2019

What lies beneath? : removal sampling to test for biases in surface activity of the eastern red-backed salamander (Plethodon cinereus)

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What lies beneath? Removal sampling to test for biases in surface activity of the eastern red-backed salamander (*Plethodon cinereus*)

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Honors Thesis

Submitted to:
Biology Department
University of Richmond
Richmond, Virginia

26 April 2019

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(advisor signature) 4/24/19
(date)

(reader signature) 4/20/19
(date)

Abstract: In light of the current global amphibian extinction crisis from threats such as climate change and disease, reliable and repeatable sampling methods carry heavy importance for assessing North American salamander species. Artificial coverboard sampling methods have long been employed in the study of terrestrial salamanders, such as the common eastern red-backed salamander (*Plethodon cinereus*), to provide estimates of population abundance. While consistently cited as a method that provides reliable capture numbers and systematic sampling, questions remain as to whether the surface salamanders surveyed are representative of the study population as a whole. In this study, removal sampling was incorporated into a standard artificial cover board and mark-recapture survey design to test if behavioral territoriality or density-dependent competition results in an inherent bias from the use of this sampling protocol. Differences were investigated between the characteristics of primary occupants of the cover boards and secondary occupants, which entered the cover board space upon the removal of the primary individuals. No difference was found between the two groups for sex, morph, snout-vent length (SVL), or reproductive state. Primary occupants had a higher mass, higher body condition (BC), and longer tails than secondary occupants. There was also a non-significant trend towards a higher proportion of recaptured salamanders as primary occupants compared to secondary. These data suggest that individuals captured by artificial cover objects may have more access to food and exhibit site territoriality. Despite this territoriality, my results suggest that artificial cover object survey protocols can collect a representative sample of the population for capture-recapture analysis. Reinforcing the robustness of this method has important implications for the study of amphibians with low detectability in the face of rapid environmental changes.

Introduction

The eastern red-backed salamander (*Plethodon cinereus*) is an integral component of forest floor ecosystems across the Northeastern United States and Canada. Plethodontid salamanders have been estimated to account for the largest portion of terrestrial vertebrate biomass in eastern North American forests (Burton and Likens 1975, Milanovitch and Peterman 2016). Accordingly, changes in population abundance of forest salamanders can have widespread impacts on forest food webs. In addition to their important role in community dynamics, this species is a useful indicator of environmental change as a lungless terrestrial salamander (Welsh and Droege 2001). Respiration occurs through the skin in plethodontid salamanders, and these species have been shown to be particularly susceptible to changes in moisture, temperature, pH, and soil chemistry (Feder 1983). These aspects of their physiology, in addition to their prevalence and wide geographic range, make *P. cinereus* an excellent species for indicating overall forest health. Changes in environmental conditions that impact forest health, such as shifts in temperature under global climate change, alterations to habitat with forest fragmentation, and reductions in environmental quality from pollution, are also likely to be reflected by declines in the salamander population. The broad distribution and abundance of *P. cinereus* has also resulted in a high volume of knowledge about the biology of this species (Petranka 1998, Jaeger et al. 2016), which can be useful for understanding future changes in population dynamics.

Measuring abundance and studying population changes over time requires estimation methods that account for detectability. As a largely fossorial species that surfaces mainly for feeding, *P. cinereus* requires survey methods that account for the majority of the population being unavailable for capture during any particular survey occasion (Taub 1961, Bailey 2004a, Dodd and Dorazio 2004). Mark-recapture methods provide a reliable method of determining population sizes and tracking changes through time because they estimate parameters such as abundance and survival while accounting for detection (Mazzerole et al. 2007). Bailey et al. (2004a) estimated average capture availability of Plethodon salamanders in the Great Smoky Mountains and found that only 13% of individuals were available for capture in a single sampling event. Another challenge for surveying *P. cinereus* is detecting them through the habitat complexity of the forest floor. A standard survey technique for *P. cinereus* that addresses this challenge is the placement of artificial cover objects. Grids of equidistant cover objects placed into the habitat provide the opportunity for standardized surveys of the individuals beneath each object. In the case of plethodontids, cover objects provide shelter from predation and increased moisture levels during periods of surface-activity (Grover 1998). While cover objects serve as a consistent method for capturing *P. cinereus*, potential biases created by this method need to be considered, such as the assumptions that the sample is random and representative of the population as a whole. In traditional mark-recapture methods, each individual must have equal probability of being captured and all individuals must be available for capture (Mazerolle et al. 2007). If surface activity differs between individuals and not all individuals in the population are equally catchable, then estimates of population parameters can be biased.

