**Benefits of overwintering in the conservation breeding and translocation of a critically endangered amphibian**

Natalie E. Calatayud1\*^, Talisin T. Hammond1^, Nicole R. Gardner1, Michelle J. Curtis1, Ronald R Swaisgood1 and Debra M. Shier1,2

1 San Diego Zoo Institute for Conservation Research, 15600 San Pasqual Valley Road, Escondido CA 92027

2 Department of Ecology and Evolutionary Biology, University of California, Los Angeles, 621 Charles E. Young Dr. South, Los Angeles, CA 90095

\*Current address: Taronga Institute of Science and Learning, Taronga Conservation Society Australia, Taronga Western Plains Zoo, Dubbo, NSW 2830, Australia

^Authors contributed equally to the intellectual development of this manuscript.

Phone: +612-6881-1452 ext. 5255

Corresponding author: ncalatayud@zoo.nsw.gov.au

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**ABSTRACT**

At high altitudes, amphibians brumate (over winter) during the winter months, an adaptation that provides protection from harsh weather and minimizes metabolic demand when food resources are scarce. However, brumation in *ex situ* populations is often avoided due to concerns regarding slow growth rates, compromised immunity, and increased morbidity, and to accelerate growth and sexual maturation. Running counter to these ideas is the hypothesis that husbandry that mimics the environmental conditions under which a species evolved may benefit animal health and reproduction. This may be particularly critical for animals slated for release into the wild. Here, we evaluated the effects of brumation on juvenile southern mountain yellow-legged frogs (*Rana muscosa*) in a conservation breeding and release program. Growth measurements, (weight and snout-urostyle length (SUL)), were examined in three experimental groups: Non-brumated, 1 or 3-month brumation. Post-release survival was also analyzed and compared between non-brumated and 3-month brumated frogs. This study indicates that brumated *R. muscosa* juveniles grow to sizes and weights similar to controls within 3 to 4 months following brumation. Mark-recapture models suggested that short-term post-release survival was not lower and in fact, may be higher in brumated compared to non-brumated frogs. Results of this study indicate that although brumation entails short-term costs to growth, this species possesses compensatory growth mechanisms following brumation which allow them to attain similar body size to non-brumated conspecifics in time for the next winter and that for frogs destined for translocation to the wild, brumation could improve survival outcomes.

**1.** **INTRODUCTION**

*Ex-situ* breeding has steadily occupied an expanding presence in the conservation portfolio. This tool is critical for establishing populations as assurance against extinction, for reintroduction and re-establishment of extirpated populations, to supplement small populations in need of genetic rescue, and for assisted colonization as a strategy to mitigate climate change mediated species loss (Conde *et al.* 2011; Seddon *et al.* 2014). Amphibian species are declining globally (Stuart *et al.* 2004) and due to poor understanding or irreversibility of threats, have become increasingly represented in conservation breeding programs (Harding *et al.,* 2016). However, for conservation breeding efforts to succeed, basic protocols for husbandry and reproductive management must be developed. Amphibians have proven difficult to maintain and breed and each species can require extensive experience and a long-term commitment to developing successful practices (Tapley *et al.* 2015). Lack of species-specific knowledge of natural history is an important barrier preventing the advancement of amphibian conservation breeding goals (Brady *et al.* 2017).

As with other species (Swaisgood and Schulte 2010), knowledge of behavior, ecology, and physiology in the natural habitat is vital for informing amphibian conservation breeding, improving health, welfare, reproduction, and suitability for release (Tapley *et al.* 2015). An important aspect of the natural environment to address in conservation breeding programs is phenology (Paton and Crouch 2002). Amphibian behavioral and physiological adaptations are tuned to annual phenological variation governed by geographic and environmental variables (Visser *et al.* 2010). For temperate anurans, life at high altitudes and exposure to harsh winters, and food scarcity have selected for brumation to maximize survival during overwintering periods. Similar to hibernation in mammals, brumation in herpetofauna is an adaptation that allows amphibians to enter into a lowered metabolic state when low temperatures threaten overwinter survival (Brattstrom 1979; Pinder *et al.* 1992; Morrison and Hero 2003).

Unlike its closely related counterpart in the north, *Rana sierrae*, which has been comparatively well studied and is beginning to recover (Knapp et al. 2016), much less is known about the ecology and conservation of *R. muscosa.* Since the 1970’s the population has declined from 166 reported locations in Southern California to nine, representing a loss of >99 % of its historical range (Hammerson 2008; Backlin *et al.* 2015). The most recent estimates indicate fewer than 200 adult individuals remain in the wild (Backlin *et al.* 2015). Decline of the species is attributed to various factors, including chytrid fungus, habitat loss and degradation, and the introduction of non-native predators (Backlin *et al.* 2015). In response to these threats, an *ex situ* population was established as an assurance colony and to provide individuals for release to re-establish or supplement populations that have declined or been extirpated (Santana *et al.* 2015).

