, particularly because *R. boylii* females only lay one egg mass per year (Kupferberg et al. 2008, Lind and Yarnell 2008). The type of river regulation most likely to cause these large changes in flow is hydroelectric power generation, which requires complicated systems of dams, reservoirs, and tunnels to move large amounts of water through a watershed (or exported to other watersheds) in order to produce power.

Flow modification has pervasive effects on river ecosystems (Renofalt et al. 2010), and ultimately riverine processes are driven by flow variables (Poff et al. 1997). Mimicking natural flows in regulated systems has been used to enhance and restore ecological health in rivers, but increasing demands for renewable energy often counter these mitigation efforts (Renofalt et al. 2010). Hydroelectric power generation is considered a renewable source of energy, and as California's population increases, so does the demand for electricity. California has more hydropower dams than any other state (Hall 2006), and the population of California is expected to increase by approximately 9% by 2025 (US Census Bureau, Population Division, Interim State Population Projections, 2005). Residential electricity demand is expected to increase by 24% by 2035, due to growth in population and continued population shifts to warmer regions with greater cooling requirements (US EIA, Annual Energy Outlook 2010). Hydroelectric power generation comprises over half of all renewable energy generation in California (California Energy Commission 2010), and in order to meet various power demands and carbon emission goals, it has become increasingly important politically and economically. Therefore it is unlikely that riverine species like R. boylii will encounter reductions in river regulation in the future, and conservation of current populations becomes even more critical for this species' survival.

*Rana boylii* is caught in the crux of hydroelectric river regulation, population growth, and increasing pressure for greater use of renewable energy sources. Although a single species should not determine conservation management policies for an entire watershed (Puth and Wilson 2001, Olden 2007), understanding landscape and community interactions for functional groups or species can be informative for resource management

(Fuller and Lind 1992). As most research has focused on short-term affects of river regulation on *R. boylii* breeding, there has been no long-term research on the effects of river regulation on gene flow, genetic diversity, and connectivity. The maintenance of genetic diversity and gene flow is a driving force behind population structure and the process leading to speciation or extinction (Avise 1994). Patterns of genetic divergence have been observed to correlate with landscape features (Slatkin 1985, Vos et al. 2001, Vigneiri 2005, Wang et al. 2008), and discerning important connections between *R. boylii* habitat and population genetic structure is critical for effective conservation of the species (Moritz 2002). *Rana boylii* occupies a unique niche in the ecosystem, functioning as periphyton grazers and prey for aquatic macroinvertebrates and snakes when in the tadpole form, and as predators of arthropods post-metamorphosis. From perspectives of conservation and ecosystem services, populations with more genetic variation may have greater resilience in the face of environmental change (Luck et al. 2003). Conservation efforts ultimately must focus on preserving genetic diversity and population connectivity in order to provide species with the greatest chance for survival.

This thesis research supports evidence that landscape fragmentation is occurring at spatial and genetic scales in *R. boylii* within these regulated systems. It also indicates that reductions in genetic diversity and gene flow have occurred over short evolutionary timeperiods in these study populations. Long-term conservation goals must focus on restoring and enhancing genetic connectivity within *R. boylii* populations, in addition to limiting flow fluctuations during the breeding and rearing periods in regulated rivers. Conservation must include entire river networks to preserve genetic diversity and promote gene flow, which ultimately is the most critical component for species survival. Further research is required to understand whether reductions in gene flow and genetic diversity are associated with measures of performance, such as limiting plasticity of traits promoting survival in this species over longer time periods, particularly in the stochastic riverine environments *R. boylii* have evolved.

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# Chapter 1: The effects of river regulation on *Rana boylii* distribution and population connectivity

#### INTRODUCTION

Connectivity occurs at many scales, and is defined as interactions between landscape structure and individual animal movement behavior (Taylor et al. 1993). Connectivity is critical for the persistence of ecological and evolutionary processes. In rivers, connectivity promotes linkage of organisms at various scales (Puth and Wilson 2001, Power and Dietrich 2002, Wiens 2002), reduces genetic divergence and increases gene flow (Slatkin 1987, Vignieri 2005, Raeymaekers et al. 2008), and protects biodiversity (Fahrig 2003). Restoring riparian connectivity may be a key factor in promoting adaptation and resilience to climate change (Seavy et al. 2009). Amphibian communities have been used to analyze landscape fragmentation and connectivity, and sensitive amphibian species have shown strong landscape patterns of extinction due to habitat degradation and fragmentation (Ficetola and De Bernardi 2004). Vos et al. (2001) found barrier elements like roads and railways reduced connectivity in moor frog (Rana arvalis) populations, and these elements gave a higher explanatory value for genetic distances compared with geographic distance. Therefore amphibians are suitable targets for analyses of connectivity, and provide valuable data on the influence of landscape connectivity on animal dispersal, particularly when combined with genetic techniques.

Connectivity and habitat fragmentation can directly and indirectly affect spatial patterns of a species (Fagan 2002, Fahrig 2003). The stream breeding foothill yellow-legged frog (*Rana boylii*) is experiencing range wide population declines, and has disappeared from over 50% of its historic range (Davidson et al. 2002). *Rana boylii* has a complex life history involving migration of adults from over-wintering refugia in low order streams and seeps to breeding sites in higher order rivers, where channels are broad and sun-lit, providing warm conditions and abundant algal food for successful recruitment of tadpoles into frogs. Because this species inhabits rivers, it is directly affected by the anthropogenic stressors most common on the west slope of the Sierra Nevada, including river regulation, recreation, development, and agricultural activities. For example, Lind (2005) observed *R. boylii* population absences were positively correlated with proximity to large dams. Both flow regulation and landscape features like dams or reservoirs can fragment the riverine landscape and reduce connectivity within and among frog populations.

Furthermore, species with more fragmented distributions are more prone to extinction (Hanski 1998, Fagan et al. 2002, Raeymaekers et al. 2008), and spatial rarity of an organism is an underlying factor relating to extinction theory (Soulé 1983, Caughley 1994, Simberloff 1998, Purvis et al. 2000, Channel and Lomolino 2000). Although the small population paradigm may explain how a rare species can go extinct (Simberloff 1986, Caughley 1994), factors causing range wide declines are often very different than those that ultimately eliminate the final population (Caughley 1994, Channel and Lomolino 2000). Without a prolonged reduction in distribution and number, it is unlikely

that an abundant and widespread species will become extinct (Raup 1994). Unfortunately *R. boylii* has experienced large-scale declines, and current populations rarely occur in abundance, particularly in regulated rivers. It is unknown if *R. boylii* functions within a metapopulation model or a stepping stone model, but it is likely that local source-sink population dynamics are important for this species persistence.

River landscapes are more severely affected by habitat fragmentation compared to linear geometry found in agriculture and forestry because of the dendritic pattern of stream networks (Fagan 2002, Wiens 2002, Ficetola et al. 2004). Dendritic connectivity is unique from a metapopulation standpoint because as the number of patches (or subpopulations) increase in a metapopulation, the number of potentially isolated patches remains constant; however, in a linear landscape, an increase in metapopulation patches reduces the number of potentially isolated patches (Fagan 2002). Therefore additional fragmentation (via flow regulation, dams, or reservoirs) of dendritic watersheds already fragmented by natural landscape features (i.e., mountains, waterfalls) may further splinter ecosystems that have limited connectivity and increase species extinction risks (Fagan 2002, Magilligan et al. 2003). These fragmentation variables may impact species differently, but all trophic and ecological interactions are inexorably linked within these ecosystems (Power et al. 1996, Puth and Wilson 2001, Wiens 2002, Olden 2007). For example, dams may represent direct barriers to fish population connectivity (Schick and Lindley 2007), but can also negatively affect other riverine organisms such as benthic macroinvertebrates indirectly, through altered substrate composition, nutrient delivery rates, and habitat availability (Hart and Finelli 1999).

For *R. boylii*, river regulation and habitat fragmentation within dendritic networks may limit potential dispersal and range expansion, particularly in small isolated populations. Limited connectivity within fragmented populations can affect patterns of migration and dispersal, as well as genetic differentiation (Deiner et al. 2007). Even low levels of fragmentation in riverine landscapes can compound local extirpation events by reducing potential recolonization and altering stream habitat (Fagan 2002, Riley et al. 2005). Raeymaekers et al. (2008) found that riverine barriers not only impacted genetic diversity in three-spined stickleback, but they also controlled the balance between gene flow and genetic drift. In particular, Raeymaekers et al. (2008) observed connectivity issues in small tributaries and upstream river stretches were more likely to have a greater impact on genetic connectivity in fish compared to mainstem/downstream reaches. Determining how river regulation affects fragmentation and connectivity for *R. boylii* is critical for conservation management, and species persistence in regulated watersheds.

Regulation of rivers and streams has been a prominent theme in California since the discovery of gold in 1848 (Starr 2007). As a result, there are few rivers remaining that have not been fragmented at some level due to factors such as dams, reservoirs, and flow regulation (Mount 1995, Ligon et al. 1995, Brown and Bauer 2009). An increasing amount of research is being conducted on the impacts of river regulation, particularly in relation to dams and fish (Heggenes and Røed 2006, Deiner et al. 2007, Schick and Lindley 2007, Brown and Bauer 2009, Wassens and Maher 2010). However, ascertaining how regulation affects *R. boylii* has only recently gained attention (Lind et

al. 1996, Lind 2005, Kupferberg et al. 2008). Because *R. boylii* breeds in rivers and streams of sufficient size for hydroelectric power generation to occur, recent research has identified key issues relating to negative impacts of pulse flows on breeding and breeding habitat (Kupferberg et al. 2008, Lind and Yarnell 2008). However, there has been no research on spatial connectivity of *R. boylii* populations, particularly comparisons of regulated rivers and unregulated rivers. Understanding how underlying spatial connectivity patterns (i.e., population structure and distribution in the river network, habitat distribution, habitat use) are affected by river regulation requires integrating fine-scale analysis with appropriate time-scales.

#### Importance of Tributaries for <u>R. boylii</u>

Tributaries and tributary confluences are key riverscape components (both biologically and geomorphically) in all lifestages of *R. boylii*. Geomorphically, tributary confluences are critical components of mesohabitat formation and an underlying source of river habitat diversity (Frissell et al. 1986, Benda et al. 2004, Yarnell et al. 2006), and research has shown *R. boylii* occur in streams with higher habitat heterogeneity (Van Wagner 1996, Yarnell 2005, Haggarty 2006). Biologically, tributaries provide refugia for *R. boylii* during high mainstem flows as well as potential breeding and foraging habitat (Kupferberg 1996). Tributary confluences are important landscape components for *R. boylii* and tributaries have been used as a functional metric to assess genetic connectivity within watersheds for this species (Dever 2007).

Breeding has been observed near tributary confluences (Kupferberg 1996), however, quantitative measurement and spatial analysis of distributions in relation to confluences has not been formally tested. Studies of dispersal and connectivity are difficult to conduct on cryptic and vagile species such as *R. boylii*. There have been few movement studies conducted on *R. boylii* or related species, and all studies have been limited by available technology (i.e., size of radio tags is too large for individuals below certain stage, size, or age) and are labor intensive (Matthews and Pope 1999, Bulger et al. 2003, and Bourque 2008).

#### Study Questions

To understand how riverscape connectivity within *R. boylii* distributions may differ among regulated and unregulated watersheds, a geo-spatial analytical framework was used to test connectivity between breeding locations, juvenile and adult locations, and tributary confluences. A network analysis of riverscape connectivity based on *R. boylii* population distributions in relation to tributary confluences, which are important landscape components, was used to address this question. This study, along with the riverscape genetics analysis discussed in the next chapter, provides two unique approaches to help inform conservation decisions relating to this species and future watershed management in regulated rivers. This study focused on the following question:

Do regulated rivers limit riverscape connectivity and fragment *R. boylii* spatial distributions in relation to tributary confluences?