In a study examining the effects of cover and moisture on plethodontid activity, Grover (1998) found that juveniles have lower surface activity in dry conditions. Both juveniles and adults were more abundant on plots with higher cover object density. He suggests that the source for this disparity is the desirability of the cover objects as a prime habitat for this species, which supports higher levels of surface activity by using artificial cover objects that increase the density of this resource on the forest floor. Marsh and Goicochea (2003) examined the introduction of bias from artificial cover object arrays for sampling *P. cinereus* and found that while daily sampling of the boards decreased the number of individuals captured, weekly sampling did not, suggesting that artificial cover boards can provide an accurate population estimate if they are not sampled repeatedly over short periods of time. Additionally, they tested for differences between individuals captured from artificial cover objects versus natural cover objects. Here, they found a higher proportion of adults to juveniles under the artificial cover objects, but no difference in sex ratios or snout-vent length within the age classes.

Cover objects provide a prime habitat for *P. cinereus* by supplying a moist place for hunting and mating that is safe from predators. Thus, this sampling methods provides a resource that creates conditions for competition. Therefore, the territorial behavior of this species should be considered in assessing the use of cover object arrays as they can be a limited resource for *P. cinereus*. Several studies have implicated territoriality as a factor that may impact surface activity of this fossorial species. Jaeger (1980) established that surface-active salamanders are almost always in a state of higher energy use than intake, and that competition also occurs over prey as a limiting resource. Taken in conjunction with a later study finding that *P. cinereus* exhibits extreme site tenacity across seasons (Gergits and Jaeger 1990), competition for access to prey is a likely mechanism driving territoriality in this species. Competitive interactions that determine territoriality have been explored in many studies. Jaeger et al. (1982) performed a series of intruder experiments in the laboratory and found that site holders defended against intruders using agonistic posturing as well as biting. Once sufficiently supported in an experimental environment, evidence of territoriality in the natural environment was supplied by Mathis (1990), finding that removal of an occupant from a cover object allowed for the uptake of the space by a new, smaller salamander and that size conferred a competitive advantage to individuals defending cover object territory.

Another strategy for measuring population composition is removal sampling, where an area is repeatedly sampled and individuals are not returned until after several consecutive surveys. The change in capture numbers over consecutive removal surveys can provide information on population abundance and composition (Petranka and Murray 2001, Bailey et al 2004b). This study combined mark-recapture techniques with removal methods to test for biases in surface activity in the *P. cinereus* population in the James River Park system in Richmond, Virginia. Individuals in this population are annually surveyed via cover object sampling and mark-recapture is used to account for detection in estimating population abundance. In this study, characteristics of surface-active individuals were investigated in regard to shifts when primary occupants were removed for mark-recapture measurements. After primary occupants were temporarily removed, a second survey was conducted 48 hours later to collect secondary cover object occupants. If a factor such as territoriality, sex-specific surface activity, or morph-linked behavior or dominance is present within the population, differences in the characteristics of the primary and secondary occupants would be expected. Testing for differences between the primary and secondary occupants determines whether there is an inherent bias in this sampling method and may indicate if site fidelity or territoriality are impacting the estimation of population parameters for this species. Evidence of this kind of bias may require a reassessment of sampling methods for this and other fossorial species. Given the current rate of decline and increasing need for population statistics on the Plethodontid taxon, accurate methodologies for acquiring these statistics are essential.

Methods and Materials

Study Site

This study was conducted in the James River Park system in Richmond, Virginia at the 42nd Street entrance to the Buttermilk Trail (37.524783 N, -77.476708 W). The study site was a rock quarry in the early 20th century and has since been converted into a recreational public park. This riparian mixed hardwood and deciduous suburban forest is mostly comprised of oaks, pawpaws, tulip poplars, and maples. To one side, the James River and train tracks border the forest, and to the other, a suburban housing development estimated at over 1,000 people/km^2 (CIESIN Data, 2016).

Study Design

Three 5 x 10 m plots were established within the park system in December 2015: T1 (33m elevation), T2 (30.3 m elevation), and H2 (29.8 m elevation). Each plot contained an array of 50 artificial pine cover boards (5cm x 30.5cm x 35.5cm) placed 1 m apart in a 5 x 10 m grid. Plots were sampled on a weekly rotating basis in the order of T1, T2, H2. Air and ground temperature were recorded at the center of the plot during each survey. Due to the location of the study site near the southeastern edge of the range, *P. cinereus* is only available for surface collection in spring and fall because of constraints in temperature and moisture during the summer. Therefore, surveys were conducted from February-April and September-December 2018 when salamanders were available for capture and not completely subterranean. This protocol was based in part from that developed by the Salamander Population and Adaptation Research Collaboration Network (SPARCnet; Sutherland et al. 2016).

