A peer-reviewed version of this preprint was published in PeerJ on 14 March 2016.

View the peer-reviewed version (peerj.com/articles/1813), which is the preferred citable publication unless you specifically need to cite this preprint.

Peterman W, Brocato ER, Semlitsch RD, Eggert LS. 2016. Reducing bias in population and landscape genetic inferences: the effects of sampling related individuals and multiple life stages. PeerJ 4:e1813
https://doi.org/10.7717/peerj. 1813

# Reducing bias in population and landscape genetic inferences: The effects of sampling related individuals and multiple life stages 

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In population or landscape genetics studies, an unbiased sampling scheme is essential for generating accurate results, but logistics may lead to deviations from the sample design. Such deviations may come in the form of sampling multiple life stages. Presently, it is largely unknown what effect sampling different life stages can have on population or landscape genetic inference, or how mixing life stages can affect the parameters being measured. In this study, we sampled embryos, larvae, and adult Ambystoma maculatum from five ponds in Missouri, and analyzed them at 15 microsatellite loci. We calculated allelic richness, heterozygosity and effective population sizes for each life stage at each pond and tested for genetic differentiation ( $F_{\mathrm{ST}}$ and $D_{C}$ ) and isolation-by-distance (IBD) among ponds. We tested for differences in each of these measures between life stages, and in a pooled population of all life stages. All calculations were done with and without sibling pairs to assess the effect of sibling removal. No statistically significant differences were found among ponds or life stages for any of the population genetic measures, but patterns of IBD differed among life stages. There was significant IBD when using adult samples, but tests using embryos, larvae, or a combination of the three life stages were not significant. Further, we found that increasing the ratio of larval or embryo samples in the analysis of genetic distance weakened the IBD relationship, and when using $D_{C}$, the IBD was no longer significant when larvae and embryos exceeded $60 \%$ of the population sample. Our findings suggest that it may be possible to mix life stages to reach target sample size quotas, but researchers should nonetheless proceed with caution depending upon the goals and objectives of the study.

Reducing bias in population and landscape genetic inferences: The effects of sampling related individuals and multiple life stages

Running Head: Sampling multiple life stages

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Words: 3,709

Key Words: Ambystoma, amphibian, complex life cycle, genetic sampling, landscape genetics, microsatellite, mixing tissue samples, population genetics, salamander, sibship


#### Abstract

In population or landscape genetics studies, an unbiased sampling scheme is essential for generating accurate results, but logistics may lead to deviations from the sample design. Such deviations may come in the form of sampling multiple life stages. Presently, it is largely unknown what effect sampling different life stages can have on population or landscape genetic inference, or how mixing life stages can affect the parameters being measured. In this study, we sampled embryos, larvae, and adult Ambystoma maculatum from five ponds in Missouri, and analyzed them at 15 microsatellite loci. We calculated allelic richness, heterozygosity and effective population sizes for each life stage at each pond and tested for genetic differentiation ( $F_{\mathrm{ST}}$ and $D_{\mathrm{C}}$ ) and isolation-by-distance (IBD) among ponds. We tested for differences in each of these measures between life stages, and in a pooled population of all life stages. All calculations were done with and without sibling pairs to assess the effect of sibling removal. No statistically significant differences were found among ponds or life stages for any of the population genetic measures, but patterns of IBD differed among life stages. There was significant IBD when using adult samples, but tests using embryos, larvae, or a combination of the three life stages were not significant. Further, we found that increasing the ratio of larval or embryo samples in the analysis of genetic distance weakened the IBD relationship, and when using $D_{\mathrm{c}}$, the IBD was no longer significant when larvae and embryos exceeded $60 \%$ of the population sample. Our findings suggest that it may be possible to mix life stages to reach target sample size quotas, but researchers should nonetheless proceed with caution depending upon the goals and objectives of the study.


## Introduction

Many organisms have high fecundity, but experience extremely high mortality. Most amphibian, fish and insect species are characterized by Type III survivorship, in which a majority of young individuals will die before reaching sexual maturity, and the genetic characteristics of these life stages may differ from the few surviving adults due to the decrease in population size (Frankham 1996). While it may often be assumed that selection pressures that reduce population size act uniformly and randomly, selection may differentially affect individuals. For example, numerous studies have assessed the role of inbreeding and heterozygosity on individual fitness (e.g., Balloux et al. 2004; Ficetola et al. 2011; Harrison et al. 2011; Slate et al. 2004). Both of these population genetic attributes are particularly relevant in species of conservation concern, which often exist in small or isolated populations. Given the interaction between selection pressures and genetic diversity, it is not unreasonable to believe that population genetic measures may differ depending on the age or life stage of the sampled cohort.

