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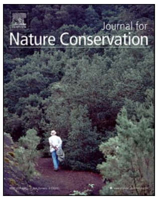

Overwinter behavior, movement, and survival in a recently reintroduced, endangered amphibian, *Rana muscosa*

Talisin T. Hammond [a,*], Michelle J. Curtis [a], Leah E. Jacobs [a], Patricia M. Gaffney [a], Meredith M. Clancy [a], Ronald R. Swaisgood [a], Debra M. Shier [a,b]

[a] *San Diego Zoo Wildlife Alliance, Escondido, CA 92027, USA*
[b] *Department of Ecology and Evolutionary Biology, University of California, Los Angeles, 621 Charles E. Young Dr. South, Los Angeles, CA, 90095, USA*

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ABSTRACT

Basic natural history information is critical for species conservation but is often deficient for endangered species, which can be rare and difficult to study. This is particularly true for behavior and natural history during the winter in temperate regions, when site access can be challenging. For translocation programs in temperate regions, however, winter may be a critical time to assess movement, survival, and threats to persistence in order to understand the causes of translocation failures. Using radiotelemetry, we monitored movement from fall through spring in reintroduced, *ex situ* bred individuals (N = 21) of the endangered mountain yellow-legged frog (*Rana muscosa)* to characterize behavior during brumation (overwinter dormancy), overwinter infection prevalence of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), and survival. We found that movement rates were variable but low in comparison to other sites. Frogs moved more in the fall when temperatures were warmer in comparison to the winter and spring. However, some frogs still moved (very small distances) even when water temperatures were below 2 °C and snow covered the ground. Frogs were difficult to detect visually, but were almost always in water, including during brumation, when they usually appeared to be in underwater rock caves and crevices in pool habitat. Frogs appeared to be spatially clustered in 3–5 groups throughout the study period and those within a group were often located <1 m from one another. Overwinter survival rates were low: over 70% of animals were confirmed dead, and all were suspected dead by mid-May. While sample size was limited, Bd prevalence and infection intensity increased during the winter and spring, which may have contributed to mortality rates. This study sheds light on a poorly understood life history phase for an endangered amphibian and will inform future management activities to protect this species.

1. Introduction

Basic natural history information and descriptive ecology are critical for effective conservation and management (Greene 1994; Bury 2006; Becker, Loyola, Haddad, & Zamudio, 2010; Able 2016; Ohmer et al. 2019) but are often unavailable for species of conservation concern (da Silva et al. 2020). Despite the rise of sophisticated statistical techniques and the use of controlled experiments, basic natural history remains highly relevant and complementary in conservation applications (Anderson et al. 2021). Availability of life history information is often biased towards certain types of species, habitats, or seasons (Marra, Cohen, Loss, Rutter, & Tonra 2015; da Silva et al. 2020). For instance, little is known about over-wintering biology for many temperate species (McMeans et al. 2020), in part because access to sites (e.g., locations with snow, heavy rains, or rapidly flowing rivers) and animals (e.g., hibernating or aestivating semi-fossorial species) can be difficult during winter. Without understanding the basic biological needs of over-wintering species that are experiencing population declines, it is challenging to make informed management decisions on a range of issues, including habitat preservation, disease mitigation, and genetics. This is particularly crucial in the context of climate change, which is expected to result in warmer winters in many regions (Williams, Henry, & Sinclair, 2015).

For amphibian species, which are currently in the midst of a global extinction crisis and are threatened by a range of factors including climate change and diseases (Sodhi et al. 2008), understanding over-wintering biology is imperative to conservation. Warmer winters driven by climate change could have costs for species that hibernate (winter

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[*] Corresponding author at: 15600 San Pasqual Valley Rd., Escondido, CA 92027, USA.
E-mail address: thammond@sdzwa.org (T.T. Hammond).

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dormancy in heterotherms) or brumate (winter dormancy in ectotherms; Humphries, Thomas, & Speakman, 2002; Turbill & Prior 2016; Cordes et al. 2020). Moreover, in many regions, the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which has caused declines in many amphibian species (Vredenburg, Knapp, Tunstall, & Briggs, 2010; Scheele et al., 2019), but see (Lambert, 2020), increases in prevalence or infection intensity during cooler seasons (e.g., fall through spring; Berger et al. 2004; Retallick, McCallum, & Speare, 2004; Ouellet, Mikaelian, Pauli, Rodrigue, & Green, 2005; Kriger & Hero 2007; Pearl et al. 2007; Lannoo et al. 2011; Ruggeri et al. 2015). To effectively manage at-risk amphibians, it is important to understand their habitat requirements and behavior during the winter months, and the extent to which anthropogenic threats may impact these needs.

Here, we evaluate post-release movement and disease dynamics across seasons in an endangered, reintroduced amphibian, the mountain yellow-legged frog (*Rana muscosa*), during fall, winter, and spring. We focus on the southern California distinct population segment of *R. muscosa*, which is thought to persist at only 8–10 localities, inhabited by fewer than 200 known adult wild frogs (Backlin, Hitchcock, Gallegos, Yee, & Fisher, 2015). These populations are threatened by introduced species, including trout, bullfrogs, and Bd, as well as habitat degradation and climate change (Backlin et al., 2015). *Ex situ* studies suggest that the overwintering period is critical to the reproductive success and survival of these populations (Santana, Swaisgood, Lemm, Fisher, & Clark, 2015; Calatayud et al. 2020). However, for southern California *R. muscosa,* field data are only available from late spring through early fall, and there is no information regarding their overwintering behavior in the wild. Detection of translocated *R. muscosa* following winter brumation is rare, suggesting that they either have low overwinter apparent survival or disperse from release sites.