Surveys and Measurements

For each week, only one site was sampled on a recurring rotation, allowing two weeks between repeated sampling of the same plot. During the first sampling event for the removal of primary occupants, cover boards were systematically searched and all *P. cinereus* individuals were collected from individual boards and placed in closed plastic containers with wet paper towels specific to each board. They were transported back to the University of Richmond where the following data were recorded for each individual: morph (red or lead), board location, snout-vent length (SVL), total length (TL), presence of cirri, number of eggs, sex, recapture status, mass, and marks. Salamanders were classified as female if eggs were detected and males if testes were detected using a standard candling method. If neither was found, the salamander was classified as "unknown." The measurement of SVL was made on the ventral side of the salamander from tip of the snout to the anterior cloaca. If the salamander had not been previously captured, it was assigned a color combination selected from five colors (red, pink, orange, green, and blue) and marked on the trunk by each limb with visible implant elastomer (VIE) tags. VIE is injected as a liquid beneath the skin and cures after injection to a pliable biocompatible solid. Though the marks are detectable in visible light, UV light assists in identification for fluorescent colors (Beausoliel 2004). This method of tagging was chosen because it has been shown to last long-term in Plethodontid individuals with low mark migration (Lunghi and Bruni 2018) and does not affect survival or growth of marked individuals (Phillips and Fries 2009). Limitations of this marking technique are the potential for mark loss, application of a mark too deep to be identified, and that proper mark application largely depends on proficiency of the operator (Beausoliel et al 2004). After removal of primary occupants, secondary occupants were collected 48 hours later in the same manner and primary occupants were then returned to the exact board from which they were collected after the second survey. Secondary occupants were also transported to the lab and the same data collection procedure was followed. Secondary occupants were returned 24 hours later to the exact board from which they were collected (Figure 1).

Data Analysis

To make comparisons between salamanders captured on primary and secondary occasions, two-sample t-tests were conducted for metrics of size and population composition. The variance within occasion was computed for each of these metrics and t-tests accounting for unequal variance were used when needed. Normality was assessed for each comparison and the criterion applied per Lumley et al. (2002) that this assumption need not be met for samples greater than 80. For non-normal samples with $n<80$, non-parametric tests were used.

Counts: The total number of individuals captured for primary and secondary occasions were counted for all 20 weeks of the study and the number of individuals captured was compared between the primary and secondary occasions.

Size: Mass, snout-vent length (SVL), tail length (TaL), and body condition (BC) were analyzed to test for differences in size between individuals captured on primary and secondary occasions across all survey weeks. TaL was calculated as total length - SVL. BC was calculated as log(mass)/log(SVL).

Composition of Captures: For each survey occasion, the proportions of each morph, sex, reproductive state, and recaptures were calculated from the total individuals captured each week that possessed the trait. These proportions were arcsine square root transformed and compared using paired t-tests to account for differences between plots from week to week. Only weeks where both primary and secondary individuals were captured were used in these analyses due to the paired nature of this statistical test; each week of the study is therefore a replicate in this analysis. For each capture occasion, if fewer than four individuals possessed a trait, the occasion was excluded from analysis. Replicates are as follows: morph (n=17), males (n=15), females (n=15), reproductive state (n=13), recaptures (n=16). Individual tests were performed for the proportions identified as males and females due to the number of unknown sex individuals in each sample. A paired t-test was performed on number of males, number of females, and recaptures, which all met the assumption of normality. A Wilcoxon Signed Rank test was performed on morph and reproductive state as they did not meet the assumptions of normality for a paired t-test and n<80.

Results

Counts

For primary occasions, 1215 salamanders were captured; for secondary occasions 408 salamanders were captured. There were, on average, 36.75 (± 18.38) more cover object occupants captured on primary occasions than secondary occasions (two-sample t-test, $t_{29.54}$=-4.08, p=0.0002, Figure 2). Averages are reported with standard error throughout.