A paired river design was used to test regulated versus unregulated system affects on population connectivity. Under a neutral hypothesis, there should be no difference between spatial patterns of *R. boylii* distribution in regulated and unregulated rivers. However, if the presence of dams and reservoirs or regulation of flow regimes limits *R. boylii* access to tributary confluences, distances between (a) breeding locations and nearest confluences, and (b) adult locations and nearest confluences would be smaller compared with distances in unregulated rivers. Fragmented distributions were expected to show greater patterns of aggregation and clustering, and if tributary confluences were critical landscape components for *R. boylii*, frog distributions would be clustered near these features.

#### **METHODS AND MATERIALS**

#### Study Area

Regions from six rivers; the North Fork Feather (NFF), Middle Fork Feather (MFF), Middle Fork American (MFA), North Fork American (NFA), Rubicon (RUB), Upper Middle Fork American (UMFA), and the North Fork Middle Fork American (NFMFA) were selected using a paired-design, each pair consisting of a regulated and unregulated watershed of similar basin area, stream size and geographic location (Table 1.1, Figure 1.1). Study reaches were selected to maximize survey data on frog populations, therefore information on accessibility, data on current population presence, and reconnaissance visits were compiled before choosing the final stream segments. All stream segments began and ended at tributary confluence locations to correlate genetic and geomorphic timescales (Frissell et al. 1986).

Within regulated rivers, three different hydroelectric power operation types were included, to analyze potential differences in the effects of operation type on genetic differentiation (Table 1.1). These general categories encompass typical components of most hydroelectric regulation operations, and a majority of hydroelectric projects consist of combinations of these types. Run-of-river operations may encompass an entire system, and are a type of hydroelectric operation that involves diverting a portion of the river flow (the diverted segments of river are referred to as "bypass reaches") to generate power through turbines before returning the water to the river downstream of the turbines and bypass reach. A peaking reach may be a component of a hydroelectric project operated to maximize peak electrical demands, and requires the use and release of large amounts of water from upstream diversions and storage locations such as reservoirs or lakes. A peaking reach may have large fluctuations in flow over short periods of time. The bypass reach may also be a component of a run-of-river project, and is the opposite of the peaking reach. Water is diverted from stretches of river for use in other parts of the watershed, and generally flow is released at a constant base flow with minimal fluctuation.

| River    |                                    | River |                          | Mainstem<br>Segment |
|----------|------------------------------------|-------|--------------------------|---------------------|
| Pair     | River                              | Code  | <b>River</b> Type        | Length (km)         |
| Footbor  | North Fork Feather                 | NFF   | Regulated (Run of River) | 12.3                |
| reather  | Middle Fork Feather                | MFF   | Unregulated              | 6.2                 |
| Amorican | Middle Fork American               | MFA   | Regulated (Peaking)      | 13.47               |
| American | North Fork American                | NFA   | Unregulated              | 13.80               |
| Unnor    | Rubicon                            | RUB   | Regulated (Bypass)       | 7.50                |
| American | North Fork Middle<br>Fork American | NFMFA | Unregulated              | 5.52                |

 Table 1.1. Study rivers and segment lengths.



Figure 1.1. Study sections of regulated and unregulated river pairs with tributary confluences.

#### Data Collection

Distributional data on *R. boylii* were collected in 2009 during long reach surveys of study river segments. Data collected included frog location, stage, sex, and habitat use. Waypoints for each frog location were logged in decimal degrees with a handheld GPS receiver using NAD83 datum and averaged for approximately 30 seconds per location to increase accuracy (generally ranging from 3–5 meters per point). Additional distribution data was used for the NFF River (GANDA 2009), and the NFA, MFA, NFMFA and Rubicon Rivers (PCWA 2007) to provide a more comprehensive picture of frog distribution within the study segments.

#### Geo-Spatial Analysis

In order to test the hypothesis, three focal questions were used to organize and build the network analysis.

1. How similar were stream networks among regulated and unregulated river pairs?

It was important to compare the underlying landscapes (i.e., among regulated versus unregulated rivers) before testing riverscape connectivity of frog distributions. Assessing the similarity between regulated and unregulated river networks was necessary to avoid erroneously attributing frog distribution patterns to landscape differences. Analysis of covariance (ANCOVA) and paired t-tests were used to compare statistical significance of any observed disparities between rivers (such as a higher number of tributaries in one river versus the comparison river). Basin area, tributary density, tributary frequency, and drainage density were used as comparison metrics.

# 2. Were tributary confluences more closely linked with *R. boylii* distribution patterns in regulated watersheds compared with unregulated watersheds?

Using statistical tests of variance and covariance (ANOVA and ANCOVA) as well as Mann-Whitney U tests, comparisons of distances between breeding locations and nearest confluences, or between adults and nearest confluences were used to compare regulated and unregulated study rivers. Rivers with higher connectivity were expected to have greater distances between these points, because frogs may be able to move more easily and disperse farther from tributary confluences within the watershed.

#### 3. Was the observed connectivity pattern different under a random dataset?

If the same analysis of distance to nearest tributary confluences was conducted with a random dataset, a neutral pattern should exist, with no difference between regulated and unregulated watersheds. A random dataset was created to test whether underlying landscape formation could be the root cause for differences in connectivity between regulated and unregulated watersheds.

To prepare and analyze the collected data, the following steps were taken to create a working map containing seven data layers (Figure 1.2).

- 1. <u>Streams</u>–A base stream network layer was created from the surveyed segments and associated tributaries using the National Hydrography Dataset (NHD) stream layer. Additional tributaries appearing in 7.5-minute quadrangle maps were digitized and merged with the NHD layer if connections with the selected mainstem segment were visible.
- 2. <u>Confluences</u>–All stream junction nodes (confluences) within the stream network were identified and categorized as perennial, intermittent, or ephemeral for the network analysis (N=103).
- 3. <u>Stream Buffer</u>–A stream buffer of the stream network was created using average stream wetted widths from ground measurements and buffering approximately 25 m from the stream channel edge. Bourque (2008) observed *R. boylii* rarely moved greater than 12 m from the stream channel.
- 4. <u>Random Points</u>–Hawth's Tools (Beyer 2004) within ArcGIS (ESRI 2007) was used to create a random point dataset within the stream buffer, with a density of 10 points per stream kilometer (N=2600) within the entire stream network.
- 5. <u>Dams</u>–A layer of all dams located within the study streams (N=3) was created.
- 6. <u>Breeding</u>-A layer of all observed breeding points (including egg masses, tadpoles, and young of year [NFF only]) was created (N=401).
- 7. <u>Adults</u>–A layer of all observed adult (N=400) and juvenile (N=102) points was created.



Figure 1.2. Example of steps required to conduct network analysis of *R. boylii* connectivity with tributary confluences for the Middle Fork American River, Peaking Reach.

Individual frog points were uniquely identified and categorized by river and river type (regulated or unregulated). Frog stage was categorized as "Breeding" (eggs and tadpoles) or "Adults" (juveniles and adults). Because longitudinal data along the NFF River were sparse, young of year were included within the "Breeding" category. All data were labeled with categorical attributes and unique numerical identifiers.

Although tadpoles may disperse from initial egg mass locations, dispersal distances and locations generally occurred within suitable breeding habitats. Egg and tadpole habitats often had similar microhabitat characteristics, with similar ranges in water depth, velocity, and substrate (Kupferberg 1997, Kupferberg et al. 2008, Lind and Yarnell 2008). Locating all egg masses within a study segment can be difficult since they are often well concealed; therefore to provide a more comprehensive analysis of *R. boylii* connectivity, tadpole locations were used as "breeding" points. By combining these stages for analysis, a broader scale understanding of the relationship between tributaries, adults, and breeding areas was possible.

All the above layers were plotted on a map and a network analysis was conducted using the Network Analyst extension of ArcGIS. Network analyses can be used to study many different types of linear networks, including roads, rivers, facilities, and canals. The analysis requires network data (i.e., a stream network) to calculate distances between points (nodes) in the network. Using the collected frog data, each data point (i.e., breeding, adult, or random) was snapped to the nearest line of the stream network layer, and the distance between each frog point and the nearest tributary confluence was calculated using the stream network. Dams were identified as barriers; therefore any point separated from the nearest confluence by a dam would move to the next proximal confluence occurring on the same side of the dam as the point. Distance data were compiled and exported for statistical analysis.

In addition, a Euclidean-based analysis was conducted, using the proportion of *R. boylii* (all lifestages) occurring within specific tributary confluence buffer intervals ranging from 50–800m (Figure 1.3). Data were compared within each river and among regulated and unregulated groups. The proportions of *R. boylii* observed at each buffer interval were arcsine-square-root transformed and analyzed using ANOVA (Sokal and Rohlf 1995, McDonald 2009, Lowry 2010).



# Figure 1.3. Example of concentric buffers around tributary confluences in the Upper Middle Fork American watershed, including all observed *R. boylii* (all lifestages).

#### Statistical Analysis

Data were log or square root transformed for data analysis to normalize distributions (Sokal and Rohlf 1995, Zar 1999). Transformation type was determined after histogram plots and Bartlett's test for homogeneity of variances were used to compare homoscedasticity, normality, and variance of the data (McDonald 2009). All datasets were normalized and were not heteroscedastic after transformation.

To determine if tributary confluence frequency or densities would influence comparisons of regulated and unregulated distances, ANCOVA ( $\alpha$ =0.05) were used to test underlying differences in riverscapes between regulated and unregulated river types (Sokal and Rohlf 1995, Zar 1999). Tributary frequency and density were used as covariates, and distance to nearest confluence was the response variable. Direct comparisons of river pairs and river types were tested using two-tailed paired t-tests ( $\alpha$ =0.05). Planned paired comparisons, which consisted of regulated versus unregulated river data, were tested using ANOVA ( $\alpha$ =0.05). However, in several groups sample sizes were small or unequal (i.e., adult females in the MFF and NFF), therefore in addition to parametric ANOVA tests, non-parametric Mann-Whitney U tests were also conducted as unplanned comparisons of individual river pairs (regulated versus unregulated). Mean distances, including 95% confidence intervals (CI), were plotted for visual and reporting comparisons.

#### RESULTS

#### Stream Network Comparison

Regulated and unregulated river pairs had similar stream sizes and watershed areas (Table 1.2–1.3). The two largest rivers, NFA and MFA, had similar basin areas (180.26 and 143.88 km<sup>2</sup>, respectively) and had basin areas approximately double the area of any other study river. Comparisons of basin area in the three regulated and unregulated pairs were not significantly different (two-tailed paired t-test, p=0.98, t= -0.03, df=4). The two smallest rivers (Rubicon and NFMFA) had the smallest drainage densities (0.16 and 0.11/km, respectively), and all rivers ranged between 0.11/km (NFMFA) to 0.66/km (NFF). Comparisons of drainage densities between the three regulated and unregulated pairs were not significantly different based on paired two-tailed t-tests (All: p=0.551, t=0.65, df=4; Mainstem Only: p=0.77, t=0.32, df=4).