We released frogs at a site in the San Jacinto mountains within the species historical range but currently unoccupied by *R. muscosa*, thus categorized as a *reintroduction* under IUCN criteria for translocation categories (IUCN/SSC, 2013). Because detection probability is low in this species (Hammond et al. 2021), it is difficult to obtain data necessary to document outcomes of releases. We describe a method for surgical implantation of radio-transmitters in this species, and use radiotelemetry to monitor individual movement of reintroduced, *ex situ* bred *R. muscosa* for approximately eight months after release to characterize relationships between post-release movement, survival, and individual (e.g., sex, body size, Bd infection status) and environmental traits (e.g. water temperature). We also describe microhabitat use before and during brumation. Our primary goal was to characterize overwinter behavior and habitat use, and to more accurately measure overwinter survival for reintroduced frogs, taking advantage of the increased detection rates enabled by our radio-transmitter implants. Although thousands of individuals from this population segment have been released, the application of this technology enables heretofore unknown details of release outcomes. Our results shed light on a little-known life history phase for an endangered species and may more generally inform conservation and management of amphibians in temperate regions.

2. Methods

2.1. Study animals

The focal individuals originated from populations in southern California that are managed under human care by the San Diego Zoo Wildlife Alliance and the Henry Doorly Zoo and Aquarium in Omaha, Nebraska. We bred all study subjects *ex situ* and head-started them to approximately three years old (hatched in spring 2016, metamorphosed in fall 2016). Each winter in the *ex situ* colony (Dec-Mar 2016–2018) the study animals were brumated at ~4.5 °C for approximately three months.

2.2. Radio-transmitter implantation

Prior to implanting radio-transmitters into study individuals, a dummy transmitter was implanted into a non-study individual to confirm the safety of the procedure. Radio transmitters (Model R1655 with internal flat ribbon antenna, Advanced Telemetry Systems) were then surgically implanted into 23 adult *R. muscosa*. Briefly, on 28 and 30 August 2019 frogs were anesthetized via immersion bath of 600 ppm tricaine methanesulfonate (MS-222) buffered to match the pH of tank water (8.1–8.2) for 5–7 min until at a surgical plane of anesthesia. The frogs were placed on cool wet gauze in dorsal recumbency with the left paramedian ventrum surgically prepped and sharply incised to a total of 8–10 mm through the skin and then body wall into the coelom. The transmitter was implanted through the body wall using digital manipulation. The body wall was closed with simple continuous pattern using 5–0 PDS, with skin closure using 1–2 horizontal mattress pattern sutures with everting technique, and tissue glue was applied topically to ensure skin closure. Average anesthesia time was 13.5 min; average surgical time was 6.25 min. Frogs were given 8–9 days to recuperate prior to reintroduction. Transmitters weighed ~1.53–1.85 g, representing 5–10% of the individual's body mass (frogs in this study weighed 22.6 ± 3.6 g, mean ± S.D.). A 10% weight limitation has been suggested for amphibian species (Baldwin, Calhoun & deMaynadier, 2006; Heyer, Donnelly, Foster, & Mcdiarmid 2014; Blomquist & Hunter 2007; Madison, Titus, & Lamoureux, 2010). Transmitters were programmed to function at 8 pulses per minute; this slow pulse rate was selected to increase transmitter longevity. Due to small body size, we implanted two individuals with smaller transmitters (Model F1010 with internal flat ribbon antenna, Advanced Telemetry Systems) with similar signal strengths that were projected to last 2–3 months.

2.3. Reintroduction and post-release monitoring

Approximately one week after the implantation procedure, 28 total frogs (21 with radio-transmitter implants: 6 males, 15 females) and 87 first year tadpoles were reintroduced into a site in the San Jacinto mountains on 6 September 2019. Prior to release all adults were tested for Bd and confirmed negative. Site selection was based on surveys by the U.S. Geological Survey and multi-partner stakeholder evaluation of alternative reintroduction sites; the site met criteria including perennial flow, suitable rocky pools, absence of introduced brown trout and bullfrogs, and mature forest canopy. Bd is omnipresent in the region and thus it was not a determining factor in site selection. This site was a narrow montane stream interspersed with shallow (<0.5 m) pools, located at ~2650 m elevation. Post-release monitoring took place the day after release and every 3–7 days until December, at which point surveys were conducted approximately every 14 days through mid-May. A handheld three-element yagi antennae (Advanced Telemetry Systems) and receiver (R-1000, Communications Specialists, Inc.) were used to triangulate the location of each individual. We collected one location per individual per survey date. Whenever possible, located frogs were captured and swabbed with a fine-tipped swab (MW113; Medical Wire & Equipment Co.) using standard methods to test for Bd (swabbing protocol available at: https://science.sandiegozoo.org/sites/default/files/sites/default/files/blogimages/Chytrid%20Guidelines%202021.pdf). Frogs were also measured and weighed, and surgical incision sites were inspected to monitor growth and health.

2.4. Environmental data collection

We deployed two data loggers (HOBO Pendant® Temperature/Light 64 K Data Logger) to collect water temperature every 6 h throughout the study period (start of September through mid May) in the two release pools. We set two remote activated cameras (Reconyx Hyperfire 2) to take 2 photos every 15 min at the two release pools. Cameras were positioned to capture ~40–70% of each pool, including the shore/rocks on at least one side of the pool, and photos were taken throughout the study period.