Size

Primary occupants weighed on average 0.14g (± 0.05g) more than secondary occupants (two-sample t-test, t_{1621}=-5.64, p<0.0001, Figure 3A). On average, secondary occupants had 1.1mm (± 0.77mm) longer SVL than primary occupants (two-sample t-test, t_{1621}=2.81, p=0.005, Figure 3B). Primary individuals had tails that were 6.312mm (± 1.62mm) longer than secondary occupants (two-sample t-test, t_{1621}=-7.64, p<0.0001, Figure 3C). Primary occupants also had better body condition than secondary occupants (two-sample t-test, t_{1621}=-9.31, p<0.0001, Figure 3D).

Composition of Captures

There was no difference between primary occupants and occupants for morph (Wilcoxon signed rank, S_{16}=8.5, p=0.703, Figure 4A), number of males (paired t-test, t_{14}=-1.34, p=0.200, Figure 4B), number of females (paired t-test, t_{14}=0.779, p=0.449, Figure 4B), reproductive state (Wilcoxon signed rank, S_{12}=-0.763, p=0.460, Figure 4C), or recaptures (paired t-test, t_{15}=-1.91, p=0.075, Figure 5).

Discussion

This study determined whether artificial cover board sampling methods for *P. cinereus* results in the collection of a representative sample of the population as a whole, fulfilling one of the classic assumptions of mark-recapture analysis. The competition for cover object habitat and the territorial behavior of this species presented the possibility that a subgroup of the population (e.g., juveniles, females, or one of the morphs) could be more excluded from surface activity under artificial cover boards. The data gathered by comparing individuals captured during primary and secondary surveys do not suggest the presence of these biases in the composition of the sampled population. However, the trend towards high numbers of recaptures for surveys using coverboards should be examined further and accounted for when creating population models. Mark-recapture models can account for this “trap-happy” response by including a behavioral term that permits initial probability of detection to differ from probability of recapture (Bailey 2004b, Nichols et al. 1984). Determining the potential bias in this sampling method is valuable because artificial coverboards are one of the most frequent methods for collecting plethodontids and other fossorial species (Grover 1998).

The salamander species at the center of this study, *P. cinereus*, is known for reaching particularly high local densities. Several studies have reaffirmed the conclusions of Burton and Likens (1975) that *P. cinereus* populations constitute a high percentage of biomass in eastern North American forests and as such are an integral part of the forest ecosystem (Petranka and Murray 2001, Milanovitch and Peterman 2016). In fact, the local density of *P. cinereus* at the site of this study is one of the highest documented (Hernández Pacheco, *in review*). As no other *Plethodon* species are present at this study site, the lack of interspecific competition coupled with extreme local density suggests the potential for intense intraspecific competition for coverboard habitat at the location of this study.

One of the most likely shifts predicted for surface active salamanders under cover boards is body size. Size was examined because previous studies have found that larger individuals can occupy artificial cover objects compared to the proportion of juveniles to adults seen in other survey methods (Mathis 1990, Marsh and Goicochea 2003). Grover (1998) also found that juveniles are less surface active in dry conditions. Therefore, artificial cover object sampling may preferentially collect larger individuals whether as a result of a better ability to defend the territory or from increased surface activity with greater availability of cover habitat.

This study found that there was a higher average mass of primary occupants. However, while there was a 1.1mm statistically significant difference between primary and secondary occupants for SVL, this difference is unlikely to be a biologically meaningful size advantage for this salamander as the average total length of this study population is 74.3 mm. Therefore, the difference in mass is not attributable to a difference in the composition of adults and juveniles captured under cover boards, which was a surprising result given the findings of previous studies (Mathis 1990, Marsh and Goicochea 2003). Despite the similarity in SVL between the two groups, primary occupants were found to have longer tails than secondary occupants. Body composition was then examined to examine whether the difference in mass between primary and secondary protocols was due to the difference in tail length. This analysis showed that the primary occupants also have a higher BC, which is either due to having higher fat percentages, mass stored in the tail, or a greater proportion of individuals with eggs. Because there is no difference in reproductive state between primary and secondary occupants, it was concluded that the higher BC in primary occupants was due to a higher percentage of fat stores. Most likely, this is a result of the increased moisture under the coverboards which affords the salamanders better conditions in which to hunt (Grover 1998). Where population studies are concerned, this method collects a representative sample of individuals in regard to size and life stage, with primary occupants having higher body condition, possibly due to higher access to food.

In addition to size, competitive interactions or territoriality could also result in one morph or sex being reduced or excluded from cover boards. This study found no differences between primary and secondary occupants amongst sex, morph, reproductive status, or recapture proportions. Other studies support this study's conclusion that there is no discrepancy in territoriality between the two sexes or morphs (Marsh and Goicochea 2003, Jaeger 1982). Similarly, Horne (1988) found that gravid females did not differ in aggression from non-gravid females or males, which supported this study's finding that there is no difference in the proportion of gravid females between primary and secondary occupants. Overall, there was no statistical difference between these two groups for population composition characteristics.