Tributaries were separated into two categories, "All" (including ephemeral, intermittent, and perennial tributaries); and "Perennial Only" (only perennial tributaries connecting to the mainstem were included). Tributary density was calculated for both categories. In general, tributary densities were similar within river pairs for both categories, with the exception of the NFF and MFF. Including all tributary types, the NFF had approximately twice the tributary density of the MFF, but both rivers had identical "perennial only" tributary densities. Mean tributary densities were larger in regulated segments for both categories (All Types: regulated=0.19, unregulated=0.12; Perennial Only: regulated=0.05, unregulated=0.05). Paired two-tailed t-tests of tributary densities between regulated and unregulated pairs were not significantly different (All Types: p=0.50, t=0.74, df=4; Perennial Only: p=0.84, t=0.21, df=4). In summary, differences in basin area, drainage density, and tributary density among regulated and unregulated segments were not significant.

|                                 | Study                               | Study Reach Stream<br>Length |   | Basin Drainage<br>Density <sup>b</sup> |                  | No. Of Tributaries<br>(Tributary Density <sup>c</sup> ) |                   |
|---------------------------------|-------------------------------------|------------------------------|---|--|------------------|---|-------------------|
| River<br>(R=reg.,<br>U= unreg.) | Basin<br>Area<br>(km <sup>2</sup> ) | Total<br>(km)                | Avg.<br>Mainstem <sup>a</sup><br>Seg. (m) | Total                                  | Mainstem<br>Only | All Types   | Perennial<br>Only |
| NFF (R)                         | 79.68                               | 52.52                        | 396.8                                     | 0.66                                   | 0.15             | 31 (0.39)   | 5 (0.06)          |
| MFF (U)                         | 68.39                               | 24.51                        | 614.8                                     | 0.36                                   | 0.09             | 11 (0.16)   | 4 (0.06)          |
| MFA (R)                         | 143.88                              | 62.55                        | 748.1                                     | 0.35                                   | 0.07             | 19 (0.11)   | 11 (0.07)         |
| NFA (U)                         | 180.26                              | 53.59                        | 766.7                                     | 0.37                                   | 0.10             | 19 (0.13)   | 9 (0.06)          |
| RUB (R)                         | 120.50                              | 19.73                        | 749.6                                     | 0.16                                   | 0.06             | 10 (0.08)   | 4 (0.03)          |
| NFMFA (U)                       | 98.53                               | 11.18                        | 919.7                                     | 0.11                                   | 0.06             | 6 (0.06)  | 3 (0.03)          |

 Table 1.2. Comparison of stream network metrics for stream segments selected for connectivity analysis.

<sup>a</sup> The mean length of a single mainstem segment within the study reach, measured from node to node.

<sup>b</sup> Drainage density was calculated as (total stream length [km] / basin area [km<sup>2</sup>]).

<sup>c</sup> Tributary density was calculated as (number of tributaries / basin area [km<sup>2</sup>]).

Tributary frequency was also used to compare stream network similarity. Frequencies were calculated by using the total stream length for all streams in the study basin as well as the surveyed mainstem lengths only. Tributary frequencies were calculated for both tributary categories, and were not significantly different between regulated and unregulated rivers using paired two-tailed t-tests using total stream length (All Types: p=0.85, t=0.2, df=4; Perennial Only: p=0.44, t=-0.86, df=4) and mainstem only (All Types: p=0.72, t=0.38, df=4; Perennial Only: p=0.87, t=-0.18, df=4).

 Table 1.3. Comparison of stream network metrics for stream segments selected for connectivity analysis.

| River                  | Total                 | Tributary Frequency <sup>a</sup><br>(Total Stream Length) |                   | Tributa<br>(Mair | ry Frequency<br>1stem Only) |
|------------------------|-----------------------|---|-------------------|------------------|-----------------------------|
| (R=reg.,<br>U= unreg.) | Stream<br>Length (km) | All   | Perennial<br>Only | All              | Perennial<br>Only           |
| NFF (R)                | 52.52                 | 0.59  | 0.10              | 2.52             | 0.41                        |
| MFF (U)                | 24.51                 | 0.45  | 0.16              | 1.79             | 0.65                        |
| MFA (R)                | 62.55                 | 0.30  | 0.18              | 1.41             | 0.82                        |
| NFA (U)                | 53.59                 | 0.35  | 0.17              | 1.38             | 0.65                        |
| RUB (R)                | 19.73                 | 0.51  | 0.20              | 1.09             | 0.53                        |
| NFMFA (U)              | 11.18                 | 0.54  | 0.27              | 1.33             | 0.54                        |

<sup>a</sup> Tributary frequency was calculated as (number of tributaries / stream length [km]).

#### Analysis of Covariance (ANCOVA) of Tributary Distances

Using ANCOVA, transformed distance data were used with tributary frequency and density measurements for respective rivers, to determine if tributary type affected *R*. *boylii* distance to confluences, between regulated and unregulated river groups. Tributary frequencies and densities were included as covariates and tributary distances were used as the dependent variable. Significant differences in distances to nearest confluence were observed between regulated and unregulated adult and breeding data (Table 1.4).

Table 1.4. ANCOVA tests of regulated versus unregulated data for distance from *R. boylii* to nearest confluence,  $\alpha$ =0.05, p-values (\*\*=highly significant, \*= significant).

|  | Tributary Density        |                   | <b>Tributary Frequency</b> |                   |  |  |
|--|--------------------------|-------------------|----------------------------|-------------------|--|--|
|  | Test for<br>Homogeneity  |                   | Test for<br>Homogeneity    |                   |  |  |
| <i>R. boylu</i> Data Set<br>(Tributary Category) | of Regression<br>p-value | ANCOVA<br>p-value | of Regression<br>p-value   | ANCOVA<br>p-value |  |  |
| All Adults (All)                                 | 0.730                    | < 0.001**         | 0.972                      | <0.001**          |  |  |
| All Adults (Perennial)                           | 0.176                    | <0.001**          | 0.292                      | <0.001**          |  |  |
| All Breeding (All)                               | 0.200                    | 0.001**           | 0.345                      | 0.012*            |  |  |
| All Breeding (Perennial)                         | 0.700                    | <0.001**          | 0.001**                    | < 0.001           |  |  |

Underlying differences in tributary density or frequency did not influence frog distributions near tributary confluences, in any dataset. However, perennial tributary

frequency did appear to affect distances from breeding locations (Figure 1.4a–b). In this case, although breeding distances to nearest perennial confluence were significantly different between regulated and unregulated rivers (ANCOVA, p<0.001), the homogeneity of regression of tributary frequencies between these groups was also significantly different (p=0.001). Since regulated and unregulated river comparisons may be affected when using individual tributary types, all remaining statistical tests (i.e., ANOVA, Mann-Whitney U) used distances from all tributary types combined.



Figure 1.4a. Square-root transformed distances between adult *R. boylii* and nearest tributary confluence and tributary frequencies for all regulated and unregulated study rivers, with 95% CI, used in ANCOVA.



Figure 1.4b. Square-root transformed distances between *R. boylii* breeding locations and nearest tributary confluence and tributary frequencies for all regulated and unregulated study rivers, with 95% CI, used in ANCOVA.

#### Distance to Nearest Tributary Confluence

Distances from *R. boylii* locations to the nearest tributary confluences were significantly different between regulated and unregulated watersheds in all frog datasets except juveniles (Table 1.5). All significant comparisons had smaller mean distances in regulated rivers compared with unregulated rivers (Figure 1.5). Distances from unknown adults to nearest confluence were slightly significant at p=0.045, but all other adult datasets were highly significant (p<0.004).

| Table 1.5. Comparisons of regulated versus unregulated data for distance from R. boylii to |
|--|
| nearest confluence, $\alpha$ =0.05, p-values (**=highly significant, *=significant).       |

| Data Sat           | Regulated | Unregulated | 1-Way<br>ANOVA | ANOVA<br>E value | Mann-<br>Whitney | Mann-<br>Whitney |
|--------------------|-----------|-------------|----------------|------------------|------------------|------------------|
| Data Set           | (n-)      | (n-)        | (p-value)      | r-value          | (p-value)        | z-value          |
| All Adults         | 175       | 225         | <0.001**       | 41.16            | <0.001**         | 6.15             |
| Female-<br>Adults  | 48        | 96          | 0.004**        | 8.51             | 0.004**          | 2.91             |
| Male-Adults        | 96        | 85          | <0.001**       | 24.91            | <0.001**         | 4.41             |
| Unknown-<br>Adults | 31        | 44          | 0.045*         | 4.15             | 0.039*           | 2.07             |
| Juveniles          | 31        | 71          | 0.332          | 0.95             | 0.289            | 1.06             |
| Breeding           | 158       | 243         | < 0.001**      | 46.31            | <0.001**         | 6.71             |



Figure 1.5. Mean square-root transformed distances between random, R. boylii breeding, juvenile, or adult and nearest tributary confluence for all regulated and unregulated study rivers,  $\alpha$ =0.05, p-values (\*\*=highly significant, \*=significant, NS=not significant) with 95% CI.

Individual unplanned comparisons of each river pair (i.e., NFF vs. MFF) supported the trend of shorter distances to regulated tributary confluences versus unregulated confluences using all adults, p<0.01 for both ANOVA and Mann-Whitney U tests (Figure 1.6). Adult female and male *R. boylii* showed similar significant patterns between regulated and unregulated rivers, although females were slightly less significant than males (Figure 1.7). ANOVA of female adults and male adults (N=145, p=0.004, Fs=8.51; N=185, p<0.001, F=24.91, respectively) supported the pattern observed among all adults with significantly smaller regulated distances to nearest tributary confluence. Results were slightly more variable among stages, potentially from unequal or low sample sizes within river pairs, and no significant difference was observed between juveniles in regulated and unregulated rivers.



Figure 1.6. Mean square-root transformed distances (m) between *R. boylii* adults and nearest tributary confluence by river pair for all adults,  $\alpha$ =0.05, p-values (\*\*=highly significant, \*=significant) with 95% CI.



Figure 1.7. Mean square-root transformed distances (m) between *R. boylii* adult males and females and nearest tributary confluence,  $\alpha$ =0.05, p-values (\*\*=highly significant, \*=significant) with 95% CI.

Among all rivers, mean distance to nearest tributary confluence for all male adults ranged from 73.6–303.8 m and for all female adults the range was 189.6–363.9 m (Table 1.6). More individuals were observed in unregulated rivers than in regulated rivers, although fewer frogs were observed in the MFF River than the NFF River. Data on juvenile frogs were sparse, no juvenile frogs were observed in the MFF River than the MFF River, and less than ten individuals were observed in the NFF and NFMFA rivers.

|               | All<br>Regulated | All<br>Unregulated | Regulated |       |       | Unregulated |       |       |  |
|---------------|------------------|--------------------|-----------|-------|-------|-------------|-------|-------|--|
| Data Set      | Rivers           | Rivers             | NFF       | MFA   | RUB   | MFF         | NFA   | NFMFA |  |
| All Adults    | 184.7            | 303.4              | 98.1      | 189   | 239   | 234.5       | 298   | 325.2 |  |
|               | (175)            | (225)              | (43)      | (56)  | (76)  | (21)        | (110) | (94)  |  |
| Female-Adults | 206.9            | 310.8              | 216.4     | 189.6 | 220.7 | 299.2       | 276.6 | 363.9 |  |
|               | (48)             | (96)               | (5)       | (20)  | (23)  | (6)         | (54)  | (36)  |  |
| Male-Adults   | 172.6            | 291.4              | 73.6      | 172.2 | 241.2 | 233.8       | 303.8 | 291.5 |  |
|               | (96)             | (85)               | (29)      | (29)  | (38)  | (9)         | (42)  | (34)  |  |
| Unknown-      | 215.7            | 310.7              | 111.3     | 248.4 | 260.8 | 171         | 363.2 | 315   |  |
| Adults        | (31)             | (44)               | (9)       | (7)   | (15)  | (6)         | (14)  | (24)  |  |
| Juveniles     | 225              | 212                | 130.3     | 236.7 | 243.4 | -           | 194.3 | 374   |  |
|               | (31)             | (71)               | (4)       | (16)  | (11)  | (0)         | (64)  | (7)   |  |
| Breeding      | 166.4            | 280.3              | 80.6      | 257.5 | 214.7 | 157.9       | 271.9 | 305.4 |  |
|               | (158)            | (243)              | (63)      | (17)  | (78)  | (22)        | (85)  | (136) |  |

Table 1.6. Mean distances [in meters] between *R. boylii* to nearest tributary confluence by river with sample size (n=).