On each visit, we collected site-level environmental data (ambient temperature,wind speed, water temperature,pH, and weather) before telemetry commenced. Each time we located an individual, we recorded a GPS coordinate (Juniper-Geode submeter GNSS receiver), collected water temperature at the location, specified whether the individual was in or adjacent to stream or pool habitat, and recorded the microhabitat type where individuals were found: downed wood, vegetation, on or underneath large rocks, sand or soil, open water, fast-moving/deep water, snow. To characterize habitat use across all individuals, for each survey date we calculated the proportion of animals that were in or out of the water, in or adjacent to stream or pool habitat, and separately calculated the proportion of animals in each of the eight microhabitat categories. For each individual, we also calculated the proportion of times the frog was found in or adjacent to a pool, and the proportion of times the frog was found in water. We then used these data to examine associations between habitat use and longevity (see "Statistical analyses" section below).

2.5. *Bd and ranavirus qPCR testing*

We sampled live frogs in the field, and tested skin swabs for the presence and quantity of Bd DNA in the San Diego Zoo Wildlife Alliance Amphibian Disease Laboratory using qPCR (Taqman) as described in Boyle, Boyle, Olsen, Morgan, & Hyatt (2004). Individuals that were necropsied were also tested for Bd and ranavirus. Details are provided in the Electronic Supplementary Data.

2.6. *Postmortem examination*

Dead recovered frogs received full post mortem examination by a board certified anatomic pathologist. Frogs were examined grossly, a full set of tissues were collected, fixed in 10% buffered formalin, routinely processed, and stained with hematoxylin and eosin for microscopic examination. Skin, kidney, and liver tissues were collected sterile for Bd and ranavirus testing (see electronic supplement).

2.7. *Statistical analyses*

All statistical analyses were conducted in the R programming environment (R Core Team, 2019). A generalized linear mixed model (GLMM) implemented in the lme4 package (Bates, Sarkar, Bates, & Matrix, 2007) was used to test for relationships between individual movement between surveys and individual and environmental traits. For the response variable, we normalized measures of distance to the number of days since the previous survey (i.e., converted the units to meters moved per day) and then log-transformed them so that the GLMM would not violate assumptions of uniformity. Snout-to-vent length (SVL), sex, and water temperature were included as fixed effects. A GLMM (binomial family) was also used to test for relationships between survey date (fixed effect) and inhabitation of stream or pool habitat (response variable: pool = 0, stream = 1). For both GLMMs we rescaled continuous variables prior to analysis to allow for comparisons of effect sizes within models, included individual identity as a random effect, and used the DHARMa package (Hartig 2017) to assess model residuals. The R packages ggplot2 and ggmap were used for figures and data visualization (Wickham 2011; Kahle & Wickham 2013).

To assess spatial clustering of frogs we used k-means cluster analysis (using Euclidean distances) implemented in the NbClust package in R (Charrad, Ghazzali, Boiteau, Niknafs, & Charrad, 2014). In NbClust we used Friedman's index (Friedman & Rubin 1967) to determine the optimal number of clusters. We applied cluster analysis to animal location data from each survey date before 7 February 2020, by which point 7 of the 21 transmittered animals were confirmed dead (prior to this date only 1 animal was confirmed dead).

To test for relationships between sampling date, Bd infection status and intensity, we used GLMs that included date as a fixed effect and Bd infection status (Binomial family) or log-transformed Bd infection intensity (Gaussian family) as the response variable (due to limited sample size we were not able to control for individual identity in these models, however, there were only 5 individuals with repeated samples, and 4 of these 5 individuals moved from negative to positive Bd infection status over the course of the study period). We assessed predictors of longevity using a Cox proportional hazards model (using the 'survival' package in R, Therneau & Lumley, 2015). Because most individuals were significantly decayed at the time we found their carcasses and all individuals were thought to be dead by the end of the study, for all animals we treated the last date that they were confirmed alive (visually or via documented upstream movement) as the date of death, and included sex, distance moved from release site (prior to the first mortality observed), and body condition (mass divided by SVL) as covariates (continuous variables were mean-rescaled prior to analysis to allow for comparison of model coefficients). Bd status could not be included in this model because it was only available for a subset of individuals. We used the 'cox.zph' function to confirm that the data met model assumptions.

3. Results

3.1. *Transmitter implant results*

Two of 24 frogs died after surgery. Both animals were the final individuals to receive a transmitter implant on their surgical date. Apart from these two individuals, the frogs successfully recovered from surgery. Frogs that were recaptured alive in the field (N = 12 unique individuals, 20 total recaptures) generally maintained or gained weight and surgical incisions did not show any signs of inflammation or infection.

Transmitters were detectable from a distance of ~50–100 m, depending on the terrain. Most transmitters functioned through May, other than one smaller-sized transmitter that lost power by mid-November (the other smaller-sized transmitter was in one of the two individuals that died soon after surgery). In most cases all individuals were successfully located on each post-release survey, but when water temperatures were very cold (~1–2 °C) a small number of transmitters appeared to stop working temporarily (this impacted 2–3 transmitters per survey for two surveys in January).

3.2. *Post-release movement*

Movement was variable across individuals (Fig. 1), but in general there was relatively little post-release movement in comparison to other reintroductions of this species, which have mainly used younger age classes (Shier et al. 2021). Based on their final recorded location, individuals settled 32.8 ± 51.1 m (mean ± S.D.) from their release pool (range: 1–179 m) and moved a total cumulative distance of 117.0 ± 48.1 m across the entire study period. Frogs moved almost exclusively upstream; we did not detect any individuals moving downstream of the original release pools (Fig. 1). The majority of movement occurred in the fall within one month of release (Figs. 1-2).