Similar to other high-altitude temperate amphibians, such as the Columbia Spotted frog (*Rana luteiventris*; Pilliod *et al.* 2002) and the southern Rocky Mountain boreal toad (*Anaxyrus boreas boreas)* (Muths and Corn 2000), *R. muscosa* can brumate for 6 to 9 months (Bradford 1983). Previous research with adult *R. muscosa* demonstrated that brumation increases reproductive success (Santana *et al.* 2015). In common and boreal toads brumation influences fat deposition, sexual maturation, and reproduction (Dulleman and Trueb, 1986; Jørgensen 1992; Roth, TL *et al.* 2010; Calatayud *et al.* 2015).

Brumation is a complex process that relies on important genetic, molecular, biochemical, and cellular changes interacting with the environment to enhance fitness and survival. Little is known about the role of brumation in the first year proceeding metamorphosis and whether brumation in early life affects fitness. Studies of brumation have focused on size prior to overwintering (Boone 2005) or the relationship between metamorphic timing and size as predictors of future fitness (Semlitsch *et al.,* 1988). However, research on some toad species, e.g. *Bufo vidris, Anaxyrus boreas,* and *A. boreas boreas,* have shown that exposure to cold temperatures can promote growth, which has a positive effect on long-term survival and reproductive viability post-brumation(Jørgensen 1992; Roth *et al.* 2010; Calatayud *et al.* 2015)*.* Amphibians inhabiting higher altitudes and colder climates show positive selection in favor of strong compensatory growth during the short active periods proceeding brumation (Metcalfe and Monaghan, 2001; (Dahl *et al.* 2008). In juvenile and 1-year old common toad *(Bufo bufo),* common frog*, (Rana temporaria) (Tattersall and Ultsch 2008),* and European green toads *(Bufo viridis) (Jorgensen 1986)* brumation is important for growth and fat deposition. Furthermore, some high-altitude species experience growth during brumation, indicating that growth is not exclusively driven by nutrition but that other internal processes such as circadian rhythms are at play (Calatayud *et al.* 2018). Despite growing evidence for the importance of brumation in amphibians, it is often omitted from husbandry practices due to lingering concerns that brumation may be associated with increased mortality (Carey *et al.* 2005). However, there is no evidence in the literature supporting higher mortality rates following brumation in *ex-situ* or natural environments.

Our experiment examined whether brumation of juvenile *R. muscosa* affected pre-release growth rates and survival following translocation to the wild. Given that cool winter temperatures exert a variety of selective pressures on frogs, particularly first-year juveniles, we anticipated that any detrimental effects during brumation would be short-lived and that post-brumation compensatory growth would negate these effects. To test the effects of brumation, we assessed differences in the following traits for experimentally brumated and non-brumated frogs: 1) changes in weight and snout-urostyle length (SUL) in animals housed in captivity for the duration of the 32-week study, 2) growth after release into the wild and 3) survival probability after release into the wild. We predicted that: 1) compensatory changes in weight and growth (changes in SUL) in brumated frogs would match non-brumated frogs by the end of the study (32 weeks) following the end of brumation and, 2) brumated frogs would have higher survival probability following release.

**2.** **METHODS**

**2.1 Study species**

*R. muscosa* is a member of the Mountain yellow-legged frog complex comprising two distinct species, the other being *R. sierrae*. Itis a medium-sized sexually dimorphic amphibian of the family Ranidae with a combination of brown-olive skin with distinct yellow coloration through the legs and brown and black markings (Fig. 1). Sexual maturity is reached between the ages of 3 and 5 and is variable between captive and wild-caught animals. Based on estimates of adult survival probability in the field (~0.75) and a presumed age at reproductive maturity of 3-5 years, the life expectancy for this species is estimated to be between 6-8 years in the wild (Russell *et al.* 2019) and, based on the age of the founding population held at San Diego Zoo, 14 years in captivit*y* (Jacobs *et al.* 2019)*.* Mature individuals measure approximately 5 – 8 cm (snout-urostyle-length, SUL) and weigh 15 – 70 g. Adult males can be distinguished from females by the presence of large nuptial pads on the thumbs of the forelimbs and are generally smaller in size than females when fully grown. Froglets typically weigh 1.5 - 2.5 g at the completion of metamorphosis and measure 22 – 30 mm in snout-urostyle length (SUL).