#### Distance to Nearest Tributary Confluence for Dataset of Random Points

Distances from random points to nearest tributary confluence were not significantly different between regulated and unregulated watersheds (Figure 1.8). Varying sample sizes were tested using ANOVA and Mann-Whitney U tests, including random points equal to total number of adult *R. boylii* observed, and three random selections of 100 points per river, no significance was observed in any dataset (Table 1.7). Individual comparisons with ANOVA and Mann-Whitney U tests of each river pair (i.e., Rubicon vs. NFMFA, MFA vs. NFA, and NFF vs. MFF) were not significant. Overall, the random point dataset was not significantly different between regulated and unregulated rivers for distance to nearest confluences, regardless of sample size or river pair, indicating *R. boylii* distribution patterns are not random, and are influenced by differences between regulated and unregulated rivers.

Table 1.7. Planned variance tests of regulated versus unregulated data for distance from random points to nearest confluence, p-values (\*\*=highly significant, \*=significant).

|                                      |           |               | 1-Way     | Mann-     |
|--------------------------------------|-----------|---------------|-----------|-----------|
|                                      | Regulated | Unregulated   | ANOVA     | Whitney   |
| Data Set                             | (n=)      | ( <b>n</b> =) | (p-value) | (p-value) |
| All Observed Adults (n) = Random (n) | 175       | 225           | 0.954     | 0.887     |
| Random (100 points per river)        | 600       | 600           | 0.649     | 0.873     |
| Feather (NFF vs. MFF)                | 100       | 100           | 0.313     | 0.346     |
| American (MFA vs. NFA)               | 100       | 100           | 0.166     | 0.078     |
| Upper American (RUB vs. NFMFA)       | 100       | 100           | 0.610     | 0.468     |



Figure 1.8. Mean square-root transformed distances (m) between random points and nearest tributary confluence by watershed, α=0.05, p-values (\*\*=highly significant, \*=significant, NS=not significant) with 95% CI.

#### Proportion within Confluence Buffer Intervals

Tributary confluences were buffered from the centroid of the confluence at equal intervals, and the proportion of frogs within each centroid was calculated. There was a strong correlation of the proportion of *R. boylii* observed within increasing tributary confluence buffer intervals, or more simply, there were more frogs observed closer to tributary confluences. Approximately 80% of all observed frogs were within 300 meters of a tributary confluence in regulated rivers, and within 400 meters of a confluence in unregulated rivers (Figure 1.9). All *R. boylii* were observed within 600 meters of a tributary confluence, regardless of regulation type, indicating tributary confluences are important for all lifestages. Slight differences between regulated and unregulated curves were observed, but were not significant using ANOVA (Assuming independent samples, regulated rs. unregulated regulated: r=0.982; z=1.01, two-tail p=0.3125). Because *R. boylii* data were pooled for this analysis, individual lifestages were not analyzed separately, and further analysis may show more discrete differences between regulated and unregulated study rivers.



Figure 1.9. Proportions of adult and breeding *R. boylii* observed within Euclidean buffer distances of tributary confluences.

#### DISCUSSION

*Rana boylii* population distributions in regulated rivers are more closely associated with tributary confluences compared to those in unregulated rivers. Although analysis of a greater dataset (including more regulated and unregulated rivers) may provide greater acuity, spatial clustering of *R. boylii* near tributary confluences suggests these riverscape components are important for population longevity. As other studies on *R. boylii* have shown, tributary confluences provide a wide range of habitat types within a small area, which may reduce energetic costs associated with frog movement while maximizing resources for all lifestages (Kupferberg 1996, Benda et al. 2004, Yarnell et al. 2006). The strong statistical significance of both adults and breeding locations with tributary confluences in regulated rivers indicates tributaries are important spatial components for this species.

Existing research has shown flow regulation can limit the distribution and abundance of riverine species (Power et al. 1996, Poff et al. 1997), as well as homogenization of river habitat and ecosystem function (Poff et al. 2007, Baker et al. 2010). Hydroelectric operation type (i.e., peaking reach vs. bypass reach) can certainly magnify the regulation impacts on habitat diversity by eliminating the necessary hydrologic variability required for creation and maintenance of riverine habitat. Rana boylii distributions in the MFA peaking reach were starkly different than in any other reach, regulated or unregulated, and breeding was only observed within tributaries. In addition, the MFA and unregulated NFA had comparable tributary frequency and density, indicating flow regulation is the critical difference in *R. boylii* distribution for this river pair. The bypass reach (RUB) and associated unregulated river pair (NFMFA) supported a similar pattern of frogs occurring closer to tributary confluences in the regulated reach, but it is difficult to compare results from the bypass reach with the peaking reach because of underlying differences in watershed size. Differences in watershed size and number of tributaries made it difficult to use data from the Feather watershed, and survey data from the MFF were difficult to collect due to rugged terrain. Nonetheless, R. boylii exhibit a stronger affinity for tributary confluences in regulated rivers compared with unregulated rivers.

Habitat, dispersal, and population size are three main factors that may explain why *R*. *boylii* distribution in regulated rivers was more closely tied to confluences than in unregulated rivers, and each factor may act synergistically to amplify the overall effect. Habitat diversity and availability play strong roles in influencing *R*. *boylii* population distributions (Yarnell 2005, Haggarty 2006). Tributary confluences may offer maximum available habitat diversity with minimal dispersal cost. Tributary confluences are generally much more geomorphically diverse stream regions (Benda et al. 2004), as well as potentially buffering perturbations from regulation to flow and water temperature regimes, which may contribute to the importance of confluences in regulated reaches for *R. boylii*. Regulation of flow limits variation required for essential river functions like habitat formation, sediment transport, and food-web linkage (Power et al. 1996, Poff et al. 1997). *Rana boylii* generally prefer breeding habitat with gravel or cobble substrates, and these substrates tend to accumulate in bars, particularly near confluences. Regulated rivers often reduce sediment supply (Poff et al. 1997, Baker et al. 2010), which is a key

component to the creation of habitat heterogeneity, such as cobble bars (Yarnell et al. 2006, Richter and Thomas 2007). Spatial habitat heterogeneity influences the ability for species persistence, and ultimately, *R. boylii* require the natural river dynamism from which they have successfully evolved for continued survival.

Dispersal and movement for *R. boylii* may be more difficult in regulated rivers fragmented by peaking flows, dams, and reservoirs. For example, the Middle Fork of the American River can fluctuate nearly a meter (change in overall river stage or depth) in several hours, and higher flows may be sustained for prolonged periods before returning to the original base flow. Because dispersal is important for gene flow as well as potential re-colonization of populations, and since these frogs appear to remain in the immediate vicinity of the channel (Bourque 2008), large changes in flow that do not correlate with natural weather events have a high probability of reducing successful dispersal in the river network. Connectivity between populations in regulated rivers with aseasonal fluctuations in flow may be unidirectional (i.e., downstream only) or nonexistent because of the difficulty in moving within the drainage. Although it is unlikely that movement ceases completely due to reservoir/dam presence, the probability that individuals will successfully reach neighboring subpopulations may be minimal. Furthermore, it may only require a few individuals to expand or connect a population (Ficetola et al. 2008); therefore flow management has the potential to greatly alter population dispersal and overall connectivity in R. boylii populations.

Population size and seasonality may be the factors that are most critical to conservation, and yet most overlooked. Frogs breed in or near tributary confluences first and expand or disperse out from these locations when existing habitat is occupied or no longer existent (Kupferberg 1996). In 2010, breeding was observed in tributaries to the MFA that previously were not utilized for breeding 2009. Consequently, populations that aggregate near tributary confluences and do not appear to utilize habitat on the mainstem of a river may indicate breeding was not feasible in the mainstem (due to habitat constraints, availability, movement, etc.) for that season, or tributary habitat was suitable for that given year. In 2010, breeding in the NFA was observed in Robbers Ravine, a small tributary of the NFA. Flow in Robbers Ravine during 2009 was limited and nearly dry by early July, however in a much wetter 2010, there was still flow and significant pool connectivity throughout the creek in late July. If population sizes are large enough to expand into seasonably suitable habitat, it appears tributaries may provide important habitat for breeding as well as breeding. Small populations may not have the necessary abundance for successful dispersal into adjacent habitats, and if flow regulation limits connectivity within the river network, population isolation will ensue.

If *R. boylii* populations are of sufficient size for expansion into intermittent and ephemeral tributaries during higher water years, these tributaries may provide higher probability of reproductive survival compared with the mainstem river, because they remain sheltered and more accessible earlier in the breeding season as flows recede. *Rana boylii* populations appear to have great plasticity in the habitat they can utilize, but breeding requires a specific range of environmental conditions for successful survival (Lind and Yarnell 2008). Based on connectivity patterns observed between regulated and

unregulated rivers, tributaries are exceedingly critical for *R. boylii* in regulated reaches where breeding may not be possible during any water year. The connectivity results indicate frog populations in regulated reaches are restricted to tributaries, and in the case of peaking reaches such as the MFA, successful re-colonization of suitable breeding habitat in adjacent tributaries during wet years may be difficult if movement is limited or populations are too small to support adequate dispersal. If this pattern is repeated for many generations, populations are likely to collapse, particularly if population connectivity is further restricted by other factors like habitat alteration or invasive species introductions in critical tributary habitat.

Extant populations must have sufficient plasticity (in dispersal ability, habitat diversity, and population size) to maximize survival. Channel and Lomolino (2000) found range reductions leading to wide-scale declines began in the periphery of a species historical range, yet remnant populations simultaneously contracted towards the periphery of the historical range. This means that peripheral populations most likely to be extirpated also occur in the habitat that is most critical during range contraction, and moreover, these peripheral populations may be key sources for future recolonization and range expansion. Therefore the importance of peripheral populations should not be understated and limiting connectivity with potential dispersal habitats can only compound *R. boylii* population declines. Maintaining connectivity within declining populations becomes paramount for conservation of genetic diversity, because connectivity promotes gene flow, which can reduce the impact of fragmentation in small populations (Frankham et al. 2002).

Future research should incorporate additional rivers to further assess fragmentation and connectivity for *R. boylii*, as well as similar riverine vertebrates. Incorporating more specific telemetry data into a connectivity model may provide a more accurate friction map of fragmentation and connectivity in *R. boylii* populations. In addition, a better understanding of how climate change will alter the hydrology of these regulated rivers versus unregulated rivers must be a component of future research. Creating conceptual models can be very useful for conservation management, particularly for identification of important landscape corridors and ecosystem functions.

#### CONCLUSION

For *R. boylii*, river regulation has a significant spatial effect on population distribution. Conservation management of this sensitive amphibian species requires an understanding of not only population abundance and life history requirements, but also the underlying anthropogenic landscape affects that ultimately may dictate population persistence.

Stream networks analyzed in this study were not significantly different between regulated and unregulated systems, thus underlying landscape structural differences cannot explain the observed differences in frog distribution in relation to confluences. In regulated rivers *R. boylii* distributions were significantly closer to tributary confluences compared with frogs in unregulated rivers. This pattern was not random and reflects a general reduction in population connectivity and an increase in population fragmentation in regulated systems.