After the first snowfall in late November there was a notable drop in water temperatures (Fig. 2) and movement was limited: 94% of intersurvey movements after this point were less than 10 m (Figs. 1-2). However, there were still slight movements even as water temperatures fell below 2 °C (Fig. 2). There was a significant positive relationship between water temperature and movement distances (Table S1; Fig. 2), although this relationship could not be disentangled from date, which was also highly correlated with water temperature (Fig. 2). There were no sex- or size-based differences in movement distances (Table S1).

K means cluster analysis of individual frog locations on each survey suggested that frogs were spatially clustered in 3–5 groups throughout

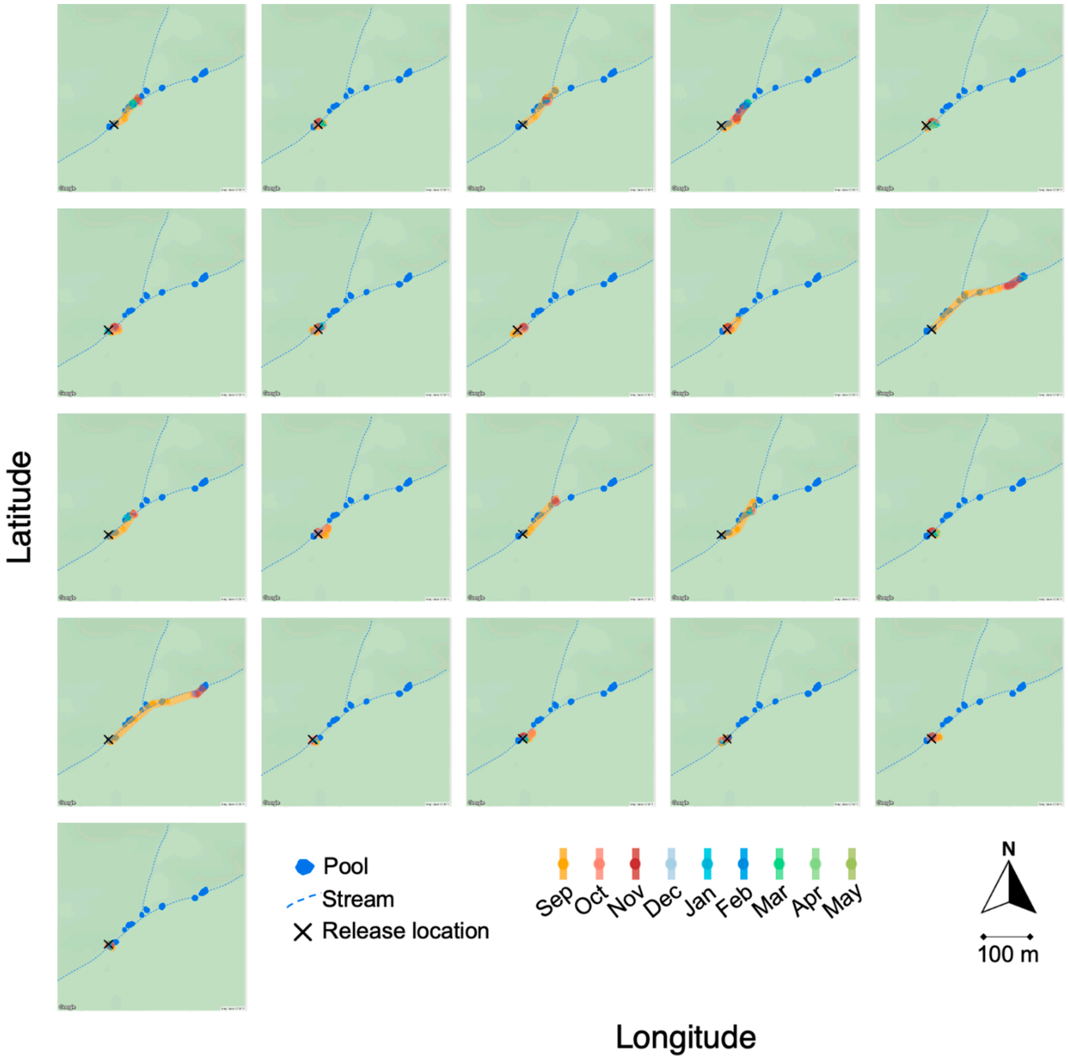

Fig. 1. Movement tracks for the 21 radio-telemetered, reintroduced frogs from September through mid-May 2020. Each panel shows data from a single individual; the black 'X' indicates the release location for that individual. Movement tracks are colored by month/season (autumn: orange/red; winter: blue; spring: green). The blue dotted line indicates the stream, and pools within the surveyed transect (any areas inhabited by frogs at any time during the study) are shown as blue polygons. The stream runs north to south, from higher to lower elevation; the majority of movements were upstream. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the study period. Across time, clusters were comprised of a mean of 4.8 (±3.1 S.D.) individuals each (range: 1–14; Supplementary Figure S1). Within a cluster the mean pairwise between-individual distance was 6.7 m, but frequently (in 33% of pairwise comparisons within clusters) frogs within clusters were found < 1 m from one another, and often individuals appeared to be grouped beneath the same rock (though visual confirmation was usually not possible).