#### **2.2 Standard housing and care**

As part of the *R. muscosa* recovery program, the San Diego Zoo Global Institute for Conservation Research (ICR) maintains an *ex situ* population designed for the production of zoo-bred offspring and head-starting of juvenile frogs for translocation to the wild. Head-starting is defined here as an *ex situ* management technique that raises early-stage amphibians (eggs, larvae, juveniles) to later life stages (sub-adults, adults) for release into the wild. The source of animals may be from the wild or breeding in *ex-situ* facilities(Semlitsch 2002; Petrovan and Schmidt 2019)*.* Standard housing, detailed below, refers to how frogs were maintained prior to and after the brumation periods. Housing tanks contained recirculating water that passed through a mechanical and biological filter, water chiller, and UV sterilizer before entering the tank. Tanks were outfitted year-round with temperature and light data loggers(Onset, HOBO models UA-002 and U22-001), platforms for basking and feeding, and accessories suitable for hiding under, such as rocks, artificial plants, and polyresin caves(Fig. S1; Supp info.pdf).

We used data loggers (Onset, HOBO) to record air and water temperature in the wild *year-round*, from occupied sites within the San Bernardino National Forest, California. We used the resulting data to modify the facility environment to emulate wild conditions. Water temperatures were seasonally adjusted to reflect approximate wild temperatures for spring (average 8 - 11°C), summer (average 13 - 16°C), fall (average 11 - 13°C), and winter (average 3 - 5°C). Air temperatures also varied seasonally (19 – 25 °C) with midday temperatures warmer than night temperatures. At midday, frogs could elect to bask under a UVB lamp. Two 1,200 cm fluorescent bulbs mounted over each tank provided ambient lighting set on a timer to seasonally appropriate light cycles that were adjusted weekly.

We recorded water parameters 2 - 3 times per week year-round in captivity, including nitrogenous waste components, pH, phosphate, hardness, chlorine, dissolved oxygen, and temperature, and water changes were conducted once a week or more accordingly.Water temperatures and relative light intensity were recorded continuously using data-loggers (Onset, HOBO). Water changes were made as needed, based on water composition analysis, to maintain parameters within appropriate ranges for this species, as established in our husbandry manual (Fig. S1-Suppinfo.pdf). Water changes used reverse osmosis water adjusted for appropriate pH and hardness. These descriptions of water quality monitoring are also applicable to the brumation periods described below.

Frogs were fed 3 - 5 times per week throughout their active period. Their diet consisted of gut-loaded crickets (*Acheta domesticus*), wingless fruit flies (*Drosophila melanogaster*)*,* black soldier fly larvae (*Hermetia illucens*)*,* mealworms (*Tenebrio molitor*), and waxworms (*Galleria mellonella*). Crickets were dusted with a vitamin/mineral powder (Repashy Calcium Plus) before feeding. Tank water was supplemented with liquid vitamins (Boyd Enterprises VitaChem Freshwater) weekly per package instructions.

**2.3 Brumation experiments: effects on growth in captivity**

To examine the effects of brumation, we first conducted a pilot experiment, in which we assigned 18 juveniles to a 4-week brumation treatment (February 18 – March 19, 2015) and 18 to a non-brumated treatment. Animals were housed together according to their treatment groups a maximum of five juveniles per 10 gallons of water (Fig. S1; Supp info.pdf). Juveniles were approximately 1.5 years of age at the commencement of the brumation experiment. The study conducted in 2015 was considered a pilot year for the experiment, and we included fewer individuals in case there were unacceptable mortality or health issues. In a second experiment in 2016 (February 6 - May 5), we tested the effects of a long brumation period of 12 weeks using 148 juveniles (74 non-brumated, 74 brumated)*.* To determine brumation effects on growth, we measured frogs once prior to brumation and every 4 weeks thereafter until release (see below section 2.4). Due to their size, animals could not be marked or reliably distinguished by individual markings during the experimental period. Due to the natural variation in individual weight and size found within the population, efforts were made to distribute the frogs evenly by weight between the groups at the onset of the experiments.