Despite the potential problems with sampling different life stages, it is not uncommon for population or landscape genetic studies to combine samples from different cohorts or life stages, either because of convenience or necessity. Early life stages are often sampled because they are accessible, abundant, and cost-effective (Heyer et al. 1994). In amphibians, the extreme decline in individuals from early life stages to adults has been well-documented. Peterson et al. (1991), found a pre-metamorphic mortality rate of $99 \%$ in ringed salamanders (Ambystoma annulatum), Shoop (1974) found that premetamorphic mortality rates ranged from $87-99 \%$ in spotted salamanders ( $A$. maculatum), and Berven (1990) recorded pre-metamorphic mortality rates ranging from

97-99\% in wood frogs (Rana sylvatica). The drastic decline in abundance can also be seen in fish and insects. Dahlberg (1979) found a mortality rate of $>99 \%$ in the eggs of many fish species, while a study of the southern green stink bug (Nezara viridula) found mortality rates to be as high as $96 \%$ (Kiritani \& Nakasuji 1967). As such, when early life stages are sampled to make inferences about the adult population, biased conclusions may result (Allendorf \& Phelps 1981; Goldberg \& Waits 2010). Obtaining unbiased estimates of genetic diversity is particularly critical for management and conservation of species.

Sampling animals from the field is often opportunistic due to the availability of the target species. Environmental factors, stochastic events, or the timing of offspring can alter when a life stage becomes available, if it can be found at all (Alford \& Richards 1999). In these cases, researchers often need to stray away from their sampling scheme and target life stage, and collect other life stages to reconcile the sample size gap (Beebee \& Rowe 2000; Lee-Yaw et al. 2008; Lee-Yaw et al. 2009; Munwes et al. 2010; Richardson 2012). Despite the relative commonness of these sampling realities, the effect of mixing life stages in population and landscape genetic analyses, however, has not been explicitly addressed. The sampling of full siblings has been shown to affect the estimates of population genetic parameters. When sampling amphibians, field researchers have the highest probability of collecting sibling pairs within larvae (Goldberg \& Waits 2010); related larvae are often spatially clustered, and samples collected at a specific location may be biased towards a single family group (Hansen et al. 1997). If researchers are unaware that family groups are being sampled, the genetic structure of the family could be misinterpreted as population structure within the panmitic population (Anderson \&

Dunham 2008). To prevent misinterpretations and avoid biased population genetic parameter estimates, samples should be screened prior to analysis, and full siblings removed (Goldberg \& Waits 2010).

To date, only Goldberg \& Waits (2010) have empirically tested the effects of sampling different life stages and quantified the importance of removing full siblings prior to analysis. The primary objective of this study was to determine the effects of pooling different life stages on population and landscape genetic inferences. Additionally, we sought to extend the findings of Goldberg \& Waits (2010) to determine the effect of sampling three life stages: adults, embryos, and larvae, on population and landscape genetic inferences. We assessed these objectives both with and without full-sibling pairs present in the data set. We predicted that the random mixing of life stages would result in genetic parameter estimates that did not differ from estimates of individual life stages. However, we predicted that there would be significant biases present when sampling different life stages as certain alleles are likely to be over-represented in the embryonic and larval life stages. Finally, we predicted that the removal of siblings from the data set would significantly alter population and landscape genetic estimates.

## Materials and methods

Ethics Statement - This research was conducted in compliance with all laws and regulations for the state of Missouri and the USA, and was conducted under Missouri Wildlife Collector's permit 15584. Sampling methods were approved by the University of Missouri Animal Care and Use Committee (Protocol 7403).

Data availability - All data and code used in this study can be accessed from Figshare at http://figshare.com/s/ee3a58909daf11e5afc206ec4b8d1f61.

Literature review - To determine how researchers are currently collecting tissue samples from amphibians with complex life cycles, we conducted a literature search of the Scopus database of population and landscape genetic studies of amphibians. We used the search terms "amphibian*" (occurring in the title, abstract, or keywords) " "microsatellite*" (occurring in all fields), and NOT "reptil*" (occurring in the title, abstract, keywords) and limited the search to findings from Molecular Ecology, Conservation Genetics, Heredity, Biological Journal of the Linnean Society, Amphibia-Reptilia, Animal Conservation, Molecular Ecology Resources, Evolution, Plos One, or Journal of Zoology. For each study, we determined if different life stages were sampled and if the study gave an indication as to whether sampling multiple life stages influenced analysis or inferences made from the data.

Sampling—Our study was conducted at Daniel Boone Conservation Area (DBCA), in Warren County, Missouri, U.S.A.(Fig. 1). This 1,424 ha area is situated on the upper Ozark Plateau physiographic region and is characterized by mature (80-100 years old) second-growth forest with an overstory dominated by oak (Quercus spp.) and hickory (Carya spp.), with varying amounts of sugar maple (Acer saccharum) and red cedar (Juniperus virginiana) in the understory (Semlitsch et al. 2009). There are $>40$ fishless manmade ponds that are, on average, separated by $2,000 \mathrm{~m}(246-3,900 \mathrm{~m})$ (Peterman et al. 2013b). We sampled adults, embryos, and larvae of Ambystoma maculatum (spotted
salamander) from five ponds at DBCA (Fig. 1). Adult salamanders were captured in mesh funnel traps in March 2013, and tissue samples were obtained by removing 0.5 cm of tail tissue. Following oviposition, we sampled embryos by collecting a single embryo per clutch in April 2013. In June 2013 larvae were captured with dip nets, and to minimize the sampling of siblings, we collected larvae from the entire perimeter of each pond. Upon collection in the field, each tissue sample was placed in $95 \%$ ethanol and stored at $-20^{\circ} \mathrm{C}$ until DNA extraction.