3.3. Post-release habitat use

Throughout the study period it was difficult to determine the exact habitat characteristics for individual locations because in most cases visual confirmation was not possible, even when a burrow probe camera was used. We visually confirmed frog locations in only 9.3% of all location events. Frogs most often appeared to be in the water beneath rocks (Figure S2). On a few occasions, frogs were visually observed wedged deep into crevices between rocks. Occasionally frogs were observed sitting in the open at the bottom of pools or in shallow streams. This was true even after water temperatures dropped in late November (N = 4 visual detections during surveys and N = 8 camera trap observations during this period). During winter a small proportion of individuals appeared to brumate out of the water, often beneath piles of dry vegetation or downed wood (though possibly there were underground water channels or water seepage in these locations, as it was not possible to determine how deep beneath the ground individuals were located; Figure S2). These individuals were always within 1 m of visible water.

During September and early October, when animals were moving most, it was common to find individuals in both stream and pool habitat, but by late October and throughout the overwinter period they were more often found in pool habitat (Fig. 3). There was a significant, negative relationship between study date and presence in stream habitat (Table S2), such that across the study period individuals were increasingly less likely to be found in stream habitat (and more likely to be found in pool habitat; Fig. 3).

3.4. Disease and mortality

Data were limited (N = 26 swabs from 18 unique individuals), but by late October (~7 weeks after release) reintroduced frogs began testing positive for Bd and all swabs collected in or after November tested positive for Bd. Later collection dates were significantly associated with

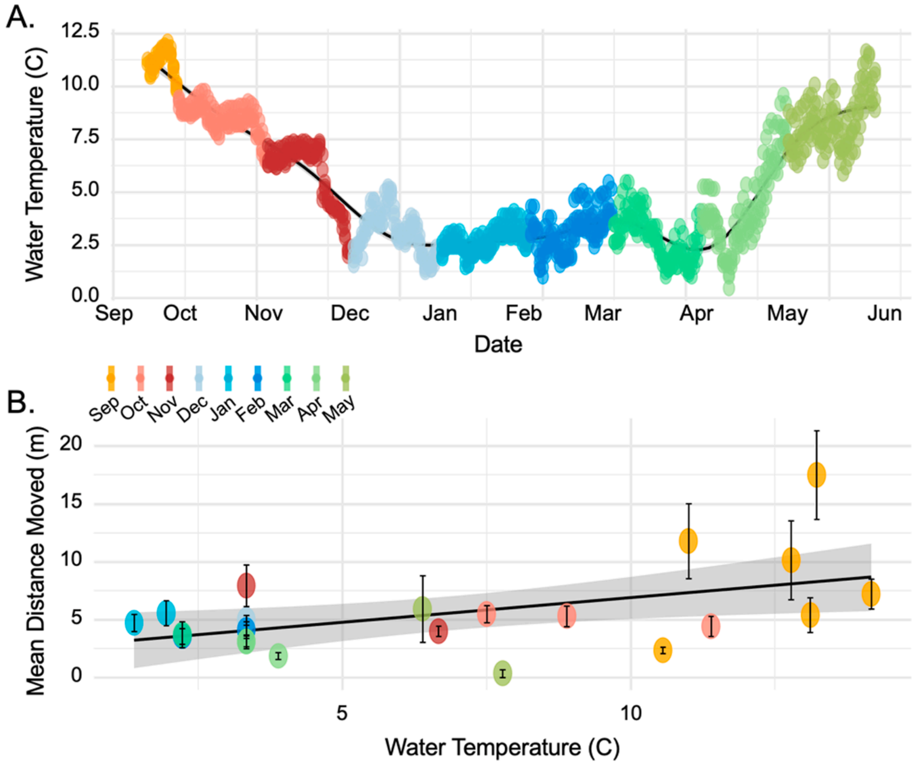

Fig. 2. Relationships between water temperature, date, and animal movement. A. Relationship between water temperature (recorded every 6 h by two data loggers) and date, colored by month. A trendline fit by a generalized additive model is indicated in black. B. Relationship between mean (±S.E) individual movement distances and water temperature on each survey date, colored by month. A trendline fit by a linear model is indicated in black.

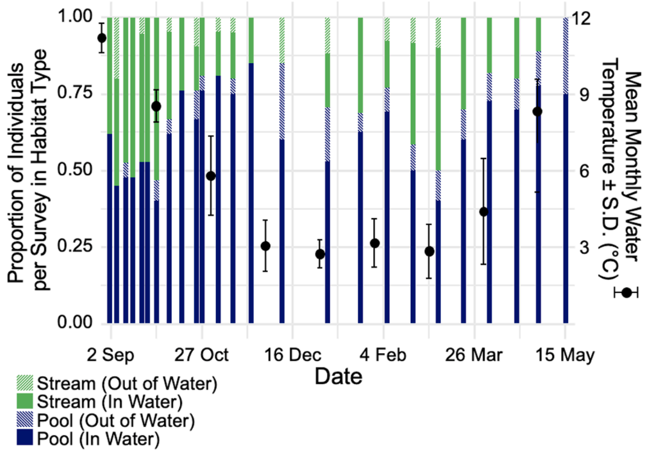

Fig. 3. Mountain yellow-legged frog habitat use throughout the study period. Stacked bars showing the proportion of individuals located in (filled) or adjacent to (cross-hatched) pool habitat (blue, darker color) vs. stream habitat (green, lighter color) on each survey date. Black points representing mean monthly water temperature (±S.D.) for September through May are overlaid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

positive Bd test outcomes (GLM: $z = 2.38$, $p = 0.02$; Fig. 4A). Bd infections also significantly increased in intensity over the course of the study (GLM: $df = 20$, $t = 8.56$, $p < 0.0001$; Fig. 4B; though again, it was not possible to disentangle the effects of study date vs. temperature). Infection intensities for live, Bd positive animals were relatively low upon recapture throughout the study period (mean ZE per swab for Bd positive, live frogs ± S.D: 1523 ± 2379; range: 2–6958). We were unable to test for relationships between longevity and Bd status because Bd data were available for only a subset of individuals/dates.