For the non-brumated group, water temperatures averaged 15.5 – 19 °C in 2015 and 13 - 16 °C in 2016. We made this small husbandry modification to the non-brumated group in 2016 to accommodate new information we gathered in the wild with temperature loggers (HOBO Pendant UA-002 and Hobo Water Temperature Pro v2 U22-001) indicating that spring and fall water temperatures averaged 13.59°C during this period when wild frogs were not brumating. These lower temperatures in the non-brumated treatment in 2016 did not induce brumation as determined by continued activity and continued appetite. For brumation treatments, water temperatures were lowered at a rate of approximately 1 °C per day until temperatures were maintained at a range of 3 - 5°C using an Aqua Logic Delta Star Chiller DS-3TXV (Fig. S1; Supp info.pdf). Frogs were fasted for 1 week prior to reaching brumation temperature. We covered tanks with dark plastic to help with insulation. Atmospheric temperatures during this period (December – March) were maintained at approximately 19°C, yet brumated tanks typically reached air temperatures at or below 10°C. We switched ambient lighting sources to lower intensity bulbs (e.g. ExoTerra Repti Glo 2.0 18-inch 15W bulb) and maintained southern California winter light cycles during the brumation period.

**2.4 Translocation and post-release monitoring**

Froglets were translocated within *R. muscosa* historical range into an unoccupied, slow-flowing stretch of the North Fork San Jacinto River, and tributaries thereof, in the San Bernardino National Forest in California. Because we had not yet developed and tested individual identification methodology for froglets in 2015, it was not possible to evaluate brumation's effects post-release in this group. Thus, all data regarding post-release outcomes come from the 2016 release. Before the 2016 release, froglets measuring over 35 mm in SUL were implanted with Trovan 8 mm PIT tags to facilitate individual identification. Froglets below 35 mm were photographed to allow identification via spot pattern, which can be used reliably in this species to facilitate identification. Froglets were transported to the release site by vehicle in food-grade plastic buckets with approximately 2.5 cm of water in the bottom and carried to the release site on foot for distances up to 500m from the road. Before releasing the frogs into the pools, water from the pools was gradually added into each bucket over a 10 min acclimation period to allow the frogs to adjust to the river water temperature and pH. We selected release pools based on size, depth, and cover. Pools were separated by an average of 100 meters (range 20 -250 meters). Sixteen to 32 frogs were released into each pool. To control for effects of pool characteristics on release success, in three of the seven pools, we released frogs from both treatments into the same pool.

To balance the need to release froglets early season and allow us to continue to collect monthly weights and SUL measurements throughout the summer in 2016, we released froglets at two time points: May and September. One hundred and eleven froglets, (67 non-brumated and 44 brumated froglets (111) were randomly selected to be translocated back into the wild on 27 May 2016. The remaining 60 frogs (30 brumated and 30 non-brumated) were held back for another 16 weeks (32 weeks from the initiation of the study, including brumation) to continue collecting growth data. After week 32 the remaining 60 froglets were also translocated into the wild on 29 Sep 2016.

Following the May release, post-release surveys were conducted weekly for four weeks, then once monthly through October. Following the September release, recapture surveys were conducted weekly for four weeks, after which low seasonal temperatures precluded further surveys due to frog inactivity and commencement of brumation. Surveys began 50 m downstream of the first release pool and extended upstream beyond the final release pool for a total length of 930 m.

We attempted to capture each frog that was sighted during a survey. Once captured, we identified the frog (via PIT tag or spot pattern), sexed, weighed, and measured SUL. Individuals not previously PIT-tagged that had grown beyond 35 mm in SUL were PIT-tagged in the field. Skin swabs were collected from a subset of recaptured individuals to test for the amphibian chytrid fungus *(Batrachochytrium dendrobatidis)* and *Ranavirus*. After processing, each frog was released back to the location from which it was captured. We recorded GPS coordinates for each frog sighted, whether or not capture was successful. Frogs not captured could not be uniquely identified and thus were not included in statistical analysis.

**2.5 Data and statistical analysis**

*2.5.1 Juvenile brumation (four- and twelve-week brumation)*

Linear modeling (ANCOVA) was conducted to examine differences between treatments in juvenile growth rate as measured by weight and snout-urostyle length (SUL) in three distinct models:

1. Frogs brumated for 4-weeks or not brumated.
2. Frogs brumated for 12-weeks versus not-brumated and maintained ex-situ for 32-weeks.
3. Frogs brumated for 12-weeks and then translocated to the wild.

This analysis in the third model was used to determine changes in weight and SUL in frogs that remained ex-situ for the 32-week study separately from frogs translocated halfway through the study (at week 16).