Lab techniques- DNA was extracted from tissue using chelex-based resin (InstaGene, BioRad). Approximately $2.5 \mathrm{~mm} \times 2.5 \mathrm{~mm}$ of tissue was finely chopped with a sterile razor and was incubated at $60^{\circ} \mathrm{C}$ for 2 hrs in $250 \mu \mathrm{~L}$ of InstaGene, vortexed, incubated for 20 min at $100^{\circ} \mathrm{C}$, then vortexed again. Following centrifugation, a $100 \mu \mathrm{~L}$ aliquot was removed and used as template DNA and the remainder was kept at $-20^{\circ} \mathrm{C}$ (Peterman et al. 2012). Nineteen tetra-nucleotide microsatellite loci were amplified using PCR; primers were fluorescently 5' labeled with FAM, NED, VIC, and PET and arranged into two multiplex reactions (Peterman et al. 2013a). Negative controls were included in all reactions to detect contamination of reagents. Amplification products were sized on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using Liz 600 size standard at the University of Missouri DNA Core Facility, and results were scored using GENEMARKER (v.1.97; Softgenetics, State College, PA, USA).

Differences among life stages-Before proceeding with analyses we created a data set free of full sibling pairs using COLONY (Wang 2012). We excluded siblings from the
analysis such that all sites only had one individual per family group. Values for observed heterozygosity were calculated in the MS toolkit Microsoft Excel add-in (Park 2001), allelic richness was calculated and rarefied to our smallest sample in HP Rare (Kalinowski 2005), FST was calculated in FSTAT 2.9.3 (Goudet 2001), chord distance ( $D_{\mathrm{C}}$ ) was calculated with the R package adegenet (Jombart \& Ahmed 2011), and effective population size estimates $\left(\mathrm{N}_{\mathrm{e}}\right)$ were made using the linkage disequilibrium approach implemented in COLONY (Wang 2012). The proportion of siblings removed from each life stage at each pond was also calculated. To determine the effect of sibling removal, we also calculated summary statistics $\left(\mathrm{H}_{\mathrm{o}}, \mathrm{A}_{\mathrm{r}}, F_{\mathrm{ST}}, D_{\mathrm{C}}\right)$ for each life stage with siblings present. All population genetic measures were compared among life stages and between estimates made with and without siblings using analysis of variance (ANOVA) and paired t-tests. Due to small sample sizes, we bootstrapped our ANOVA analyses and conducted permutation $t$-tests to more robustly assess differences among life stages and removal of siblings.

Effect of mixing life stages- Prior to pooling life stages together, we conducted a second removal of related individuals using COLONY (Wang 2012). Specifically, we found and removed parent-offspring and embryo-larvae sibling pairs within each pond. All unrelated individuals of all life stages were pooled by pond of origin to make five mixedtissue populations. From these populations, we randomly sampled 25 individuals using the R package hierfstat (Goudet 2013) in R (R Core Team 2013). This bootstrap resampling procedure was repeated 1,000 times (both with and without siblings), and the mean and $95 \%$ confidence intervals were calculated for $\mathrm{H}_{\mathrm{o}}, \mathrm{A}_{\mathrm{r}}, F_{\mathrm{ST}}$, and $D_{\mathrm{C}}$.

Isolation-by-distance analysis- For the isolation-by-distance (IBD) analysis we conducted simple Mantel tests correlating the genetic distance $\left(F_{\mathrm{ST}}\right)$ with the Euclidean distance between ponds. This test was repeated for all life stages, with and without siblings, and significance was assessed using 100,000 permutations using the R package ecodist (Goslee \& Urban 2007). We tested for IBD in the mixed sample population, and calculated the mean and $95 \%$ confidence interval for both the Mantel $r$ correlation statistic and the associated P-value based on the 1,000 bootstrap iterations. Because we found a significant IBD relationship when using adult-only tissue samples (see Results), we further assessed how the IBD relationship changed with the inclusion of larval and embryo samples. For this analysis, we varied the proportion of larval and embryo samples included with our adult samples. This was assessed at proportions ranging from 0 (no larval or embryo samples) to 1 (only larval and embryo samples) at increments of 0.05. At each increment, we assessed the mean and $95 \%$ confidence intervals of the Mantel $r$ and the corresponding P-value based on 1,000 bootstrap samples of the data. We used the data set without siblings for this analysis and sampled each population to the minimum adult sample size $(\mathrm{n}=18)$.

## Results

Literature review - We found that 19 out of $90(21 \%)$ of studies meeting our search criteria on Scopus conducted population or landscape genetic analyses of amphibian species using mixed tissue sampling (searched on 2 September 2013). Five of these studies stated that one life stage was sampled only when the target life stage was not
available (Beebee \& Rowe 2000; Lee-Yaw et al. 2009; Lee-Yaw et al. 2008; Munwes et al. 2010; Richardson 2012). None of these studies made attempts to check or correct for the effects of mixing life stages in their analyses, although it was common for siblings to be removed prior to analysis.