Between 5 January and 16 May 2020, 15 of the transmittered frogs ($N = 3$ males, 12 females) were found dead at the release site (Fig. 4C). When possible ($N = 12/15$) carcasses were recovered for postmortem examination and Bd testing. The remaining 6 transmittered frogs were suspected dead by May 2020 based on movement patterns (e.g. no movement over a period of many months), but carcasses were never recovered. After telemetry was completed in mid-May 2020, five additional surveys took place between June and December of 2020, and an additional survey took place in fall 2021; no adult frogs were detected on these visits (though in October 2020 two young metamorphs were found dead and both individuals were positive for Bd based on qPCR testing and histology). The Cox proportional hazards model did not reveal any significant predictors of longevity in the field (i.e. no apparent relationships with sex, body size, movement characteristics; Table S3), however, statistical power to detect effects on survival was low due to low sample size, temporal clustering of mortalities, and the fact that there was 100% mortality.

Recovered carcasses ranged from mildly (3/12) to moderately (3/12) to severely autolyzed (6/12), hindering definitive determination of cause of death in all cases. Recovered carcasses included two males and 10 females; three females had active folliculogenesis in the ovaries. Seven were in fair body condition and five were in good body condition based on coelomic adipose stores. Four of 12 were PCR positive for Bd based on postmortem testing and two had histologic evidence of Bd infection in the skin. Six individuals lacked epidermis, the cornified layer which is required for examination to document ante-mortem Bd infection microscopically. In some cases, individuals tested positive for Bd prior to death, but tested negative at necropsy. Because of the advanced stages of decay, negative postmortem tests for Bd were excluded from the analyses, and those that tested positive at necropsy were included in assessments of Bd status/prevalence only (binary: positive or negative), but not in analyses of infection intensity. PCR testing for ranavirus was negative (12/12).

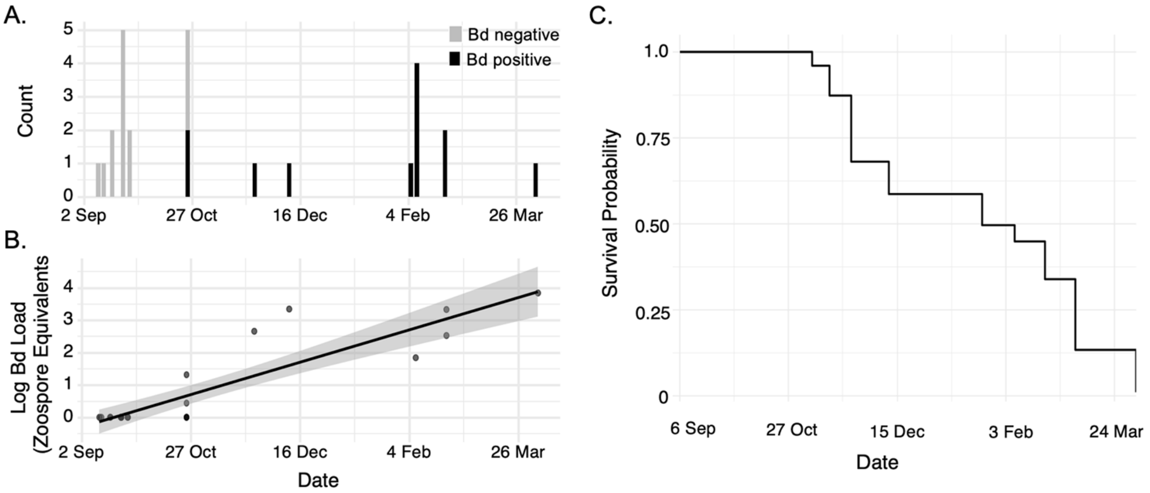

Fig. 4. Bd infection statuses (A), Bd infection intensities (B), and (C) survival probability for frogs as a function of date of sampling. (A) Stacked bar plot showing counts of infection statuses (Bd-negative in grey, Bd-positive in black) for all swabbed individuals on each survey date. (B) Infection intensity, shown as the log of the zoospore equivalents per swab, for each individual swabbed on each survey date. Each point represents an individual swab. A trendline fit by a linear model is indicated in black. (C) Survival curve showing results from a Cox proportional hazards model.

4. Discussion

4.1. Transmitter implant procedure

Our transmitter implant procedure was generally successful. While 2 of 24 individuals died during the procedure, we suspect that this was anesthetic-related, with concerns for maintaining cool pre-anesthetic tank water and undetected changes in anesthetic solution buffering (Weick, Knutson, Kninghts, & Pember, 2005) rather than with the surgical procedure itself or response to the implanted transmitter. Increased vigilance for pre-anesthetic holding tank and anesthetic solution temperature and buffering may reduce procedure mortality in the future. The other 22 individuals (including the "pilot" individual) appeared to recover fully from the procedure. We selected an implant rather than a belt or backpack because external attachment methods can cause skin lesions and entanglement issues in amphibians (Madison et al. 2010). Moreover, for a species like *R. muscosa* where individual detection rates are low (Hammond et al. 2021), it would have been challenging to recover external transmitters prior to battery death. Optimal attachment methods will depend on the species in question, but the implant method performed well for this cryptic, largely aquatic species.