*Rana boylii* has been successful in these dynamic river environments for approximately eight million years (Macey et al. 2001), yet it has only taken the last 150 years for humans to permanently and drastically transform riverscapes in the Sierra foothills and Pacific coast-ranges. In regulated systems, poorly timed flow releases will only aggravate existing watershed fragmentation. This species requires use of all habitats from small creeks to big rivers within a basin, and if population connectivity continues to decrease in regulated systems, extant populations will be ill equipped to face stochastic changes in the future. Currently conservation management in hydro-regulated rivers does not emphasize tributaries as important components for R. boylii. Most surveys conducted for the species require only peripheral surveys in perennial tributaries, and monitoring often does not include all tributaries in a watershed, as they are often considered outside of the impact of the project nexus. Future protection and enhancement measures for extant R. boylii populations should promote greater hydrologic variability to increase habitat diversity and increase population connectivity, and analyses of population movement and dispersal in order to ensure gene flow and long-term population viability in the watershed

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# Chapter 2: Assessing Genetic Diversity and Genetic Fragmentation of *R. boylii* Populations in Regulated and Unregulated Rivers

#### **INTRODUCTION**

The foothill yellow-legged frog (*R. boylii*) is an endemic species to California and Oregon, and unique as a river dwelling species. Organisms like *R. boylii* represent the integration of millions of years of geological change and biological evolution (Karr and Chu 2000), and connect to the watershed through a wide range of aquatic and terrestrial interfaces (Kupferberg 1996, Lind 2005, Yarnell 2005, Wheeler 2007). *Rana boylii* require use of all habitats from small creeks to big rivers within a basin. Tadpoles cannot mature into frogs without access to sunlit channels with abundant algal foods, while juveniles and adults cannot survive winter floods without access to refugia like small tributaries (Twitty et al. 1967, Kupferberg 1996). This frog species is important because it links with multiple interfaces in a dynamic and complex ecosystem, and as a current California Species of Special Concern (Jennings and Hayes 1994, CDFG 2008); *R. boylii* currently lack large-scale conservation efforts implemented in federally threatened or endangered species.

#### Foothill Yellow-Legged Frog Life History

*Rana boylii* have declined from over fifty percent of their historic range (Davidson et al. 2002), and the current distribution ranges from the southern foothills of the Sierra Nevada, north to Oregon, and along the Pacific Coast Range from central California to southern Oregon (Jennings and Hayes 1994, Stebbins 2003, Lind 2005). Historically, they may have ranged from sea level to an elevation of 1,830 m (Zweifel 1955), but current populations are rarely observed over 915 m. Of the many rivers or streams within the current species range, few do not include a dam or diversion of some kind (Carle 2004, Lind 2005).

*Rana boylii* has been extant as a native species in the rivers and streams of California and Oregon for approximately 8 million years (Case 1978, Macey et al. 2001), yet it has only taken the last 150 years for human influence to permanently and drastically transform their habitat (Mount 1995, Karr and Chu 2000). Currently, many existing *R. boylii* populations in rivers on the western slope of the Sierra Nevada occur in rivers in which flow is regulated by hydroelectric projects, including the McCloud, Pit, Butte, North Fork Feather, South Fork Feather, North Yuba, Middle Yuba, South Yuba, Bear River, Middle Fork American, South Fork American, North Fork Mokelumne, and San Joaquin Rivers. It is difficult to find a higher order river without river regulation draining from the Sierra Nevada, and even fewer that contain extant *R. boylii* populations. Studies on the effects of river regulation on *R. boylii* have largely focused on the negative impacts pulse flows (or aseasonal flow fluctuations) have on *R. boylii* breeding and breeding habitat (Lind et al. 1996, Lind 2005, Kupferberg et al. 2008). Large changes in flow can scour egg masses from breeding locations, and *R. boylii* females only lay one egg mass per year,

thus loss of any egg mass can severely reduces population fecundity (Kupferberg et al. 2008, Lind and Yarnell 2008).

In addition, gene flow and habitat connectivity can be indirectly affected by the way a river is managed, not necessarily simply through dam presence. Gene flow is the successful migration of alleles between populations, and is dependent upon population size and migration rates (Frankham et al. 2002). Aseasonal high flows that restrict connectivity within a watershed (i.e., prevent movement from one tributary to another, alter habitat due to regulation of flow, allow dispersal or introduction of non-native species into a region) may have greater impacts on local population dynamics (Lind et al. 1996, Reese and Welsh 1998, Gibbs 2001, Pearl et al. 2004, Lind and Yarnell 2008, Kupferberg et al. 2008). Lind et al. (1996) found that regulated flows permitted the encroachment of riparian vegetation, which limited *R. boylii* breeding habitat by increasing shade, and causing further incision and armoring of the streambed. Kupferberg (1997) found that introduced American bullfrogs (*Rana catesbeiana*) caused a reduction in *R. boylii* size at metamorphosis.

River regulation has been shown to impact breeding in *R. boylii* (Lind et al. 1996, Lind and Yarnell 2008, Kupferberg et al. 2008), but the effects of river regulation on gene flow within and among populations, dispersal rates, movement, overwintering behavior and habitat, or development rates have not been studied. *Rana boylii* demographic data are generally lacking throughout the Sierra Nevada range, and large gaps in knowledge on the life history of this species persist. Successful conservation management strategies in regulated systems for this species depend upon filling these gaps.

#### Study Questions

Identifying potential regulation impacts on *R. boylii* genetic structure and spatial distributions are critical for future conservation and management of this species. To determine if *R. boylii* populations in watersheds regulated by hydroelectric generation (regulated) had less genetic diversity and lower gene flow compared with watersheds without dams or hydroelectric generation (unregulated), a genetic analysis was conducted. Two different genetic markers were used, mitochondrial DNA (mtDNA) and nuclear DNA (random amplified polymorphic DNA [RAPD]).

The study focused on the following question:

Do regulated watersheds with flow regulation and reservoir impoundments have limited gene flow and lower genetic diversity within and among *R. boylii* populations in comparison to unregulated watersheds?

Genetic data can be used to assess population structure and provide a comparison for future population studies. Baseline data that can be used in future comparisons are critical to understand landscape impacts at a genetic level, which are particularly relevant due to the difficulties associated with distinguishing between human-caused amphibian declines and natural population fluctuations (Semlitsch et al. 1996, Kimberling et al. 1996). Ultimately, determining a population's genetic structure will give clarity and power to restoration preservation, and re-introduction efforts.

Genetic markers have been used with increasing frequency in the last several decades to study conservation genetics issues such as species boundaries (Shaffer et al. 2004, Rowe and Beebee 2007, Gamble et al. 2008), relatedness (Wang 2004, DeWoody 2005), fragmentation (Funk et al. 2005b), gene flow (Austin et al. 2004), population connectivity (Vos et al. 2001), phylogeny (Macey et al. 2001), and diversity (Deiner et al. 2007). Limited gene flow can cause a reduction in the number of haplotypes, an increase in the number of population demes (locally interbreeding groups within a larger population in the watershed), and distinct genetic structuring within populations. Gene flow and the maintenance of genetic diversity is a driving force behind population structure and ultimately an important step in the process leading to speciation or extinction (Avise 1994, Barber 1999, Gibbs 2001, Frankham et al. 2002). Genetic structuring may be occurring among R. boylii populations in regulated and unregulated watersheds due to naturally small population sizes and potentially strong philopatric associations with breeding habitat. However, if hydroelectric river regulation limits gene flow and population connectivity due to fragmentation affects, it would be expected that demes within unregulated rivers would have greater genetic diversity when compared with populations in regulated rivers.

#### **MATERIALS AND METHODS**

#### Study Area

Regions from six rivers; the North Fork Feather (NFF), Middle Fork Feather (MFF), Middle Fork American (MFA), North Fork American (NFA), Rubicon (RUB), Upper Middle Fork American (UMFA), and the North Fork Middle Fork American (NFMFA) were selected using a paired-design (Table 2.1). Each pair consisted of a regulated and unregulated river reach of similar stream order size and geographic location (Figure 1.1). Due to difficult terrain, river access, and frogs sampled, genetic data collected in the NFF and MFF were not sufficient for comparisons, and these reaches were not included in the genetic study. Study reaches were selected to maximize survey data on frog populations, therefore information on accessibility, data on current population presence, and reconnaissance visits were compiled before choosing the final stream segments. Genetic comparisons within populations were from sampling locations less than 10 km apart, to avoid confounding patterns associated with geographic distance (i.e., for gene flow see Dever 2007, Monsen and Blouin 2003, for movement see Bourque 2008) [Figures 2.1–2.3]. All stream segments began and ended at tributary confluence locations to correlate genetic and riverscape connectivity spatial scales (Frissell et al. 1986).

Within regulated rivers, three different hydroelectric power operation types were included, to analyze potential differences in the effects of operation type on genetic differentiation (Table 2.1). These general categories encompass typical components of most hydroelectric regulation operations, and a majority of hydroelectric projects consist of combinations of these types. An additional study river was included to analyze potential reservoir and dam affects, the Upper Middle Fork American (UMFA), which was separated from the RUB and NFMFA by Ralston Reservoir (Figure 2.3).

| Divor                 |                                    | Divor |                          | Mainstem<br>Sogmont |
|-----------------------|------------------------------------|-------|--------------------------|---------------------|
| Pair                  | River                              | Code  | River Type               | Length (km)         |
| Easth on <sup>a</sup> | North Fork Feather                 | NFF   | Regulated (Run of River) | 3                   |
| Feather               | Middle Fork Feather                | MFF   | Unregulated              | 5                   |
| Amorican              | Middle Fork American               | MFA   | Regulated (Peaking)      | 10                  |
| American              | North Fork American                | NFA   | Unregulated              | 6                   |
| Unnor                 | Rubicon                            | RUB   | Regulated (Bypass)       | 5                   |
| American              | North Fork Middle Fork<br>American | NFMFA | Unregulated              | 5                   |
| N/A                   | Upper Middle Fork American         | UMFA  | Regulated (Bypass)       | 2                   |

Table 2.1. Study rivers and segment lengths.

<sup>a</sup> Insufficient samples for population genetic analysis



Figure 2.1. Study section of the regulated MFA River with locations of *R. boylii* DNA samples.



Figure 2.2. Study section of the unregulated NFA River with locations of *R. boylii* DNA samples.



Figure 2.3. Study sections of the NFMFA, Rubicon, and UFMFA Rivers with locations of *R*. *boylii* DNA samples.

#### Field Methodology

Contiguous reaches were surveyed for *R. boylii* of all lifestages between mid May and early August 2009 and for juveniles and adults in June and July 2010. Data collected included frog location, stage, sex, morphometric measurements, survey time, and habitat use. Waypoints for each frog location were logged in decimal degrees, with a handheld GPS receiver using NAD83 datum, and averaged for approximately 30 seconds per location to increase accuracy (generally 3–5 meters per point).

#### **DNA Sampling**

In order to determine genetic diversity in frog populations, buccal swabs were used to collect DNA from *R. boylii* adults in 2009. The buccal cavity (mouth) of each frog was swabbed using Epicentre Catch-All<sup>TM</sup> Sample Collection Swabs following Epicentre protocols. The mouth of each frog was opened with a sterile plastic ruler, and a new buccal swab was rotated on the lower surface of the tongue for 15 seconds on each side of the buccal cavity. The swab was then air dried at ambient temperatures (generally greater than  $34^{\circ}$ C). The swab was then capped and labeled, and stored in a sterile plastic bag. Buccal swabs are considered highly advantageous compared with toe clips because swabs are less invasive and DNA is generally easier to isolate from swabs than tissue, and has been used successfully in other amphibian species (Pidancier et al. 2003, Broquet et al. 2007). Preliminary results of buccal swab functionality in a pilot study on *R. boylii* in the South Fork Eel River suggested this method provided adequate DNA and did not cause any tissue damage to the frog, however, additional lab research showed

inconsistencies in polymerase chain reaction (PCR) amplification and sequencing, as DNA levels were extremely low in several samples. Therefore, buccal swabs were only used in 2009, and tissue sampling was conducted in 2010. Tissue was collected from each post-metamorphic frog caught in 2010 following USGS (2001) toe clipping protocols. The longest toe on the left foot was clipped at the most distal joint and immediately placed in 70% ethanol in a cryogenic tube. Buccal DNA was isolated using the Epicentre BuccalAmp<sup>TM</sup> DNA extraction kit and DNA from tissue was isolated using the Qiagen DNeasy kit. DNA concentrations were quantified using a Nanodrop. Samples were stored at -80°C, and aliquots of 50  $\mu$ l were stored at -20°C for PCR reactions.