Sample summary - We collected 24-25 adults and 19-27 embryos from each of the five ponds, and 29-36 larvae from three ponds (Supplement 1). We were unable to sample larvae from two of the ponds due to high embryo mortality. Of the original 19 screened primers, two loci (Am_13, Am_60) were not polymorphic, and two loci (Am_33, Am_43) showed very little polymorphism and deviated significantly from expected heterozygosity values under Hardy Weinberg equilibrium (HWE). These four loci were removed from the dataset and all population genetic statistics were calculated using the remaining 15 loci (Supplement 2). No other loci or populations deviated from Hardy-Weinberg equilibrium or were significantly linked. Overall, we had $<0.5 \%$ missing data.

Statistical summary— For all tests, none of the population genetic parameters differed significantly among life stages (bootstrap ANOVA P-value $>0.05$ ) or within ponds and among life stages (permutation t-test P-value $\geq 0.25$; Fig. 2, Tables $1-2$ ), regardless of whether or not siblings were present in the data. The proportion of samples removed due to sibship was nearly significant (bootstrap P-value $=0.053$, Fig. 2f), with a greater proportion of field-collected samples being omitted from larvae. There was also an increase in the estimated mean $F_{\text {ST }}$ calculated in the larval and embryo data as compared to adults (Fig. 2c), but we note that this increase was not significant. However, this trend
was not observed when genetic distance was measured using allele frequencies $\left(D_{\mathrm{c}}\right.$, Fig. 2d). Values of population genetic summary statistics calculated on data sets with siblings removed are given as the mean ( $\pm$ standard deviation). Effective population size among ponds averaged $87.4( \pm 25.28)$ for adults, $82.4( \pm 25.58)$ for embryos, and $64( \pm 4.32)$ for larvae. Average rarefied allelic richness of adults was $3.83( \pm 0.22), 3.95( \pm 0.23)$ for embryos, and $4.13( \pm 0.20)$ for larvae. The average observed heterozygosity was 0.53 $( \pm 0.01)$ for adults, $0.51( \pm 0.03)$ for embryos, and $0.51( \pm 0.01)$ for larvae. On average, we removed $33.2 \%( \pm 0.09)$ of larval samples due to sibship, while only $13 \%( \pm 0.084)$ of adult and $14.98 \%( \pm 0.09)$ of embryo samples were removed. Pairwise genetic distances between ponds measured using $F_{\text {ST }}$ averaged $0.011( \pm 0.008)$ in adults, $0.021( \pm 0.007)$ in larvae, and $0.019( \pm 0.014)$ in embryos (Table 3).

When comparing each life stage at each summary metric, we found no significant differences between data containing the sibling pairs and data with removed sibling pairs (permutation t-test P -value $\geq 0.19$; Fig. 2, Tables $1-2$ ). We found that the mixing of life stages resulted in genetic estimates of $\mathrm{A}_{\mathrm{r}}, \mathrm{H}_{\mathrm{o}}, F_{\mathrm{ST}}$, and $D_{\mathrm{C}}$ that, on average, did not differ from estimates made for each specific life stage (Tables 1-2). There were, however, up to three pond-pair $F_{\text {ST }}$ values that fell outside of the bootstrapped $95 \%$ confidence intervals (Table 3). When genetic distance was measured using $D_{\mathrm{c}}$, only one pond-pair fell outside of the bootstrapped confidence interval. In general, there was a greater frequency of pairwise genetic distance measures based on embryo samples to fall outside of the mixed sample confidence interval. For both $F_{\mathrm{ST}}$ and $D_{\mathrm{C}}$, the removal of siblings resulted in more pairwise estimates falling outside of the mixed sample confidence interval (Table 3). Due to sample size, clear inferences cannot be drawn from larvae. With regard to IBD, only
tests using adult samples (with and without siblings) resulted in significant relationships (Table 4), and the IBD relationship was stronger when genetic distance was measured using $D_{\mathrm{C}}$ (Fig. 3). IBD tests from embryos or larvae had much lower Mantel $r$ correlations and were not significant. The mixing of tissue samples resulted in nonsignificant IBD tests when using $F_{\mathrm{ST}}(p>0.05$; Table 4 ), but had little effect when using $D_{\mathrm{C}}$ (Table 4).

Our assessment of increasing the proportion of larval or embryo tissue samples clearly demonstrated that the calculated Mantel $r$ decreases as the proportion of larval and embryo samples increases (Fig. 3). This pattern was consistent regardless of whether genetic distance was measured using $F_{\mathrm{ST}}$ or $D_{\mathrm{C}}$. Correspondingly, the average P-value of the Mantel test increased as the proportion of larval and embryo samples increased. In this analysis, the mean bootstrapped P-value for tests using $F_{\text {ST }}$ started at 0.057 (0.0550.60 ) for adult-only samples, and increased to 0.231 (0.223-0.239) for larvae/embryoonly samples (Fig. 3b). In contrast, the P-value for Mantel tests with $D_{\mathrm{C}}$ started at 0.019 (0.018-0.020) when only adults were included and increased to 0.146 ( $0.140-0.152$ ) without adult samples (Fig. 3d). The 0.05 threshold is passed when the proportion of larvae and embryos in the sample reaches $0.60-0.65(\mathrm{P}$-values $=0.047-0.054$, respectively).