The proportion of recaptured individuals on each of the survey occasions is particularly of interest in this study because this metric indicates whether the same individuals maintain hold over the territory over repeated sampling, which could lead to an unrepresentative sample. Although there was no significant difference between the proportion of recaptures for primary and secondary occupants, there was a clear trend towards a higher proportion of recaptured primary occupants. This trend, when taken in consideration with the difference in tail length between the two groups, suggests that primary occupants are better defenders of the coverboards, therefore retain their tails and occupy the coverboards more often. While a difference in recapture proportions between capture groups could pose a problem for population estimates, spatial capture-recapture (SCR) methods accommodate these differences by including the behavioral effect in the statistical models to provide robust and accurate demographic estimates (Muñoz et al. 2016, Sutherland et al. 2016, Bailey et al. 2004a, Bailey et al. 2004b). While SCR methods estimate population parameters accounting for detection discrepancies, they still depend on samples that are characteristically representative of the study population. In species such as *P. cinereus,* which are fossorial and have low surface detectability, SCR methods are amongst the most unbiased of estimation methods provided the sampling method does not underrepresent parameters such as sex, age class, or morph due to behavioral competitive exclusion or density-dependent competition (Muñoz et al. 2016, Sutherland et al. 2016, Bailey et al. 2004a, Bailey et al. 2004b).

As in any ecological study, considerations need to be made when interpreting the findings. Firstly, due to the fossorial nature of *P. cinereus*, one of the few systematic ways of sampling a population is through the use of cover boards. Although natural cover object searches may have yielded a sample more closely representative of the true population than the secondary occupants, these searches are difficult to conduct systematically. For the purposes of direct comparison, artificial cover objects were chosen for this experiment to eliminate confounding variables such as amount of leaf litter, search area, and soil type which present more variation in natural cover object searches. Because the comparison group (secondary occupants) was also collected through the use of artificial cover boards, it is still possible that an accurate comparison to the true population was not made. However, through the removal of primary occupants and the 48-hour time allowance between collections, this protocol provides as accurate a comparison as possible given the constraints from studying a fossorial species. As other studies have suggested a higher rate of competitive exclusion for juveniles than adults (Mathis 1990, Marsh and Goicochea 2003), it is also possible that juveniles were so excluded from the cover boards that they were not present even after the removal of the primary occupants. This result, and the results of the study overall, should be taken in light of the high density of *P. cinereus* at this study site (Hernández Pacheco, *in review*). Given that many of the cover boards presented piles of salamanders numbering as many as 13 under a single board, it is possible that behavioral and territorial characteristics vary from other *P. cinereus* populations. This scenario would explain the discrepancy between this study's findings and others that suggest intense exclusion of other salamanders, which have largely been conducted in more northern or mountainous portions of the range of this species (Jaeger et al. 2016). Additionally, the suburban forest in which this population resides is highly fragmented and frequented for recreational use, which could further exacerbate a departure from territorial behavior seen in more forested landscapes.

The results from this study indicate that the population characteristics of sex, morph, life stage, and reproductive state are not different between the two capture groups. The potential for site territoriality may be indicated by the difference in tail length between the groups and possible trend towards higher recaptures in primary occupants, but broad differences in the composition of captures were not found. Therefore, the artificial coverboard protocol captures a representative sample of salamanders that can be analyzed using SCR to provide an accurate estimate of population parameters. The reinforcement of the ability of such sampling methods to produce robust estimates of amphibian population dynamics is vital to the study of terrestrial salamanders and the conservation of species with low detectability in the currently unstable and rapidly shifting environmental conditions.

Acknowledgments

Thank you first and foremost to Dr. Kristine Grayson for her mentorship and guidance throughout the completion of this work. Thanks to the University of Richmond research team, Lily Thompson and Raisa Hernández Pacheco for data management and schedule coordination. For the collection, marking, and data entry of these hundreds of salamanders thank you to Grayson lab members Sarah Timko, Christian Law, Alexis Porter, Meghan Leber, Dennon Hoernig, Petra Hafker, Ashley Appolon, Josie Anderson, Maddie Hair, Charlotte Lee, Maria Seitz, and Khaela Sanchez. I am grateful to Emily Boone for serving as second reader for this thesis and for her help throughout its writing. Thank you to SPARCnet for sharing the standardized artificial coverboard sampling procedure. Permission for plot establishment was obtained through Nathan Burrell and Tyler Twyford with the James River Park System. Partial funding for this study was provided by the University Research Council grant from the University of Richmond. Research approved by the Virginia Department of Game and Inland Fisheries and the University of Richmond IACUC.