#### Molecular Methodology

Choice of molecular marker for the examination of genetic structure is important as different markers can produces incongruent results (see Milá et al. 2010) due to differences in mutation rate and inheritance pattern. For this study, two types of genetic markers, mtDNA and RAPDs were used to determine genetic haplotype variability, gene flow, and genetic structuring within and among *R. boylii* populations in regulated and unregulated watersheds.

#### Mitochondrial DNA

Mitochondrial DNA has a much higher rate of nucleotide substitution relative to nuclear DNA, because it does not contain the proofreading machinery that nuclear DNA does (DeWoody 2005), and it has several mutational hot spots (Galtier et al. 2005). In addition, mtDNA does not deviate significantly from expectations of neutral evolution (Bos et al. 2008). Thus mtDNA is a fairly stable marker not influenced by environmental selection that can be used to determine gene flow among populations, as it is distributed among individuals rather than within and among individuals (Avise 1994, Moritz 2002, DeWoody 2005). Since mtDNA is maternally inherited and is haploid, it provides a robust indicator of genetic drift and has been used extensively in determining lineage and population structure (Shaffer et al. 2004, DeWoody 2005, Nielsen et al. 2006).

For this study, a region of the NADH dehydrogenase subunit-2 gene (ND2) mtDNA was amplified and sequenced. This region was chosen because substitution rates are elevated at ND2 relative to other regions of the mtDNA genome (Bos et al. 2008). Following methods described in Dever (2007), PCR primers yielded a 1000 base pair (bp) amplicon, and final PCR conditions included 5.0  $\mu$ L of each primer (tRNAtrp\_R: 5'-TTA AAG GGC CTG AGT TGC ATT-3' and tRNAmet\_F: 5'-AAG CTT TCG GGC CCA TAC C-3', Lind 2005), 5.0  $\mu$ L of PCR buffer (Tris-Cl), 4.0  $\mu$ L of dNTP, and 0.24  $\mu$ L of *Taq* polymerase in a final volume of 50  $\mu$ L. The reaction consisted of 30 cycles of 30s at 94°C, annealing for 30s at 56°C, and elongation for 1 min at 72°C. Amplified product was separated and visualized on an agarose gel stained with ethidium bromide, samples were then cleaned with a Millipore kit and sent to Davis Sequencing Lab for sequencing.

Sampled mtDNA sequences were aligned and used to calculate descriptive population genetic statistics including Nei's (1987) haplotype diversity (h), and Tajima's D (to test

gene neutrality) (Tajima 1989). Intersite variation was calculated using pairwise genetic distances from  $G_{ST}$  (Nei 1973), which is comparable to Wright's F-statistic,  $F_{ST}$ , and is a measure of differentiation among subpopulations (Wright 1943). The level of dispersal and gene flow between subpopulations was also estimated using a variance-partitioning algorithm (AMOVA, Excoffier et al. 1992) calculated in ARLEQUIN (Excoffier et al. 2005). Mantel's test (Mantel 1967) was also used to compare correlations in genetic and geographic distance matrices with 10,000 randomizations, to test for isolation-by-distance.

#### RAPDs

Random amplified polymorphic DNA markers were used to estimate genetic diversity within and among populations. RAPD markers are suitable for estimating genetic diversity in a population, and are a useful and cost-effective marker in population genetic studies (Lynch and Milligan 1994, Gibbs and Weatherhead 1994, Bagley et al. 2001, D'Surney et al. 2001, Karuppudurai et al. 2007). RAPDs are highly abundant within most species, and many polymorphic loci have been reported (as many as several hundred) (D'Surney et al. 2001, Wang 2004). RAPDs have the ability to detect microdifferentiation between genetically similar subpopulations (by examining loci with high number of alleles) (Nei 1973), and have been demonstrated to quantify levels of diversity in R. boylii (Dever 2007). Compared with mtDNA, RAPDs have higher mutation rates, which may reflect more recent changes in the genetic structure of a population (DeWoody 2005, Dever 2007). In addition, RAPDs are much easier to develop, generate greater numbers of loci for genetic analysis, require less DNA than other markers, and are an inexpensive PCR-based technique for generating a DNA banding profile for each individual (Lynch and Milligan 1994, Kimberling et al. 1996, Dever 2007). This makes RAPDs a good alternative for studying rare species, or populations that have very small population sizes, as the study impact is minimal (Kimberling et al. 1996). Nonetheless, RAPDs have certain limitations as they are not locus specific, and are dominant markers, which means estimation of diversity cannot be as accurate (i.e., only possible to estimate a single parameter when there may be two in actuality) as other locus specific and co-dominant markers (Lynch and Milligan 1994, Wang 2004). Co-dominant markers are generally preferred over dominant markers, because all alleles in a genotype are observable phenotypically, so allele frequencies and relatedness are more accurately estimated (Lynch and Milligan 1994, Wang 2004). RAPDs are also not as easily repeatable as markers like microsatellites or AFLPs (Lynch and Milligan 1994, Kimberling et al. 1996).

In order to avoid biased parameter estimates, Lynch and Milligan (1994) and Wang (2004) outlined several steps that can be taken, including sampling more individuals, equalizing sampling sizes in different populations, using a similarity estimator, and standardizing the amplification protocol. Nei (1987) observed that small sample sizes do not have as adverse an effect in estimating genetic diversity and genetic distance when large numbers of loci are examined (Gorman and Renzi 1979, Kimberling et al. 1996). Assumptions that were required for RAPD analysis include: 1) each marker must represent a Mendelian locus in which the amplified marker allele is in Hardy-Weinberg equilibrium with a null recessive allele; and 2) marker alleles from different loci do not

co-migrate to the same position, or no linkage disequilibrium (Lynch and Milligan 1994, Kimberling et al. 1996). It has been noted that RAPDs provide useful estimates of genetic variation if awareness of the limitations and assumptions are taken into account (Lynch and Milligan 1994, Kimberling et al. 1996, D'Surney et al. 2001).

PCR reactions for each sample utilized the Ready-To-Go<sup>™</sup> RAPD Analysis Beads Kit (Biosciences) since it included all the necessary PCR components in a dried bead optimized for RAPD amplification (Wagner et al. 2005, Dever 2007). Following methodology described in Dever (2007), RAPD primers #2, #4, and #5 were used as they produced identifiable polymorphic bands. Banding patterns were created for each individual using a stratified sampling system, and each band was identified using a binary code. Three different individuals conducted separate band counts to provide an accurate estimate of band diversity. Allele frequencies for RAPDs were estimated following Lynch and Milligan (1994), as described in Dever (2007). Genetic differentiation was calculated using the program TFPGA (Miller 1998) to calculate  $\theta$  (Weir and Cockerham 1984), which is a measure of genetic distance that has little bias and can be used across many genetic data, and a bootstrap procedure consisting of 1000 replicates over loci to determine 95% confidence limits (Dever 2007). F<sub>ST</sub> and pairwise genetic distances between subpopulations were estimated using Reynolds et al. (1983) coancestry distance in ARLEOUIN (Excoffier et al. 2005). Coancestry distance is an inbreeding coefficient. used as the basis for measurement of genetic distance in short-term evolution, when divergence between populations with a common ancestral population is considered solely due to drift (Reynolds et al. 1983). Mantel's tests were used to analyze isolation-bydistance with 10,000 randomizations in the web program IBD (Jensen et al. 2005) following methods outlined in Dever (2007).

#### RESULTS

Buccal swabs were collected from 160 individuals in 2009. Successful PCR amplification and sequencing of mtDNA from buccal swabs was extremely limited, ND2 sequences were obtained from only 15 samples provided for analysis. Various PCR methods were used to improve and refine the mtDNA PCR amplification, however, success rates were negligible. DNA quantities were low and an unknown inhibitor may have been present in the buccal extraction kit, but specific causes were not identified. An additional 15 samples were utilized from tadpole tissue samples collected by A. Lind, in 2009, however the overall low sample size prohibited statistically valid mtDNA analyses for samples collected during the 2009 field season (Table 2.3). Additional field sampling in was conducted in the summer of 2010 to provide sufficient samples for mtDNA analysis.

#### Mitochondrial DNA

In total, 62 *R. boylii* were sampled and sequenced (Table 2.2). An alignment of a segment of 583 base pairs yielded a total of 20 haplotypes and had a haplotype diversity of 0.625 for all study rivers. Tajima's D neutrality test was significant for non-synonymous sites, indicating this variation was not neutral. The MFA had the highest haplotype diversity (0.956), and the RUB had the lowest (0.295) among all study rivers (Table 2.3). Among river pairs, the unregulated NFMFA had higher haplotype diversity, number of polymorphic sites, and haplotypes compared to the regulated RUB. The NFA had lower haplotype diversity compared with its regulated river pair, the MFA.

| Regulation  |  | Adult  | Adult |          |         |    |
|-------------|--|--------|-------|----------|---------|----|
| Туре        | River                                      | Female | Male  | Juvenile | Tadpole | N= |
| Peaking     | Middle Fork American (MFA)                 | 4      | 5     | 1        | 0       | 10 |
| Unregulated | North Fork American (NFA)                  | 8      | 4     | 3        | 8       | 23 |
| Bypass      | Rubicon (RUB)                              | 1      | 5     | 2        | 5       | 13 |
| Unregulated | North Fork Middle Fork<br>American (NFMFA) | 3      | 5     | 8        | 0       | 16 |

Table 2.2. Demographic data on *R. boylii* individuals sequenced for mtDNA analysis.

| Table 2.3.  | Rana boylii mtDN | sequence data from  | n NADH 2 gene fron   | 1 583 hn segment. |
|-------------|------------------|---------------------|----------------------|-------------------|
| 1 abic 2.5. | Mana obym midin  | a sequence data nom | i i widhi a gene non | 1 Job op segment. |

|                    |  | Samples             | рі                          | Pars.              |                |                      |
|--------------------|--|---------------------|-----------------------------|--------------------|----------------|----------------------|
| Regulation<br>Type | River                                      | Sequenced 2009–2010 | Poly.<br>Sites <sup>a</sup> | Sites <sup>b</sup> | h <sup>c</sup> | $\mathbf{H}_{d}^{d}$ |
| Peaking            | Middle Fork American (MFA)                 | 10                  | 9                           | 6                  | 8              | 0.956                |
| Unregulated        | North Fork American (NFA)                  | 23                  | 7                           | 1                  | 4              | 0.383                |
| Bypass             | Rubicon (RUB)                              | 13                  | 2                           | 1                  | 3              | 0.295                |
| Unregulated        | North Fork Middle Fork<br>American (NFMFA) | 16                  | 12                          | 6                  | 9              | 0.817                |
| All Rivers         |  | 62                  | 21                          | -                  | 20             | 0.625                |

<sup>a</sup> Polymorphic sites in ND2 *R. boylii* mtDNA sequences

<sup>b</sup> Parsimony informative sites in ND2 *R. boylii* mtDNA sequences

<sup>c</sup> Number of haplotypes within population

<sup>d</sup> Haplotype diversity

#### RAPDs

Buccal swabs were successful for analysis of RAPD markers, and 98 individuals were selected for analysis, yielding 199 total loci (Table 2.4). Sample sizes obtained in 2009 in the NFF and MFF were not large enough to provide  $F_{ST}$  estimates, as no subpopulations could be compared within these rivers. However, estimates of the number of polymorphic sites (or band variation) indicated a greater diversity of bands in the MFF than in the NFF.