## Discussion

Our literature search revealed that mixed tissues have been used in about one fifth of amphibian population genetic studies using microsatellites, despite the lack of knowledge concerning the effects that this may have on population or landscape genetic inferences.

Collection of genetic samples from the field is likely influenced by numerous factors, such as the timing of life stage development and accessibility to sampling sites. Many of the studies that mixed tissues did so when the target life stage was not found and minimum sample requirements had to be met. Decisions made during sampling can undoubtedly influence the inferences made, as genetic variation within spatially structured populations can vary spatially, temporally, or as a result of life history (Anderson \& Dunham 2008; Schwartz \& McKelvey 2009; Scillitani et al. 1996).

In our study, however, we found little evidence for adverse effects of including siblings or with mixing tissue samples collected from different life stages when assessing levels of allelic richness, heterozygosity, or effective population size. Despite this finding, we feel that it is best practice to remove all full-sibling pairs so that only a single family group is represented (Goldberg \& Waits 2010). We found that estimates of pairwise $F_{\text {ST }}$, although not statistically different among life stages, differed substantially between life stages as well as from the mixed-tissue sample. However, this pattern was not observed when pairwise genetic distance was measured using $D_{\mathrm{C}}$. The greatest impact of mixing life stages was evident in our tests of IBD among pond pairs. Only the tests using adults were significant, while larvae-only, embryo-only, and mixed-tissue samples showed little correlation with distance. The IBD relationship was strongest when genetic distance was measured using $D_{\mathrm{C}}$, and we found that the IBD relationship seen in adultonly samples decreased as the proportion of larvae and embryos included in the population sample increased. In our dataset, the IBD relationship, when assessed with $D_{\mathrm{C}}$, remained significant until the frequency of larvae and embryos in the population sample exceeded $60 \%$. As such, our results suggest the greatest effects of mixing different life
stages may be evident in landscape genetic analyses, with different conclusions potentially being drawn from adult-only samples as compared to larvae, embryo, or mixed-tissue samples. Further, such relationships may be sensitive to the genetic distance statistic used.

Contrary to our predictions, we did not observe significant biases in our population genetic measures among life stages or with the exclusion of full siblings from the data. This is counter to the findings of Goldberg \& Waits (2010) who found that skewed estimates between larval and adult population genetic measures were eliminated or reduced when full siblings were removed from the larval sample. As in the analysis of mixed-tissue samples, the greatest differences were observed in relation to genetic differentiation measured by $F_{\mathrm{ST}}$, which increased (insignificantly) after the removal of full siblings from the data, as well as from adults to embryos, to larvae. These increases in genetic differentiation in the embryonic and larval stages, as compared to the adults is predicted by population genetic theory (Allendorf \& Phelps 1981), and has been empirically demonstrated in Columbia spotted frogs (Rana luteiventris) (Goldberg \& Waits 2010).

The clearest result from our study was the proportion of samples that had to be removed from each life stage due to redundancy of siblings. We note that it is actually quite unlikely that we sampled full sibling adults given their life history and longevity (Petranka 1998), but we chose to remove these putative siblings from our data set for consistency of methods among life stages. We note, however, that Goldberg \& Waits (2010) did not test for or remove sibling pairs from their adult samples. In our study, up to $40 \%$ of larval samples collected in the field had to be removed. Although larvae are
often the most readily accessible and conveniently sampled life stage, this represents an inefficient use of resources, and we encourage future studies to avoid sampling larvae when possible. Given that inferences differed minimally and insignificantly between larvae, embryos, and adults, we advocate for sampling adults or embryos. However, we note that both larvae and adults can be sampled non-destructively, which may become a factor in deciding which life stage to sample for some species.

In our study, we assessed IBD through the use of simple Mantel tests. We readily acknowledge the limitations and criticisms of the Mantel test for making robust inference (e.g., Guillot \& Rousset 2013; Legendre et al. 2015). However, we feel that for our limited data set and ultimate goal of assessing relative differences and patterns between life stages, mixed life stage samples, and genetic distance measures, the simple Mantel test was sufficient and provides an appropriate cautionary caveat for future researchers using any method. More rigorous methods such as distance-based redundancy analysis (Legendre \& Anderson 1999), multiple regression of distance matrices (Holzhauer et al. 2006), distance-based Moran’s eigenvector maps (Legendre \& Legendre 2012), or mixed effects models fit with an appropriate error structure (Clarke et al. 2002) should preferentially be used over Mantel tests in future studies seeking to estimate the effects of landscape features on genetic differentiation.