In all the cases, the main effect model terms were brumation group (brumated or not brumated) and time (as a continuous variable, weeks) (Wilkinson and Rogers, 1972; Chambers, 1992).  The group x time interaction was used to determine if growth rates differed between the groups. Statistical analyses were conducted using R (R-studio v1.2.5033, 2019 RStudio Inc). Residual analysis showed non-constant variability so a regression model for σ was developed (absolute value of residuals against time) and used to repeat the linear modeling with regression weights (1/σ2) (Neter et al. 1996). This analysis was conducted for the 4- and 12-week studies separately.

Since growth in the treatment groups was further affected by the environment in which they were placed, *in*- versus *ex-situ*, we analyzed the trajectories of weight gain and increases in body length in reintroduced brumated and non-brumated animals separately from those held *ex situ*. Mean weight and SUL in captive and translocated groups of brumated and non-brumated frogs were analyzed from week 16 -32 using a two-tailed Welch t-test (GraphPad Prism V 8.4.2, 2020).

*2.5.2 Effect of brumation on short-term post-release apparent survival*

We used the package R2ucare (Gimenez et al. 2017) to confirm goodness of fit for our modeled capture history data. For the first release group (N = 111) we fit Cormack-Jolly-Seber mark-recapture models in Program MARK (White & Burnham 1999) run through RMark (Laake and Rexstad 2008). We did not include the second release group (i.e. the animals released on 29 Sep 2016 after being held an additional 16 weeks in captivity to collect comparative data on growth rates; see section 2.4) in models due to the limited number of post-release surveys and recaptures for this group and we excluded from analysis the three surveys that took place after the second release (these surveys resulted in a cumulative total of only two recaptures for the first release group). We used uneven survey-intervals (~1 week between the earlier surveys, ~4 weeks between the later surveys) and generated estimates for monthly survival rates. We fit models with and without one or both of two covariates for survival (brumation treatment and/or release pool) and with/without one covariate for recapture probability (brumation treatment). This resulted in a total of 8 models (including those that modeled survival and/or recapture probability as a constant). We ranked models by AIC. Due to low **△**AIC values between models (all <4), we used model-averaging (implemented through the ‘model.average’ function in RMark) to calculate real parameter estimates of survival and recapture probabilities.

1. **RESULTS**
   1. **Effects of brumation on juvenile growth (weight gain and body size (SUL) increases)**

During 4 and 12-week brumation treatments, there was a cessation of growth in both SUL and body mass. While constant for non-brumated frogs, growth in brumated frogs resumed within eight weeks of their emergence from brumation and followed a similar linear trajectory to that observed in non-brumated animals. Brumated frogs attained comparable SULs to non-brumated frogs within eight weeks of emergence, regardless of whether they had overwintered for 4 or 12 weeks. For brumated frogs, reaching body lengths equivalent to those observed in non-brumated animals appeared to come at the expense of weight (see sections 3.2.1 and 3.3.1). Frogs that were brumated for 12-weeks and were translocated into the wild on week 16 (i.e. four weeks from emergence from brumation) reached body weights and lengths equivalent to their non-brumated counterparts faster than frogs that remained in ex-situ for the duration of the study (32 weeks).

* + 1. *Mortality in brumated and non-brumated frogs maintained ex-situ*

Mortality rates during this pilot experiment (4-weeks) were low and were approximately evenly distributed across treatment groups (3 in the non-brumated group, 2 in the brumated group) while in the 12-week study, 3 mortalities occurred in the non-brumated group. None of the animals in the pilot study were released and this study was analyzed independently of the 12-week study.

**3.2 Morphological assessment of brumation and post-brumation growth in juveniles overwintered for 4-weeks while maintained *ex-situ***

*3.2.1 Changes in weight*

Upon emergence from brumation, frogs did not reinitiate weight gain immediately. Weights did not differ significantly across time within the brumated group until the last sampling point (Fig. 2A; Table 1). Non-brumated frogs had significantly greater weights (Table 1) as well as a greater growth rate compared to brumated frogs from the time of emergence throughout the entire study (Table 1; Fig. 2).

*3.2.2 Changes in SUL*

In contrast to the results observed for weight, increases in SUL within the brumated group became significant at week 17 and continued to increase significantly until the end of the study (Table 2; Fig.2B. Growth rates between brumated and non-brumated frogs only differed at week 9 (t-value= 2.886; P=0.005) four weeks after their emergence from brumation. However, both groups showed linear growth and no significant difference in growth rates for the remainder of the study (Table 2; Fig. 2B).