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Figure 1: A temporal schematic of salamander removal from cover board plots. A) Red dots represent primary occupants present at first collection. B) Plot after removal of primary occupants. C) 48 hours after removal of primary occupants. Blue dots represent second occupants that emerged in the absence of primary occupants. D) Plot after removal of secondary occupants. E) Return of primary occupants to original location after removal of secondary occupants. F) Return of secondary occupants to original location. Green dots represent new individuals not surveyed in either capture event.

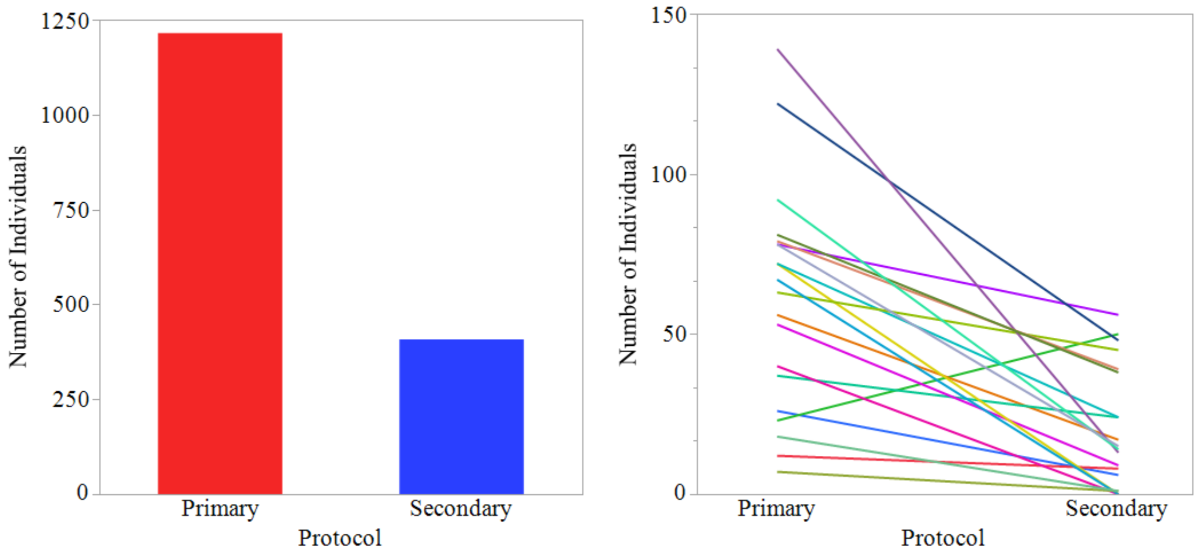

Figure 2: Capture numbers for primary and secondary occupants. A) Total capture numbers (n_p=1215, n_s=408). B) Total number of captured individuals each week (weeks 8, 10, and 12 omitted because zero secondary occupants were captured).