To our knowledge, our study is the first attempt to determine how the sampling and mixing of different life stages affects genetic parameter estimates. Our findings with the common and widespread $A$. maculatum should be broadly generalizable to other poolbreeding amphibian species, especially those that exhibit similar life history characteristics. The life history of A. maculatum is not unlike that Ambytoma spp.
(Petranka 1998). It is unclear how differences in life history (e.g., life span, breeding site fidelity, reproductive strategy, etc.) alter the effects of sampling different life stages, but our results could be applicable to insects or fish that also exhibit Type III survivorship. We found that mixed-tissue samples can lead to different conclusions when conducting spatial analyses, such as IBD, and these results would likely extend to more complex landscape genetic analyses as well. As such, we caution researchers to carefully consider the implications of mixing samples collected from multiple life stages. Our finding that population genetic parameters differed little with the removal of siblings or mixing of life stages needs to be interpreted cautiously. First, we had a relatively small sample size from which to draw inference, although we note that it is not much smaller than that used by Goldberg \& Waits (2010) who assessed eight populations of R. luteiventris and four populations of A. macrodactylum. We do note that the power to infer differences is likely greater in our study due to the large number of polymorphic microsatellites used. Perhaps of greater importance is the fact that our populations are relatively close together (maximum distance $=3,200 \mathrm{~m}$ ) and situated within continuous forest habitat. In contrast, Goldberg \& Waits (2010) sampled populations separated by $2.7-18.5 \mathrm{~km}$ of agricultural matrix. Further, the populations included in our study are very robust, with recorded breeding aggregations of several hundred individuals (Semlitsch unpublished data). Like Goldberg \& Waits (2010), we suggest that future studies include a pilot phase to assess the effects of sampling different life stages to meet the objectives of the specific project. An important consideration for any population or landscape genetic study is the target demographic group for which inferences are desired. If quantifying movement or connectivity of populations is the main objective of a study, then sampling adult life
stages may provide the most accurate inferences. If the study objectives are to quantify the distribution of genetic diversity, then perhaps larvae or embryos will suffice.

## Acknowledgements

We thank T. Luhring for assistance in the field.

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|  | $\mathrm{N}_{\mathrm{e}}$ |  |  |
| :--- | :--- | :--- | :--- |
| Pond | Adult | Embryo | Larvae |
| 1 | 120 | 92 | 62 |
| 2 | 67 | 114 | - |
| 3 | 100 | 93 | - |
| 4 | 100 | 55 | 70 |
| 5 | 50 | 58 | 60 |
| Avg | 87.4 | 82.4 | 64 |


| SD | 25.28 | 22.58 | 4.32 |
| :--- | :--- | :--- | :--- |


| Proportion of samples removed |  |  |  |
| :--- | :--- | :--- | :--- |
| Adult | Embryo | Larvae | Combined <br> life stages |
| 0.08 | 0.08 | 0.21 | 0.40 |
| 0.16 | 0.05 | - | 0.25 |
| 0.00 | 0.12 | - | 0.24 |
| 0.16 | 0.20 | 0.39 | 0.41 |
| 0.25 | 0.30 | 0.40 | 0.37 |
| 0.13 | 0.15 | 0.33 | 0.33 |
| 0.08 | 0.09 | 0.09 | 0.07 |

506 Table 2. Rarefied allelic richness and observed heterozygosity estimates at each pond for both the full data set, and with siblings type indicates values that fall outside of the bootstrapped $95 \%$ confidence interval.

Full data set

|  | $\mathrm{A}_{\text {r }}$ |  |  |  | $\mathrm{H}_{\text {o }}$ |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Pond | Adult | Embryo | Larvae | Mixed samples $(95 \% \mathrm{CI})$ | Adult | Embryo | Larvae | Mixed samples $(95 \% \mathrm{CI})$ |
| 1 | 4.11 | 4.22 | 4.21 | 4.22 (3.99-4.44) | 0.54 | 0.49 | 0.52 | 0.53 (0.49-0.57) |
| 2 | 3.94 | 4.10 | - | 4.08 (3.86-4.27) | 0.51 | 0.45 | - | 0.50 (0.47-0.53) |
| 3 | 3.65 | 3.68 | - | 3.67 (3.44-3.89) | 0.50 | 0.47 | - | 0.49 (0.46-0.53) |
| 4 | 3.77 | 3.63 | 3.90 | 3.73 (3.50-3.98) | 0.52 | 0.50 | 0.49 | 0.51 (0.47-0.54) |
| 5 | 3.66 | 3.88 | 4.07 | 3.92 (3.64-4.17) | 0.50 | 0.54 | 0.52 | 0.54 (0.50-0.57) |
| Avg | 3.83 | 3.90 | 4.06 | 3.93 (3.51-4.35) | 0.52 | 0.49 | 0.51 | 0.52 (0.48-0.56) |
| SD | 0.20 | 0.26 | 0.15 | - | 0.01 | 0.03 | 0.02 | - |