4.2. Movement before and during brumation in *R. muscosa*

As has been reported for other herpetofauna (Tattersall & Ultsch 2008; Nordberg & Cobb 2016), our results suggest that *R. muscosa* may maintain some limited activity during brumation, as we documented very short-distance movements even when ice was in the water and occasionally observed animals out of hibernacula during the winter months. Fine-scale space use in southern California *R. muscosa* has not been published previously. Previous work characterizing seasonal movements in the northern sister species, *R. sierrae* (previously classified as *R. muscosa*), documented higher movement in August and September in comparison to October, when frogs appeared to enter winter dormancy (Matthews & Pope 1999; Pope & Matthews 2001). By the end of October, *R. sierrae* individuals were described as being exclusively underwater, never out in the open, and were often located under ledges and in rock crevices. *R. sierrae* gathered in a smaller number of lakes to overwinter and were sometimes found aggregated in large groups (>8 individuals; Matthews & Pope 1999).

While direct temporal comparison is limited because the focal *R. sierrae* site was at a higher elevation than our study site (3470 m vs. 2650 m), these patterns are similar to what we found for *R. muscosa* in southern California. We may have in part documented more movement in September simply because animals were reintroduced in September and dispersal is often elevated immediately following release. We did occasionally see individuals out in the open during winter. While our total sample size was limited and visual confirmation usually was not possible, we also regularly located multiple individuals brumating within close distances (<1 m) of each other. Throughout much of the study period, frogs appeared to be spatially clustered at the field site in 3–5 groups. In general, previous work with both translocated and wild *R. sierrae* has documented longer-distance movements than we found (Matthews & Pope 1999; Matthews 2003; Matthews & Preisler 2010; but see Brown et al. 2019; Keung, Lawler, Yarnell, Todd, & Brown, 2021 for studies of *R. sierrae* in streams), including some over-land movements (Pope & Matthews 2001), which we never observed, but overwintering behavior was comparable between southern California *R. muscosa* and *R. sierrae*.

4.3. Mortality and Bd in reintroduced *R. muscosa*

We failed to document any significant predictors of longevity (e.g. body size, sex, movement, or habitat use characteristics), largely because mortalities were clustered during late winter, yielding little inter-individual variation in survival. While our data cannot determine cause of death with certainty, results share features with other Bd-related die-off events in *R. muscosa* and *R.sierrae* (Rachowicz et al. 2006; Woodhams et al. 2007). Sample size was low, but a large proportion of individuals tested positive after late October, with infection intensities increasing over the course of the study (though some work has suggested that Bd positive yellow-legged frogs may be more detectable, Joseph & Knapp 2018). During the study period, water temperatures were generally lower than most optimal ranges reported for Bd (Stevenson et al. 2013). However, Bd's thermal optimum is known to vary with strain (e.g. Stevenson et al. 2013), there can be interactions with host physiology (Sonn, Berman, & Richards-Zawacki, 2017; Hettyey et al. 2019; Siddons & Searle, 2021), and higher Bd prevalence has been reported in cooler times of year (e.g. fall, spring) in many regions (Berger et al. 2004; Retallick et al. 2004; Kriger & Hero 2007; Pearl et al. 2007; Lannoo et al. 2011). Moreover, *Rana sierrae* and *R. muscosa* are known to develop and maintain Bd infection at low temperatures and infected individuals exhibit reduced overwinter survival (Joseph & Knapp 2018; Rachowicz & Briggs 2007). While the infection intensities in the individuals we recaptured tended to be relatively low, it is possible that once infection approached clinical significance it became less likely for the frog to be detected and captured. There were no instances of individuals clearing infection. Studies across taxonomic groups have documented differences between the microbial communities of *ex situ* reared vs. wild animals (reviewed in Trevelline et al. 2019; Williams et al., 2018), and it is possible that skin microbiota of *ex situ* reared frogs could have contributed to Bd susceptibility, as has been documented for other species.

Other potential causes of death include complications with the transmitter implants, water oxygenation issues at the field site, the age at release, other unmeasured environmental alterations (e.g., pollutants), or potentially depredation (particularly for the six individuals for which we did not find carcasses). Postmortem examination and health assessments did not suggest significant complications from the transmitter implants. Transmitters did slightly displace ovarian follicles in gravid females; however, there was no inflammation, infection, or scarring to suggest a physiologic response to the transmitter. Additionally, a test transmitter was implanted in an individual that remained in the breeding facility; that frog fully recovered, is brumated every winter in temperatures that mimic those in the wild, and remains alive as of October 2021, having shown no issues related to the transmitter. While previous work has found that transmitters and other animal-borne technology can have fitness costs (e.g. Hooijmeijer et al. 2014), other studies have found limited impacts (e.g. in amphibians: Johnson 2006; Schmidt, Indermaur, & Tockner, 2008). The surgical implantation method we used has been recommended for amphibian species over external attachment methods, which can cause skin trauma, entanglement, and can be difficult to remove for cryptic species (Dervo et al. 2010; Madison et al. 2010).