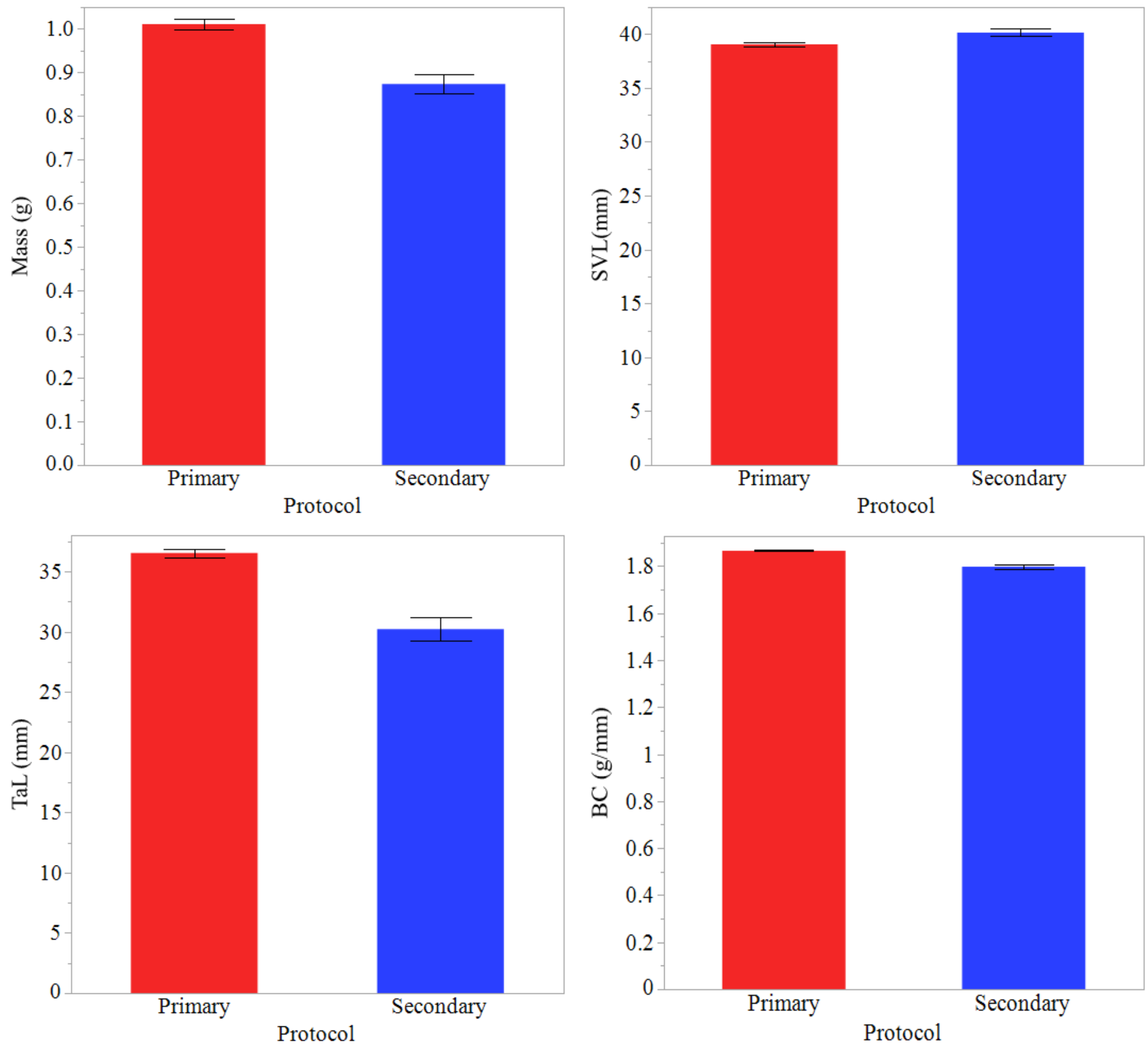

Figure 3: Differences in size between individuals captured on primary (n=1215) and secondary (n=408) occasions. A) The mean mass B) The mean snout-vent length (SVL) C) The mean tail length (TaL) D) The mean body condition (BC). Error bars represent 1 standard error.