Siblings removed

|  | $\mathrm{A}_{\mathrm{r}}$ |  |  |  | $\mathrm{H}_{\text {o }}$ |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Pond | Adult | Embryo | Larvae | Mixed samples $(95 \% \mathrm{CI})$ | Adult | Embryo | Larvae | Mixed samples $(95 \% \mathrm{CI})$ |
| 1 | 4.16 | 4.20 | 4.36 | 4.22 (4.00-4.41) | 0.55 | 0.53 | 0.51 | 0.54 (0.50-0.57) |
| 2 | 3.98 | 4.15 | - | 4.19 (4.05-4.31) | 0.52 | 0.48 | - | 0.51 (0.49-0.53) |
| 3 | 3.65 | 3.72 | - | 3.77 (3.57-3.95) | 0.50 | 0.48 | - | 0.50 (0.47-0.53) |
| 4 | 3.77 | 3.64 | 3.88 | 3.81 (3.57-4.03) | 0.54 | 0.52 | 0.50 | 0.52 (0.49-0.56) |
| 5 | 3.57 | 4.04 | 4.16 | 3.98 (3.71-4.21) | 0.53 | 0.54 | 0.53 | 0.54 (0.51-0.57) |
| Avg | 3.83 | 3.95 | 4.13 | 3.99 (3.62-4.35) | 0.53 | 0.51 | 0.51 | 0.52 (0.48-0.56) |
| SD | 0.22 | 0.23 | 0.20 | - | 0.01 | 0.03 | 0.01 | - |

511 Table 3. Pairwise genetic distances ( $F_{\mathrm{ST}}$ and $D_{\mathrm{C}}$ ) between ponds for each life stage and for mixed-tissue life stages. Mixed is the
512 bootstrap mean and $95 \%$ confidence interval from randomly sampling all life stages together. Bolded type indicates values that fall 513 outside of the bootstrapped 95\% confidence interval.

Full data set

| Pond-pair | $F_{\text {ST }}$ |  |  |  | $D_{\text {C }}$ |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Adult | Embryo | Larvae | Mixed (95\% CI) | Adult | Embryo | Larvae | Mixed (95\% CI) |
| 1 _2 | 0.000 | 0.000 | - | 0.003 (-0.006-0.014) | 0.168 | 0.195 | - | 0.190 (0.163-0.216) |
| 1 -3 | 0.012 | 0.002 | - | 0.011 (0.002-0.023) | 0.212 | 0.208 | - | 0.235 (0.206-0.267) |
| 1_4 | 0.019 | 0.020 | 0.020 | 0.008 (-0.001-0.020) | 0.205 | 0.247 | 0.193 | 0.237 (0.204-0.268) |
| 1 -5 | 0.015 | 0.024 | 0.029 | 0.022 (0.008-0.037) | 0.227 | 0.250 | 0.204 | 0.237 (0.208-0.267) |
| 2_3 | 0.005 | 0.006 | - | 0.019 (0.007-0.032) | 0.196 | 0.201 | - | 0.209 (0.183-0.237) |
| 2_4 | 0.011 | 0.023 | - | 0.004 (-0.006-0.017) | 0.188 | 0.230 | - | 0.218 (0.192-0.244) |
| 2_5 | 0.013 | 0.030 | - | 0.022 (0.008-0.038) | 0.216 | 0.233 | - | 0.211 (0.183-0.240) |
| 3-4 | 0.000 | 0.004 | - | 0.019 (0.007-0.032) | 0.151 | 0.165 | - | 0.171 (0.142-0.200) |
| 3-5 | 0.012 | 0.023 | - | 0.010 (0.000-0.022) | 0.242 | 0.207 | - | 0.221 (0.192-0.250) |
| 4_5 | 0.005 | 0.012 | 0.011 | 0.010 (0.000-0.023) | 0.213 | 0.197 | 0.181 | 0.223 (0.194-0.252) |
| Avg | 0.009 | 0.014 | 0.020 | 0.013 (0.002-0.026) | 0.202 | 0.213 | 0.193 | 0.215 (0.187-0.244) |
| SD | 0.006 | 0.011 | 0.009 | - | 0.027 | 0.027 | 0.011 | - |

Siblings removed

| Pond-pair | $F_{\text {ST }}$ |  |  |  | $D_{\text {c }}$ |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Adult | Embryo | Larvae | Mixed (95\% CI) | Adult | Embryo | Larvae | Mixed (95\% CI) |
| 1 _2 | 0.000 | 0.000 | - | 0.002 (-0.005-0.011) | 0.191 | 0.199 | - | 0.187 (0.164-0.212) |
| 1_3 | 0.015 | 0.001 | - | 0.011 (0.003-0.020) | 0.240 | 0.218 | - | 0.230 (0.206-0.257) |