Historical overwinter die-offs in northern *R. muscosa* and *R. sierrae* have been previously attributed to water oxygenation issues (Bradford 1983). This appears to mainly be a problem facing smaller lakes and ponds that freeze over, which were uncharacteristic of our field site. Camera trap images suggested that two pools at this site froze over, but only for three days, and temperature loggers showed that water temperatures at the bottom of these pools stayed above freezing. We also documented many individuals alive after the freezing event. Moreover, previous work has documented successful overwintering of *R. sierrae* in similar shallow pools, where individuals were often found in rock crevices containing an air pocket that might have assisted with oxygen availability (Pope & Matthews 2001; Matthews & Pope 1999). Finally, this was one of the first times three-year-old, *ex situ*-bred *R. muscosa* had been released in southern California. Other systems have found that when animals spend longer periods under human care, they may have lower post-release survival (Mathews, Orros, McLaren, Gelling, & Foster, 2005). However, because all study individuals appeared to maintain or gain weight and to successfully hide in their habitat, we do not have any evidence that compromised behavioral competency was an issue in this case. It is possible that this die-off may be related to amphibian chytridiomycosis, possibly acting in combination with unknown environmental or individual traits.

4.4. Conservation significance & conclusions

Although based on a relatively small sample size, there are a number of lessons learned that can be applied to *R. muscosa* and other species reintroduction programs. By deploying a diversity of biologging and monitoring techniques (including telemetry, remote triggered cameras, disease monitoring, and water temperature loggers), we were able to gain new insights into the species' natural history and conservation management (Wassmer, Jensen, Fahlman, & Muuray, 2020). Although *R. muscosa* is relatively small to deploy radiotelemetry, releasing older frogs with surgically implanted transmitters allowed us to better characterize post-release dispersal, morbidity, and habitat use. We now have more evidence, for example, that *R. muscosa* may be unlikely to risk over-land dispersal. We also documented limited activity during brumation in the wild.

Perhaps the most important lessons are related to the high mortality we documented. This was particularly concerning given the relatively high apparent survival rates that have been documented in adult *Rana muscosa* and *Rana sierrae* (e.g., Russell et al. 2019; Brown et al. 2020). Although necropsies of recovered bodies did not allow us to pinpoint a cause of death, they provided insights, suggesting that Bd infection may be at play, and ruling out some other causes of death (e.g., no evidence for predation). The die-off we observed is consistent with overwinter die-offs observed in northern California *R. sierrae* and *R. muscosa* where Bd was implicated (Fellers, Bradford, Pratt, & Wood, 2007; Woodhams et al. 2007). Bd is a known threat for this species (Rachowicz et al., 2006; Vredenburg et al., 2010; Backlin et al., 2015), but it remains unclear whether it is the main factor limiting recovery and reintroduction success in southern California. The observation that released frogs thrived initially suggests that they were in many ways well-prepared for life in the wild (e.g. adequate foraging behaviors).

Because all habitat in their native range is likely compromised by Bd presence, re-establishing this species may be challenging. Our findings suggest that the strategy of releasing frogs of unknown Bd susceptibility into Bd positive habitat may result in reintroduction failures. However, it is possible for translocations of individuals from amphibian populations that have experienced Bd-related declines into Bd positive environments to be successful. Some of our previous reintroductions of related, *ex situ* bred *R. muscosa* into other sites in southern California have resulted in inter-annual survival and even reproduction (Shier et al. 2021). This suggests that release site conditions for this study may have been suboptimal for *R. muscosa* persistence; this may have involved higher densities of Bd or factors that influenced Bd susceptibility. Additionally, in the Sierra Nevada mountains scientists and conservation managers have documented inter-annual survival, reproduction, and recruitment of translocated *R. muscosa*/*R. sierrae* into areas where Bd is present, mainly when the individuals originate from populations that have experienced Bd-related declines but have subsequently shown signs of recovery (Knapp et al. 2016; Joseph & Knapp 2018; Knapp et al. 2021). This makes it critical to consider the ecological and evolutionary history of hosts and pathogens during reintroduction of disease-threatened species (Mendelson, Whitfield, & Sredl, 2019).

Unfortunately, as is the case for many species (Brannelly et al. 2021), the history and mechanisms by which southern California *R. muscosa* persist with Bd are poorly understood. Several small populations of *R. muscosa* in southern California have persisted with relatively low Bd infection prevalence in many years (Backlin et al., 2015; Russell et al., 2019). However, these dynamics vary spatially and temporally and many of the few remaining *R. muscosa* populations in southern California are in a state of active decline (Gallegos, Backlin, Wong, Hitchcock, & Fisher, 2020). It is unclear whether Bd is the primary cause of decline, as these populations also frequently face drought and wildfire-related threats. Even if Bd is a primary threat, the extent to which this may vary across populations is unknown. Given the precarious state of southern California *R. muscosa*, *ex situ* programs and reintroductions remain a critical tool for safeguarding these populations from extinction; at some sites reintroduced frogs represent a significant proportion of remaining animals on the landscape (Gallegos et al., 2020), and thus will remain important for meta-popuation management and to help buffer against species extinction. However, for future reintroductions to achieve more consistent success, additional research is needed to characterize Bd dynamics. It will be critical to describe inter-individual/population genetic variation in Bd resistance/tolerance (Savage & Zamudio 2011, 2016), the role of the skin microbiome in reducing susceptibility (Woodhams et al. 2007; Williams et al. 2018), and other strategies for mitigating the impacts of Bd on this species (Scheele et al. 2014). It will also be important to study environmental features associated with Bd prevalence as well as the seasonality, inter-annual variation, and biogeography of Bd for this species/region to optimize disease risk analysis during reintroduction site selection (Sainsbury, Amstrong, & Ewen, 2012). By selecting refugia sites that are less habitable to Bd or by better understanding between-population differences in Bd susceptibility we may be able to better work towards recovering this species. Our results here suggest that Bd could be a major issue facing recovery of *R. muscosa* at certain locations in southern California, and thus addressing this threat must assume prominence in management strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnc.2021.126086.

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