|            |                               |            | Useable | No. of                   |
|------------|-------------------------------|------------|---------|--------------------------|
| River      | Location                      | N=         | Loci    | <b>Polymorphic Sites</b> |
| NFF        | Pulga                         | 8          | 64      | 64                       |
| MFF        | Milsap Bar                    | 12         | 90      | 90                       |
| MFA        | American Canyon               | 6          | 99      | 39                       |
|            | Gas Canyon                    | 6          | 99      | 38                       |
|            | Todd Creek                    | 5          | 99      | 39                       |
|            | Slug Gulch                    | 4          | 99      | 33                       |
| NFA        | Shirttail Creek               | 4          | 120     | 31                       |
|            | Robbers Ravine                | 15         | 120     | 102                      |
|            | Secret Ravine                 | 4          | 120     | 41                       |
| RUB        | Upstream of Ralston Reservoir | 8          | 99      | 63                       |
|            | Long Canyon                   | 7          | 99      | 72                       |
| NFMFA      | Skunk Canyon                  | 7          | 85      | 50                       |
|            | El Dorado Canyon              | 8          | 85      | 59                       |
| UMFA       | Near Campground               | 4          | 99      | 44                       |
| All Rivers |                               | <i>9</i> 8 | 199     | 199                      |

| Table 2.4.  | Rana bovl | <i>ii</i> sample d | ata from | 2009 | RAPD   | markers.   |
|-------------|-----------|--------------------|----------|------|--------|------------|
| 1 abic 2.4. | Munu voya | <i>i</i> sample u  | ata mom  |      | IVII D | mai Kei 5. |

#### North Fork American (NFA) and Middle Fork American (MFA)

Based on TFPGA estimates of average  $\theta$ -value with 1000 bootstrap replicates, there was greater genetic differentiation between NFA subpopulations compared with peaking reach MFA subpopulations (NFA:  $\theta = 0.0418$ , MFA:  $\theta = 0.0175$ ), while estimates of overall F<sub>ST</sub> obtained from AMOVA indicated greater genetic differentiation in MFA subpopulations [NFA = 0.084, MFA = 0.129] (Table 2.5). Mantel tests of isolation-bydistance indicated differentiation between subpopulations in the NFA were strongly correlated with geographic distance (Z =0.195, r = 0.994, p < 0.0001; Figure 2.4). The river distance between NFA (1 to 2) and MFA (1 to 4) subpopulations was approximately 8.5 km, measured from tributary confluence to tributary confluence along the river network (Figure 2.1–2.2). However, isolation-by-distance was not a significant factor for MFA subpopulations (Z = 0.059, r = 0.097, p > 0.462; Figure 2.5), despite an overall  $F_{ST}$ value indicating more differentiation within subpopulations than any other river. Results suggest a significant population structuring effect occurring in the peaking reach of the MFA, and compared with all other rivers, frogs were only observed in tributaries to the MFA (versus the mainstem and tributaries). Distribution of R. boylii within the peaking reach appears to support the high level of genetic drift ( $F_{ST}$ ) observed, and no breeding was observed in the mainstem portion of the river (the peaking reach).

|                |           | Total    | Arlequin        |         |
|----------------|-----------|----------|-----------------|---------|
|                | Number of | Loci     | AMOVA           | TFPGA   |
| Comparisons    | Subgroups | Observed | F <sub>ST</sub> | θ (avg) |
| NFF            | 1         | 199      | n/a             | n/a     |
| MFF            | 1         | 199      | n/a             | n/a     |
| MFA            | 4         | 108      | 0.1290**        | 0.0175  |
| NFA            | 3         | 120      | 0.0843**        | 0.0418  |
| NFMFA          | 2         | 85       | 0.0366          | 0.0130  |
| RUBICON        | 2         | 99       | 0.0410          | 0.0370  |
| UMFA-NFMFA-RUB | 3         | 103      | 0.0834**        | 0.0294  |

Table 2.5. Estimates of  $F_{ST}$  and  $\theta$  from 2009 *R. boylii* RAPD markers,  $\alpha$ =0.05. (AMOVA p-values: \*\*=highly significant ≤0.01, \*= significant ≤0.05).



Figure 2.4. Unregulated NFA River subpopulations: Influence of isolation by geographic distance log transformed (km) on genetic distance from Reynolds et al. (1983) coancestry in RAPD variation. Mantel test: Z = 0.195, r = 0.994, p < 0.0001, based on 10,000 replicates.



Figure 2.5. Regulated MFA River (in peaking reach) subpopulations: Influence of isolation by geographic distance log transformed (km) on genetic distance from Reynolds et al. (1983) coancestry in RAPD variation. Mantel test: Z = 0.059, r = 0.097, p > 0.462, based on 10,000 replicates.

#### North Fork Middle Fork American (NFMFA) and Rubicon (RUB)

Comparison of  $\theta$  and  $F_{ST}$  indicated little difference in subpopulation differentiation between the smallest river pair (in watershed size), although the average  $\theta$ -value was much greater in the RUB compared with the NFMFA. The RUB is a bypass reach, and frogs were distributed much more evenly through the reach, compared with the peaking MFA reach. Distributions of *R. boylii* observed during surveys of the NFMFA and RUB were similar, yet the average  $\theta$ -value of subpopulations in the RUB was 0.037, compared with 0.013 in the NFMFA. The  $F_{ST}$  p-value was nearly significant in the RUB, indicating there may be genetic drift occurring between RUB subpopulations, but this pattern may not be clearly observable without additional genetic data. Subpopulations in both NFMFA and RUB did not indicate significant isolation-by-distance affects, and in both cases the subpopulations analyzed were within 1–2 km of one another.

Fine scale analysis of the three subpopulations separated by a reservoir and dam (UMFA, lower NFMFA, and lower RUB) indicated significant genetic differentiation among subpopulations; overall  $F_{ST}$  from AMOVA was 0.083 (Table 2.5), which was significant (p<0.002) and was evidence of divergence due to genetic drift. Isolation-by-distance was not a significant factor in any of the Mantel tests of the subpopulations in the UMFA watershed (Z = 0.281, r = 0.445, p > 0.123; Figure 2.6). This indicates that factors unassociated with distance are responsible for genetic differentiation within populations separated by less than 3–4 km and that dispersal and gene flow is limited among these groups.



Figure 2.6. Subpopulations upstream of Ralston Reservoir (2=NFMFA, 2=Rubicon, 1=UMFA): Influence of isolation by geographic distance log transformed (km) on genetic distance from Reynolds et al. (1983) coancestry in RAPD variation. Mantel test: Z = 0.059, r = 0.097, p > 0.462, based on 10,000 replicates.

#### Regulated vs. Unregulated

Overall comparisons of regulated and unregulated rivers show significant differences in genetic structure. The AMOVA F<sub>ST</sub> values from RAPD data were higher in each regulated river compared with the unregulated pair (Table 2.5). Although the AMOVA was not significant for the NFMFA vs. RUB pair, the regulated bypass reach (RUB) had a nearly significant AMOVA F<sub>ST</sub> p-value of 0.052 compared to the unregulated NFMFA AMOVA  $F_{ST}$  p-value which was 0.105. Comparison of  $\theta$  values between regulated and unregulated indicated the peaking MFA reach had lower genetic divergence compared to the unregulated NFA, however, the test of isolation by distance showed geographic separation was correlated with high genetic drift observed ( $\theta$ =0.0418). Interestingly, the smaller RUB regulated bypass reach had a similar  $\theta$ -value to the unregulated NFA  $(\theta=0.037)$  yet isolation-by-distance was not a factor for this regulated reach. The three populations that were analyzed for potential reservoir affects (UFMFA, NFMFA, and RUB) showed significant correlations with genetic drift and isolation, and these populations were not significantly affected by isolation by distance. Therefore, in the cases where unregulated rivers had high  $F_{ST}$  or  $\theta$  values, all were significantly correlated to genetic isolation by distance, whereas in regulated rivers with high  $F_{ST}$  or  $\theta$  values, isolation by distance was not a significant factor.

#### DISCUSSION

The nuclear DNA data illustrate that river regulation limits gene flow in R. boylii populations. Gene flow is the gradual exchange of alleles between two populations, and it occurs via dispersal or migration of individuals that reproduce in local populations (i.e., genetically effective dispersal) (Slatkin 1985, Dobson 1994, Frankham et al. 2002). For example, small fragmented populations can function as single large populations with sufficient gene flow from migration and dispersal by preventing inbreeding and maintaining variation (Frankham et al. 2002). RAPD analysis indicated significantly lower levels of migration and high levels of genetic drift were observed based on AMOVA F<sub>ST</sub> values for frogs inhabiting the regulated river regions (MFA and RUB). Geographic distance was not a significant variable in explaining divergence in population structure, and the Mantel tests of isolation by distance did not substantiate the high  $F_{ST}$ values observed in the peaking MFA and the populations separated by Ralston Reservoir. The RAPD data illustrate limited gene flow in these regulated study reaches, and gene flow within and among populations is a driving force behind population demographics and phylogenetic structure and can reduce fragmentation effects in small populations by connecting isolated subpopulations (Barber 1999, Vos et al. 2001, Gibbs 2001). Dever (2007) found nine haplotypes and a haplotype diversity of 0.824 within R. boylii populations in seven different tributaries along a section of the Eel River, compared to the 20 haplotypes and haplotype diversity of 0.625 observed in the four study rivers (MFA, NFA, RUB, and NFMFA), two of which are regulated. Gene flow is particularly important for R. boylii in the Sierra Nevada, as many populations are sparsely distributed and often in low abundance, which may exacerbate fragmentation or connectivity impacts from river regulation, such as flow fluctuation and habitat alteration.

The discrepancy between the  $F_{ST}$  estimate for the MFA (0.129) from RAPD data and the high haplotype diversity (0.956) observed from the mtDNA may be due to several factors. First, RAPD markers have higher mutation rates compared to the ND2 region in mtDNA and therefore are indicative of more recent patterns of genetic structure (Vandewoestijne and Baguette 2002). Second, differences in dispersal between males and females may cause nuclear markers such as RAPDs to become fixed at a faster rate than mtDNA markers because mtDNA is a haploid, maternally inherited marker (Ballard and Whitlock 2004). This also supports previous research by Dever (2007), which found mtDNA and RAPD markers gave divergent estimates of R. boylii genetic structure in the Eel River. In addition, *R. boylii* populations within the peaking reach of the MFA may have consisted of a much larger population prior to construction of the hydroelectric project based on the high number of haplotypes and the high haplotype diversity. illustrating the potential differences in time scales between RAPDs and mtDNA. The mtDNA data may indicate historical patterns of gene flow and genetic diversity for R. boylii populations in the MFA prior to the influence of hydroelectric river regulation, while the RAPDs illustrate more recent patterns of genetic drift. Third, sampling size and location can influence mtDNA haplotype distribution and diversity, as larger sample sizes may include rare haplotypes that change the population haplotype diversity and differentiation patterns. Samples collected in the same region of the stream may include siblings from the same clutch of eggs, which would affect mtDNA haplotype diversity. In

this study, mtDNA samples from the unregulated NFA were collected in geographically close proximity to one another and several were from adjacent tadpoles, which may likely be from the same clutch; whereas mtDNA samples from the peaking MFA segment were collected at more spatially distinct locations, greatly decreasing the likelihood that they were from closely related frogs. Consequently, it is important to utilize multiple genetic markers in any study of population structure, as each marker can provide uniquely different data.

River regulation effects may be compounded by both structures and flow operation. For example, the stark difference in *R. boylii* distribution and gene flow observed between populations in the unregulated NFA ( $F_{ST}$ =0.084) and peaking reach of the MFA ( $F_{ST}$ =0.129), indicate populations in the MFA are experiencing significantly higher reductions in gene flow compared with the Rubicon bypass reach ( $F_{ST}$ =0.041). Flows during the spring and summer in the peaking reach of the MFA can fluctuate in magnitude and frequency at much higher rates than fluctuations observed in the bypass reach of the RUB. However,  $\theta$  values indicate greater genetic divergence in the RUB compared with the MFA. Thus, flow regulation may be a causal factor in explaining genetic divergence in *R. boylii* regardless of the operation type. Discrepancies between the RUB and MFA may be explained by the smaller sample sizes in the RUB, but overall the contrast in flow operation is important, because flow management can be adjusted, and if flow is the driving factor in the reduction in gene flow and genetic divergence.