| 1_4 | $\mathbf{0 . 0 2 4}$ | $\mathbf{0 . 0 2 1}$ | $\mathbf{0 . 0 2 4}$ | $0.008(0.000-0.017)$ |  | 0.243 | $\mathbf{0 . 2 5 8}$ | 0.234 | $0.229(0.200-0.256)$ |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| $1 \_5$ | 0.020 | $\mathbf{0 . 0 3 6}$ | 0.028 | $0.017(0.006-0.030)$ |  | 0.242 | $\mathbf{0 . 2 7 3}$ | 0.249 | $0.224(0.195-0.251)$ |
| 2_3 | $\mathbf{0 . 0 0 5}$ | 0.010 | - | $0.016(0.007-0.027)$ |  | 0.222 | 0.233 | - | $0.214(0.193-0.234)$ |
| 2_4 | 0.013 | 0.023 | - | $0.006(-0.004-0.018)$ |  | 0.220 | $\mathbf{0 . 2 5 9}$ | - | $0.213(0.189-0.235)$ |
| 2_5 | 0.015 | $\mathbf{0 . 0 4 0}$ | - | $0.019(0.007-0.031)$ |  | 0.226 | $\mathbf{0 . 2 7 6}$ | - | $0.203(0.178-0.228)$ |
| $3 \_4$ | $\mathbf{0 . 0 0 0}$ | 0.006 | - | $0.016(0.007-0.028)$ |  | 0.179 | 0.191 | - | $0.173(0.146-0.203)$ |
| 3_5 | 0.013 | $\mathbf{0 . 0 3 7}$ | - | $0.007(-0.001-0.017)$ |  | $\mathbf{0 . 2 6 2}$ | $\mathbf{0 . 2 5 6}$ | - | $0.212(0.189-0.237)$ |
| $4 \_5$ | 0.004 | 0.020 | 0.012 | $0.008(-0.002-0.020)$ |  | 0.232 | $\mathbf{0 . 2 4 0}$ | 0.227 | $0.213(0.187-0.239)$ |
| Avg | 0.011 | 0.019 | 0.021 | $0.011(0.002-0.022)$ |  | 0.226 | $\mathbf{0 . 2 4 0}$ | $\mathbf{0 . 2 3 7}$ | $0.21(0.185-0.235)$ |
| SD | 0.008 | 0.014 | 0.007 | - |  | 0.025 | 0.030 | 0.011 | - | intervals of the bootstrap iterations. Mantel P-values were estimated from 100,000 permutations.

Full data set

| Life stage | $F_{\text {ST }}$ |  | $D_{\text {c }}$ |  |
| :---: | :---: | :---: | :---: | :---: |
|  | Mantel $\boldsymbol{r}$ | P-value | Mantel $r$ | P-value |
| Mixed | 0.38 (-0.04-0.71) | 0.18 (0.03-0.54) | 0.775 (0.505-0.943) | 0.031 (0.016-0.100) |
| Adult | 0.715 | 0.034 | 0.731 | 0.033 |
| Embryo | 0.164 | 0.316 | 0.687 | 0.033 |
| Larva | -0.125 | 0.666 | -0.055 | 0.668 |

Siblings removed

|  | $F_{\mathrm{ST}}$ |  |  |  | $D_{\mathrm{C}}$ |  |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Life stage | Mantel $\boldsymbol{r}$ | P-value |  | Mantel $\boldsymbol{r}$ | P-value |  |
| Mixed | $0.310(-0.10-0.65)(0.22(0.05-0.54)$ |  | $0.758(0.467-0.936)$ | $0.035(0.016-0.100)$ |  |  |
| Adult | 0.704 | 0.033 |  | 0.794 | 0.016 |  |
| Embryo | 0.093 | 0.417 |  | 0.427 | 0.118 |  |
| Larva | 0.190 | 0.667 |  | -0.186 | 0.667 |  |

Figure 1. Map of Daniel Boone Conservation Area depicting the locations of the five ponds used in this study. Wildlife ponds are ponds readily used by amphibians, such as Ambystoma maculatum, for reproduction.

Figure 2. Bar plots representing mean values of a) observed heterozygosity, b) rarefied allelic richness $\left(\mathrm{A}_{\mathrm{r}}\right)$, c) genetic distance $\left(F_{\mathrm{ST}}\right)$, d) genetic distance $\left(D_{\mathrm{C}}\right)$, e) effective population size $\left(\mathrm{N}_{\mathrm{e}}\right)$, and f) proportion of samples removed due to sibship. Solid bars represent values containing full siblings, patterned bars represent values after sibling removal, and error bars represent standard deviations.

Figure 3. Change in Mantel $r$ when using $F_{\text {ST }}(a)$ and $D_{\mathrm{C}}(b)$, and the corresponding change in the P -value ( $\mathrm{c}=F_{\mathrm{ST}} ; \mathrm{d}=D_{\mathrm{C}}$ ) with increasing proportion of tissue samples coming from larvae and embryos. The dashed line in c and d is drawn at 0.05 to indicate the traditional threshold for significance. Mean (solid black line) and $95 \%$ confidence intervals (gray shading) were estimated at 0.05 increments between from 0 to 1 . A proportion of 0 represents an adult-only sample, while a proportion of 1 represents a larvae/embryo-only sample. At each 0.05 increment, 1000 bootstrap samples were conducted and Mantel P-values were estimated from 100,000 permutations.


## 541 Fig. 1



Fig. 2


Fig. 3