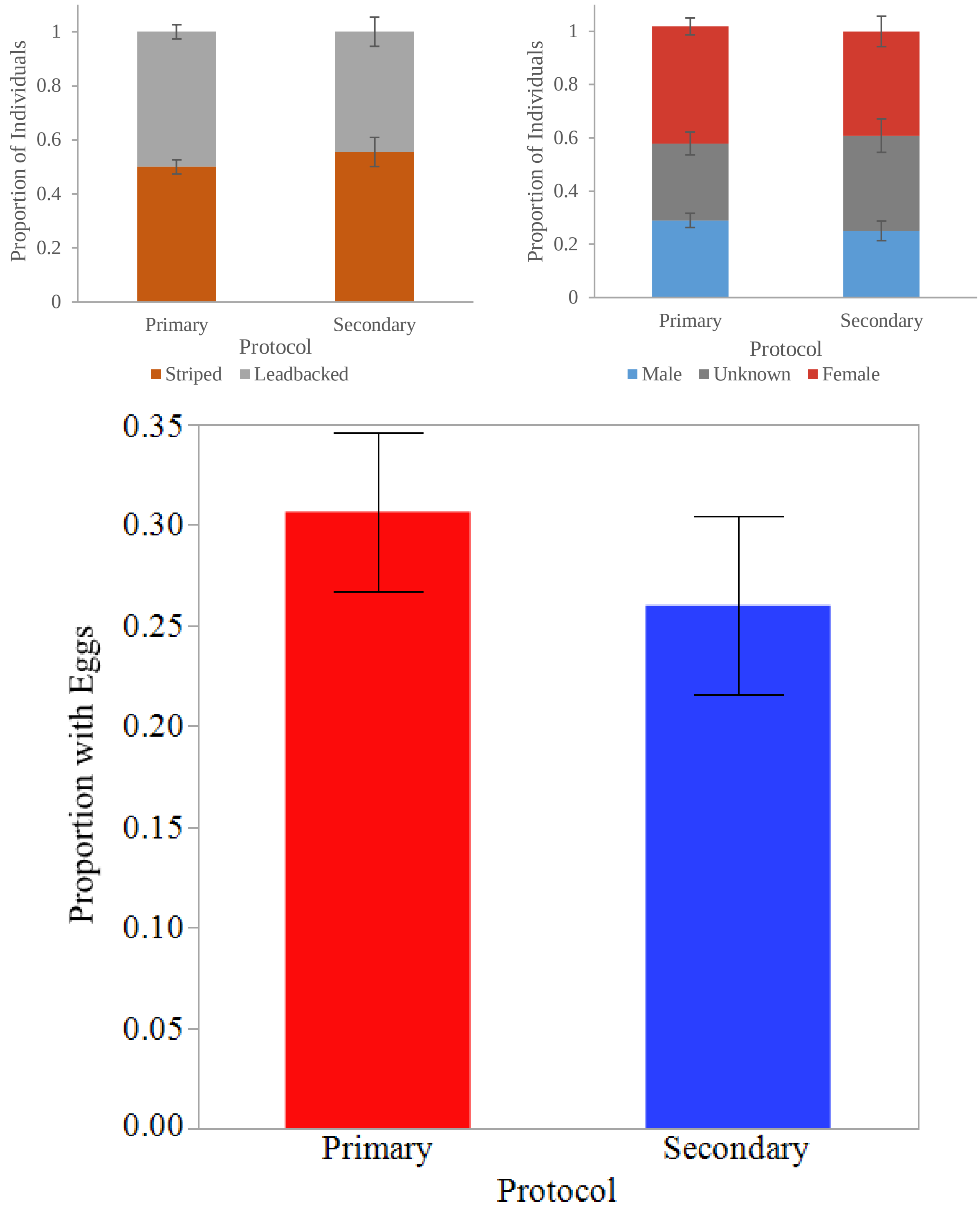

Figure 4: Differences in composition of captures for primary and secondary occasions based on morph (A) (n=17), sex (B) (n_{males}=15; $n_{females}$=15), and reproductive state (C) (n=13). For morph, individuals were categorized as either striped (S) or lead backs (L). All graphs show each characteristic as a mean proportion of individuals possessing that trait from total captures each week. Error bars represent 1 standard error.

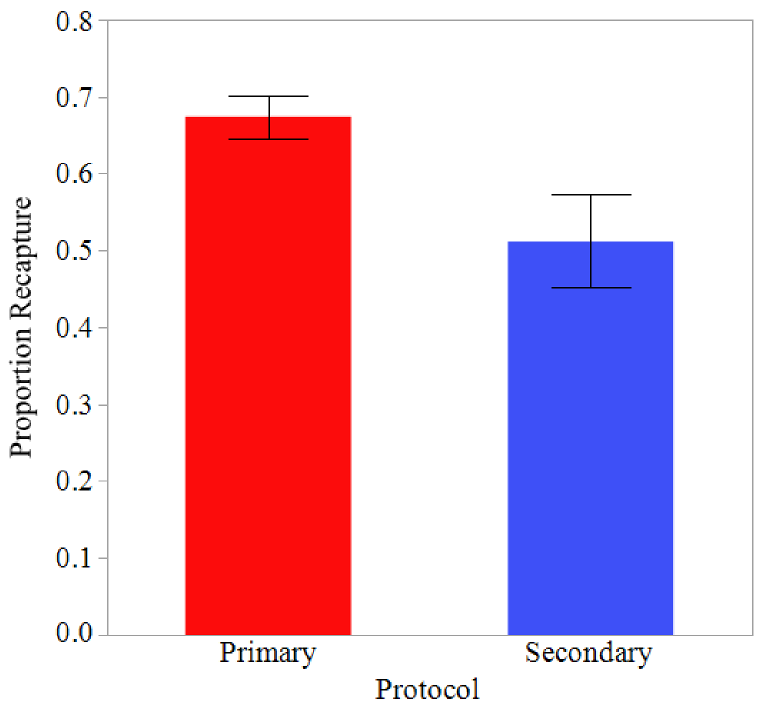

Figure 5: The mean proportion of recaptured individuals each week of primary and secondary occupants (n=17). Error bars represent 1 standard error.