Impacts from physical structures associated with river regulation may not be as straightforward to mitigate or alter. Dams, reservoirs, and powerhouses are generally permanent landscape components. Although *R. boylii* may be fairly motile aquatic vertebrates (see Bourque 2008), it was not known whether a dam or reservoir serves as a barrier to movement. The significantly high  $F_{ST}$  observed among the three subpopulations separated by Ralston Reservoir (a long narrow reservoir dividing the NFMFA, RUB, and UMFA) demonstrates an anthropogenic structure can have a geographically and genetically isolating effect. Furthermore, Ralston Reservoir was constructed in 1966, which means genetic drift and limited gene flow is occurring among these *R. boylii* populations over a short evolutionary time frame. Since the  $F_{ST}$  statistic is based on an island model, isolation by distance in natural populations from similar geographic regions unless gene flow is limited. In addition, small population sizes are at greater risk of genetic drift, particularly if connectivity between subpopulations is restricted.

In small populations, very few migrants may be required to maintain gene flow among populations (Funk et al. 2005b, Ficetola et al. 2008). At the same time, small populations are subject to higher rates of genetic drift, and if gene flow between populations is severed due to fragmentation or limited connectivity, a reduction in heterozygosity and inbreeding will likely follow (Crow and Aoki 1984, Luikart and Cornuet 1998, Ficetola and De Bernardi 2004, Funk et al. 2005b, Schmeller et al. 2007). Lowered

heterozygosity and increased inbreeding leads to lowered genetic fitness, and may lead to greater risk of extinction in small, isolated populations (Gibbs 2001, Beebee and Griffiths 2005, Schmeller et al. 2007), while a large population with high rates of gene flow and genetic homogeneity may promote considerable plasticity of phenotypes, and provide a larger "reservoir" of potential traits that increase the overall "fitness" of the population (Dobson 1994). Deiner et al (2007) observed fish populations in coastal California streams that occurred upstream of natural barriers had lower genetic diversity and larger  $F_{ST}$  values, but microsatellites did not observe differentiation between populations separated by a dam. Using different genetic markers can yield different results, and RAPD data in this study illustrate flow regulation and water impoundment (dams and reservoirs) affects *R. boylii* populations at a genetic level, and if small populations in these regions continue to contract, successful recolonization or expansion may be insufficient for long-term survival.

Historically, conservation efforts to counteract fragmentation often place higher priorities on large habitat patches versus small habitat patches, and efforts to protect single, large habitat patches containing high population densities may not be appropriate, as this strategy unintentionally selects against long distance dispersers (Driscoll 1998, Fellers and Kleeman 2007). Successful conservation of R. boylii populations requires consideration of all subpopulations within the riverscape, and maintaining the greatest amount of genetic diversity possible within these rivers. Rivers are dynamic systems, and although a single stochastic event has a greater probability of wiping out a single large population with no chance of recolonization, a group of many small populations may be able to disappear and recolonize suitable patches over time (Frankham et al. 2002). Therefore, although small populations living in stochastic environments such as rivers or streams may be naturally fragmented and disjunct (Vos et al. 2001, Kupferberg 1997, Lind 2005, Welsh and Lind 1996), it only takes a few individuals to disperse and connect different sub-populations, thereby maintaining gene flow and genetic diversity (Austin et al. 2003, Funk et al. 2005, Dever 2007). Since individuals that disperse the farthest are most likely to genetically connect distant breeding sites, conservation of many "connected" smaller habitat patches may be more important for amphibian populations (Ficetola and DeBernardi 2004, Fellers and Kleeman 2007).

It is evident that *R. boylii* populations are fragmenting at genetic and spatial scales in regulated rivers. Based on the higher  $F_{ST}$  values observed in the peaking reach of the MFA and in the populations separated by Ralston Reservoir, fragmentation may be occurring at multiple levels within the watershed. Fragmentation of a population, and the decline or loss of a highly sensitive species is one of the first signs of an ecosystem in stress (Welsh et al. 2005), thus promoting ecological connectivity of aquatic and terrestrial habitats is very important for maintaining population structure, particularly in smaller populations (Funk et al. 2005 and 2005b). Furthermore, if fragmentation precipitates a reduction in habitat connectivity, gene flow, or patch size, the ramifications may occur at both ecological and evolutionary levels (Vignieri 2005, Schick and Lindley 2007). Schick and Lindley (2007) found that fragmentation events lower (more downstream) in a watershed had a significant effect on the stability of fish populations

upstream. Unfortunately, there has been minimal empirical research on genetic and demographic bottlenecks of lotic amphibians (Lind 2005).

Future research should incorporate additional landscape data to assess genetic fragmentation such as road densities, habitat diversity and frequency (particularly in relation to cobble/gravel bars that *R. boylii* use for breeding), as well as potential climate change affects on the hydrology of these rivers. Using data from this study to model changes in phylogeographic structure over time will provide resource managers critical information necessary for successful conservation.

#### CONCLUSION

*Rana boylii* populations are being affected at a genetic scale by river regulation in these study rivers. Based on the above research, it appears flow regulation (or dam operation type) may have the greatest impact on population differentiation in *R. boylii*. However, the structural presence of reservoirs and dams may be limiting gene flow. In general, regulated study rivers had limited gene flow and higher genetic divergence among subpopulations compared with unregulated study rivers, and these differences were not attributable geographic distance. Hydroelectric operation is unlikely to dwindle. If river regulation has altered populations in *R. boylii* at a genetic level over the last 50 years since most dams in California were constructed, the long term effects may be exponentially greater, particularly given *R. boylii* have small population sizes in many regulated systems. Flow regulation is guaranteed to continue given California's growing population and the need for renewable energy, drinking water, and irrigation. Therefore, preserving genetic diversity within *R. boylii* populations must be paramount to conservation efforts for this species. Protection of the river environments *R. boylii* inhabit alone will not be sufficient for long term sustainability of populations.

These findings about the genetic isolation among one frog species may be applicable to other frog species endemic to California that are also experiencing declines, but not receiving the public attention that follows more economically significant species (e.g., salmon) (Jennings and Haves 1994, Lannoo 2005, Lind 2005, Wake and Vredenburg 2008). In California, there are approximately 12 native frog species of which only R. boylii and Pseudacris regilla are distributed across a wide remnant of their former range (Lannoo 2005). All 12 species are considered Species of Special Concern in California (California Department of Fish and Game 2008), Rana muscosa in southern California is endangered under the federal Endangered Species Act (ESA), R. draytonii is threatened under the ESA, and most species appear to be declining in distribution and abundance throughout their ranges (Jennings and Hayes 1994, Lannoo 2005, CDFG 2008). For R. *boylii* that occur in regulated systems, conservation management should insist on a more natural flow regime, and make all tributaries and tributary confluences important features in monitoring and restoration efforts. Understanding how landscape features effect genetic variation (through gene flow, population divergence, and inbreeding) is critical for biological conservation of a species (Manel et al. 2003, Funk et al. 2005).

Conservation of biodiversity must occur at both ecological and genetic scales. Frogs may be unlikely candidates for conservation; despite overwhelming evidence indicating global amphibian declines (Wake and Vredenburg 2008). Many amphibians are elusive, cryptic, and often difficult to study (Bernal et al. 2005, Vignieri 2005). For *R. boylii*, most data collected is used for estimates of relative abundance and distribution, which may have limited use in discerning how fragmentation is affecting population fitness over time. Currently, it is not clear whether habitat management is more important than population management for conservation of a species, particularly given the brief evolutionary time frames to which land managers and biologists are often constrained (Gibbs 2001). However, future research should focus on both long and short-term affects of anthropogenic habitat fragmentation on genetic diversity, as conservation efforts ultimately must focus on preserving diversity and connectivity in order to provide species such as *R. boylii* with the greatest chance of survival.

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# **Summary Conclusion**

*Rana boylii* has inhabited rivers and streams in California for millions of years, yet changes in landscape, biota, and water use threaten to extirpate the species from all but the most remote locations. This is the first study to combine genetic population analysis of a riverine frog with a landscape connectivity analysis within the stream network in order to more fully examine fragmentation at multiple scales. River regulation continues to have the greatest impact on this species, directly and indirectly. It is evident that tributaries and tributary confluences are critical landscape components for *R. boylii*, and that without these riverscape features, current populations in peaking reaches such as the MFA may be extirpated. For *R. boylii*, tributaries provide the requisite habitat plasticity for survival within these stochastic river environments. Flow regulation and barriers (i.e., dams, reservoirs, and diversions) compound the ability for animals to access these habitat areas, which further limits dispersal among *R. boylii* populations.

Regulation impacts frog distribution within rivers, with adults being significantly more limited to tributaries than the mainstem river. Additionally, results from this study show populations in regulated rivers exhibit lower levels of genetic diversity (fewer polymorphic loci and haplotypes) and greater levels of genetic subdivision due to genetic drift compared with populations from unregulated rivers. Rana boylii populations in regulated study rivers had significantly higher F<sub>ST</sub> values, and restricted landscape distributions, indicating lower gene flow, genetic diversity, and higher population fragmentation compared with populations in unregulated rivers. Additional research has shown the short-term adverse impacts of flow fluctuation on R. boylii breeding (Lind et al. 1996, Kupferberg et al. 2008), which likely compounds the effects of genetic fragmentation by reducing fecundity and reproductive fitness. Using both genetic and spatial analyses has illustrated the complex interactions between landscape features and dispersal in *R. boylii*. Although analysis of population genetics does help explain spatial structure, extrapolation of these findings into other watersheds is not a simple task. Dever (2009) observed high levels of genetic diversity in coastal R. boylii populations in an unregulated section of the Eel River, implying larger populations do sustain significant gene flow within the river network and among tributaries. However, R. boylii populations in rivers on the western slope of the Sierra Nevada appear to have lower genetic diversity and higher levels of fragmentation based on results from this study, and in support of values of within locality variation observed by Lind (2005). The simplifying assumptions of genetic models may limit their utility in quantifying the effect of landscape structure on connectivity without additional spatial data on a species habitat and distribution. However, by combining analyses of spatial distribution, landscape structure, and population genetics, it is possible to link multiple scales of evolution and ecology, and hopefully provide much greater understanding of a species.

Management of *R. boylii* populations in regulated watersheds should consider patterns of spatial and genetic connectivity for any successful long-term conservation to occur. As with many riverine species, *R. boylii* require an array of different habitat types in order to survive. Flow regulation has altered the pattern of natural hydrologic variation required for species that have adapted within dynamic environmental conditions. As a result, *R*.

*boylii* populations are currently becoming isolated at genetic and spatial scales, limiting potential adaptive plasticity required to survive within these regulated watersheds.

While hydroelectric power generation provides renewable and generally clean energy for many California residents, and will continue to do so for the foreseeable future, preservation of existing biodiversity within any system must be a priority. Conservation management must balance the prioritization of species, despite the fact that these species are often linked, and similar to a house of cards, ignoring one connection risks collapse of the entire deck. Ecosystems are many times more complex, but ultimately conservation requires preservation of diversity at both genetic and spatial scales in order to succeed. Mimicking natural hydrograph patterns in regulated systems (and eliminating aseasonal flow releases) may be a simple response to a complex problem, with long-term impacts for *R. boylii* survival. Human longevity gives us a rare advantage in the natural world, as we have the ability to change our habits and learn from our experiences by observing our mistakes over time. Consequently, conservation management requires consistent and long-term monitoring of sensitive populations in river ecosystems to effectively assess and avoid future impacts from increasing water needs.