**3.3 Morphological assessment of brumation and post-brumation growth in juveniles overwintered for 12-weeks.**

Brumated frogs did not change with respect to weight or body length during their time in brumation whilst non-brumated frogs continued to grow during this period. Similar to brumated frogs in the four-week study, brumated frogs that remained in captivity for the duration of the 32-week study did not show signs of resuming growth until eight weeks after emerging from brumation (week 20) (Tables 3 & 4; Fig. 3A-B). In contrast, brumated frogs translocated back into the wild showed accelerated growth and weight gain compared to brumated frogs still in captivity (Table 5 -6; Fig. 4). Growth rates in the treatment groups was further affected by the environment in which they were placed, in- versus *ex-situ*. To break this analysis down clearly, we describe changes in growth rates in brumated and non-brumated animals housed *ex-situ* separately from those included in the translocation research.

*3.3.1 Changes in weight (ex-situ only)*

Throughout the 32-week duration of this study, frogs brumated for 12 weeks remained significantly lighter than non-brumated groups (Fig. 3A; Table 3). In the brumated group, increases in weights became apparent eight weeks after frogs emerged from brumation (Fig. 3A), highlighting a delay in which brumated frogs presumably resumed active foraging again and began gaining weight. Although average weights remained significantly different, once active, the rate of weight gain in the brumated group progressed in a linear fashion and did not differ significantly from that observed for non-brumated animals (Table 3; Fig. 3A).

*3.3.2 Changes in SUL (ex-situ only)*

Brumated frogs maintained in captivity for 32-weeks had delayed increases in body length in comparison to the non-brumated group and did not show any increase in SUL (Table 4) until eight weeks after they emerged from brumation (Fig. 3B). Although non-brumated frogs did not appear to grow much during the winter period they grew enough to remain significantly longer than brumated frogs (Table 4). Brumated frogs remained shorter than non-brumated frogs from the time of emergence through the remainder of the study (Table 4, Fig. 3B). SUL in brumated frogs appeared to increase faster than weight. Although both groups grew linearly throughout the study, brumated frogs had a slightly greater rate of increase in SUL (F-value=240.6, P=0.0326).

*3.3.3 Changes in weight (in situ)*

We conducted surveys at 1, 2, 3, 4, 8, 12, and 16 weeks post-release, and these time points are referred to herein as 17, 18, 19 20, 24, 28, and 32 weeks to reflect the time since the start of the experiment. For translocated individuals, no significant differences in weight between the brumated and non-brumated treatment groups were detected (Fig. 4A; Table 5A). Although there was an uneven distribution of frogs recaptured from each group at every time point, the results indicate that brumated frogs reached average weights equivalent to their non-brumated conspecifics shortly after release (Fig 4A). Furthermore, weight gain continued to increase similarly in both treatment groups as reflected by the frogs recaptured in subsequent surveys. By the end of the 32-week study, the weights of translocated brumated frogs were significantly greater than brumated and non-brumated frogs that remained in captivity (Table 6A). Among non-brumated frogs, translocated individuals differed, albeit non-significantly, from that maintained ex situ (Table 6A). The post-release weights of translocated brumated frogs were not significantly different from translocated non-brumated frogs (Fig 4A; Table 6A).

*3.3.4 Changes in SUL (in situ)*

Similar to the results observed for changes in SUL described previously, increases in body length changed faster overtime for the brumated frogs than did changes in weight. After 1 week in the wild (week 17), brumated animals no longer showed any significant differences in SUL compared to their non-brumated counterparts (Table 5B, Fig 4B). Of the animals recaptured for each treatment group, both had a gradual increase in SUL, and recaptured frogs had increased their weight significantly by week 24 (Fig 4B; Table 6B). Overall, during the final weeks of the study, SULs were not significantly different between treatment groups in zoo-based or translocated animals (Table 6B).

*3.4 Effect of brumation on short-term post-release fitness*

The mark-recapture data passed (χ2=0.743, df = 4, p = 0.946) the omnibus test for goodness of fit (‘overall\_CJS’ function in the R2ucare package), thus, we moved forward with fitting Cormack-Jolly-Seber mark-recapture models (Gimenez et al. 2017). The top-ranked model contained only brumation treatment as a predictor of survival probability and modeled recapture probability as constant. However, △AIC values for the full set of Cormack-Jolly-Seber models were all <4 (Table 7), thus, we implemented model averaging. Model-averaged monthly apparent survival estimates (± S.E.) were 0.72 ± 0.15 (95% confidence interval: 0.37-0.92) and 0.70 ± 0.14 (95% confidence interval: 0.39-0.89) for frogs that were brumated (one estimate is provided for each release pool). For frogs that were not brumated, mean model-averaged monthly apparent survival estimates (± S.E.) were 0.56 ± 0.16 (95% confidence interval: 0.26-0.83) and 0.48 ± 0.17 (95% confidence interval: 0.19-0.78). In the top-ranked model, the 95% confidence interval of the beta estimate for brumation treatment did not overlap with zero (0.05-2.99), suggesting a statistically significant, positive impact of brumation on survival probability. However, this effect was not robust, and in lower-ranked models that allowed survival probability to vary with brumation treatment the 95% confidence interval of the beta, estimate did overlap with zero (though these models did consistently show the same pattern of brumated animals exhibiting higher apparent survival than non-brumated animals).

Detection probability showed some differences between brumated and non-brumated frogs, with brumated frogs being slightly more detectable (mean ± S.E. of 0.11 ± 0.03, 95% confidence interval 0.07-0.18 for brumated frogs vs. 0.09 ± 0.03, 95% confidence interval 0.05-0.15 for non-brumated frogs). In the highest-ranked model that contained brumation treatment as a covariate for detection, the 95% confidence interval of the beta estimate for brumation treatment did not overlap with zero (0.11-1.48).

**4.** **DISCUSSION**

Results from this study supported our prediction that growth was delayed during brumation, but that subsequent compensatory growth occurred once frogs were returned to warmer temperatures. Unlike the non-brumated group, this pattern of compensatory growth in brumated frogs relied on a faster increase in body length than weight gain compared to the brumated frogs, which had a steady rise in both weight and body length. Although frogs from the 4-week study resumed growth earlier than frogs in the 12-week study (5 weeks and 8 weeks respectively), ultimately brumated frogs were able to reach similar weights and SULs to non-brumated frogs regardless of the length of brumation.

Release to the wild was associated with rapid growth compared to frogs maintained *ex-situ* but released brumated frogs also showed a delay in growth in the weeks immediately after translocation, as might be expected as frogs acclimate to their new environment and learn how to extract resources. Analyses of post-release weights and SULs show that translocated brumated frogs matched non-brumated frogs in weight and SUL by the end of the study. Translocated brumated frogs showed more rapid compensatory growth than brumated frogs held *ex-situ* and differences in growth between these treatment groups indicate unexplored and potentially important effects of living in the wild on an animal’s body condition. Although beyond the scope of this study, future studies should examine diet quality and availability which may be higher in the wild compared to animals maintained *ex-situ.*

Jorgensen (1992) suggests that brumation initiates physiological processes that enable adaptation to seasonal fluctuations in temperature and restricted periods in which animals can access food resources. Post-brumation growth spurts may come at the expense of weight gain when nutritional allocation is invested in growth first and fat stores later (Metcalfe & Monaghan, 2001; Pope & Matthews, 2002). For species inhabiting harsh climates with time-limited access to food and foraging-favoring temperatures, adaptive plasticity should favor individuals that adapt their structural growth, storage, and reproduction to short active periods (Bernard 1994). This strategy for cold climates may select for compensatory growth mechanisms to support growth and development during the limited growing season. In *Bufo bufo,* larger increases in appetite and compensatory growth occur when subjected to longer brumation periods, at the expense of decreased lipid deposition observed for shorter brumation periods (Jorgensen, 1986). Therefore, the observed *R. muscosa* growth “spurts” suggest a similar compensatory mechanism in this species and may explain why reduced growth rates associated with brumation did not lead to costs in health or post-release survival.

Our study did not characterize the physiological underpinnings of compensatory growth, but lessons from research with other species are informative. In *Pelophylax esculenta,* brumation elicits tissue modifications influencing the contractile performance of the heart, renal performance (to avoid dehydration), and cellular apoptosis leading to the reduction in size of several tissues including the digestive tract, kidney, and liver (Constanzo, *et al.* 1995). Such tissue modification supports rapid recovery of weight after brumation (Naya *et al.* 2009). Further, alterations to intestinal flora contribute to appetite change and nutrient uptake during overwintering (Gossling *et al.* 1982), explaining phenomena like aphagia, hypothermia, and biochemical changes and alterations to hormonal and gene expression patterns (Muir *et al.* 2007). These physiological underpinnings of brumation are stage-specific and not static throughout the period (Brenner, 1969). For example, natural temperature fluctuations provide breaks in brumation and allow episodic growth spurts (Castanet, 1990). This carefully regulated suite of physiological responses during brumation suggests that it is part of an evolved strategy that affects many aspects of organismal biology, with cascading impacts on fitness (e.g. survival).

Short-term brumation of *ex situ* animals can provide conservation breeding programs the capability to ensure that animals destined for release experience brumation, and its adaptive benefits before release, in a time-wise manner while optimizing yearly translocation plans. Due to the reality’s conservation management programs face, there are often multiple trade-offs to consider when undergoing research that seeks to maximize animal production and fitness. Future work should continue to explore the impacts of differential brumation periods as well as the effects of varying brumation temperatures which would better model ever-changing environmental and climatic conditions faced by translocated animals.

Our study provides evidence that brumation does not negatively impact survival but rather provides animals destined for release with exposure to environmental cues that they will experience in the wild. Therefore, the benefits of brumation may act to enhance short-term post-release apparent survival. Our findings are somewhat limited by low individual detection rates in combination with low-density populations but do highlight that detection of brumated frogs was slightly higher than for non-brumated frogs. Although stream occupancy (presence/absence) by *R. muscosa* is highly detectable (Backlin *et al.* 2015), our results confirm that individual visual detection/identification probability is relatively low for this species (~10%;(Hammond *et al.* 2020). Moreover, because this species is known to disperse after translocation (Matthews 2003) and mark-recapture models cannot distinguish between animals that have died and those that have migrated out of the study area, our survival estimates are likely underestimated. Improved methods for detecting translocated frogs are required to evaluate the effects of brumation and other pre-release treatments on long-term fitness. Recently radiofrequency identification (RFID) technology has significantly increased recapture rates of this species in the wild(Hammond *et al.* 2020), opening doors for an improved understanding of which pre-release treatments may enhance post-release success. Small radio-telemetry implants and scent detection dogs (Savidge *et al.* 2011; Byosiere, *et al.,* 2019) are also being tested for their ability to increase detection of this species. Statistical methods to account for uncertainty may also be implemented in the future (e.g. (Gilroy *et al.* 2012; Schaub and Royle 2014)).

**5.** **CONCLUSIONS**

Understanding and managing the rearing environment for animals intended for release to the wild has long been considered important (Kleiman 1989; Beck 1991). Our research with *R. muscosa* highlights the importance of less readily observable physiological changes that may be influenced by husbandry decisions, with potential repercussions for post-release growth and survival. Therefore, particularly when *R. muscosa* are destined for release into the wild, implementing brumation into standard husbandry procedures may be critical not only for reproduction (Santana et al. 2015), but also for post-release success more generally. Future research could reveal that changes in physiological function among animals in *ex situ* populations may underpin the success or failure of translocation programs. Such research informs adaptive management strategies necessary to move forward the field of translocation biology and provide managers with the necessary tools to re-establish at-risk species on the landscape.

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**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest exist.

**AUTHORS CONTRIBUTIONS**

NEC and NRG collected data pertaining to brumation. MJC, NRG, NEC and DMS undertook surveys and collected data pertaining to translocation and monitoring. NEC and TH analyzed and wrote the manuscript. DMS and TH wrote and analyzed the translocation and monitoring section, and TTH analyzed translocation data. NEC, NRG, MJC, DMS, and RRS were all involved in the experimental design and editing of the manuscript. Dr. Natalie Calatayud would like to thank the San Diego Zoo Institute for Conservation Research for her post-doctoral fellowship and Exploradora de Immuebles, S.A. (EISA) for awarding financial aid to the Mountain Yellow-legged program.

**DATA AVAILABILITY STATEMENT**

Data will be made available from the corresponding author upon reasonable request.

**ETHICS STATEMENT**

We performed this research ethically under the standardized auspices of the San Diego Zoo Institute for Conservation Research’s Institutional Animal Care and Use Committee, IACUC protocols 15-001 and 16-005.

**ORCID**

*Natalie E Calatayud* [*https://orcid.org/0000-0003-4890-0190*](https://orcid.org/0000-0003-4890-0190)

*Talisin T Hammond* [*https://orcid.org/0000-0003-4175-8128*](https://orcid.org/0000-0003-4175-8128)

*Nicole R Gardner* [*https://orcid.org/0000-0002-4046-9685*](https://orcid.org/0000-0002-4046-9685)

*Michelle Curtis* [*https://orcid.org/0000-0002-9852-6507*](https://orcid.org/0000-0002-9852-6507)

*Ronald R. Swaisgood* [*https://orcid.org/0000-0001-5353-2148*](https://orcid.org/0000-0001-5353-2148)

*Debra M Shier* [*https://orcid.org/0000-0003-0262-6501*](https://orcid.org/0000-0003-0262-6501)

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