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

William Peterman, Emily R Brocato, Raymond D Semlitsch, Lori S Eggert

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_{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.

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4 Running Head: Sampling multiple life stages
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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_{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.

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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 pre-metamorphic mortality rates ranged from 87–99% in spotted salamanders (*A. maculatum*), and Berven (1990) recorded pre-metamorphic mortality rates ranging from

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70 97–99% in wood frogs (*Rana sylvatica*). The drastic decline in abundance can also be
71 seen in fish and insects. Dahlberg (1979) found a mortality rate of >99% in the eggs of
72 many fish species, while a study of the southern green stink bug (*Nezara viridula*) found
73 mortality rates to be as high as 96% (Kiritani & Nakasuji 1967). As such, when early life
74 stages are sampled to make inferences about the adult population, biased conclusions
75 may result (Allendorf & Phelps 1981; Goldberg & Waits 2010). Obtaining unbiased
76 estimates of genetic diversity is particularly critical for management and conservation of
77 species.

78 Sampling animals from the field is often opportunistic due to the availability of
79 the target species. Environmental factors, stochastic events, or the timing of offspring can
80 alter when a life stage becomes available, if it can be found at all (Alford & Richards
81 1999). In these cases, researchers often need to stray away from their sampling scheme
82 and target life stage, and collect other life stages to reconcile the sample size gap (Beebee
83 & Rowe 2000; Lee-Yaw et al. 2008; Lee-Yaw et al. 2009; Munwes et al. 2010;
84 Richardson 2012). Despite the relative commonness of these sampling realities, the effect
85 of mixing life stages in population and landscape genetic analyses, however, has not been
86 explicitly addressed. The sampling of full siblings has been shown to affect the estimates
87 of population genetic parameters. When sampling amphibians, field researchers have the
88 highest probability of collecting sibling pairs within larvae (Goldberg & Waits 2010);
89 related larvae are often spatially clustered, and samples collected at a specific location
90 may be biased towards a single family group (Hansen et al. 1997). If researchers are
91 unaware that family groups are being sampled, the genetic structure of the family could
92 be misinterpreted as population structure within the panmitic population (Anderson &

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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).

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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 m (246–3,900 m) (Peterman et al. 2013b). We sampled adults, embryos, and larvae of *Ambystoma maculatum* (spotted

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139 salamander) from five ponds at DBCA (Fig. 1). Adult salamanders were captured in
140 mesh funnel traps in March 2013, and tissue samples were obtained by removing 0.5 cm
141 of tail tissue. Following oviposition, we sampled embryos by collecting a single embryo
142 per clutch in April 2013. In June 2013 larvae were captured with dip nets, and to
143 minimize the sampling of siblings, we collected larvae from the entire perimeter of each
144 pond. Upon collection in the field, each tissue sample was placed in 95% ethanol and
145 stored at -20˚C until DNA extraction.
146
147 *Lab techniques*— DNA was extracted from tissue using chelex-based resin (InstaGene,
148 BioRad). Approximately 2.5 mm × 2.5 mm of tissue was finely chopped with a sterile
149 razor and was incubated at 60°C for 2 hrs in 250 µL of InstaGene, vortexed, incubated
150 for 20 min at 100°C, then vortexed again. Following centrifugation, a 100 µL aliquot was
151 removed and used as template DNA and the remainder was kept at -20°C (Peterman et al.
152 2012). Nineteen tetra-nucleotide microsatellite loci were amplified using PCR; primers
153 were fluorescently 5' labeled with FAM, NED, VIC, and PET and arranged into two
154 multiplex reactions (Peterman et al. 2013a). Negative controls were included in all
155 reactions to detect contamination of reagents. Amplification products were sized on an
156 ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using Liz 600
157 size standard at the University of Missouri DNA Core Facility, and results were scored
158 using GENEMARKER (v.1.97; Softgenetics, State College, PA, USA).
159
160 *Differences among life stages*— Before proceeding with analyses we created a data set
161 free of full sibling pairs using COLONY (Wang 2012). We excluded siblings from the

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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), F_{ST} was calculated in FSTAT 2.9.3 (Goudet 2001), chord distance (D_C) was calculated with the R package *adegenet* (Jombart & Ahmed 2011), and effective population size estimates (N_e) 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 (H_o, A_r, F_{ST}, D_C) 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 mixed-tissue 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 H_o, A_r, F_{ST}, and D_C.

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185

186 *Isolation-by-distance analysis*— For the isolation-by-distance (IBD) analysis we
187 conducted simple Mantel tests correlating the genetic distance (F_{ST}) with the Euclidean
188 distance between ponds. This test was repeated for all life stages, with and without
189 siblings, and significance was assessed using 100,000 permutations using the R package
190 *ecodist* (Goslee & Urban 2007). We tested for IBD in the mixed sample population, and
191 calculated the mean and 95% confidence interval for both the Mantel *r* correlation
192 statistic and the associated P-value based on the 1,000 bootstrap iterations. Because we
193 found a significant IBD relationship when using adult-only tissue samples (see Results),
194 we further assessed how the IBD relationship changed with the inclusion of larval and
195 embryo samples. For this analysis, we varied the proportion of larval and embryo
196 samples included with our adult samples. This was assessed at proportions ranging from
197 0 (no larval or embryo samples) to 1 (only larval and embryo samples) at increments of
198 0.05. At each increment, we assessed the mean and 95% confidence intervals of the
199 Mantel *r* and the corresponding P-value based on 1,000 bootstrap samples of the data. We
200 used the data set without siblings for this analysis and sampled each population to the
201 minimum adult sample size (n=18).

202

203 **Results**

204 *Literature review*— We found that 19 out of 90 (21%) of studies meeting our search
205 criteria on Scopus conducted population or landscape genetic analyses of amphibian
206 species using mixed tissue sampling (searched on 2 September 2013). Five of these
207 studies stated that one life stage was sampled only when the target life stage was not

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208 available (Beebee & Rowe 2000; Lee-Yaw et al. 2009; Lee-Yaw et al. 2008; Munwes et
209 al. 2010; Richardson 2012). None of these studies made attempts to check or correct for
210 the effects of mixing life stages in their analyses, although it was common for siblings to
211 be removed prior to analysis.
212
213 *Sample summary*— We collected 24–25 adults and 19–27 embryos from each of the five
214 ponds, and 29–36 larvae from three ponds (Supplement 1). We were unable to sample
215 larvae from two of the ponds due to high embryo mortality. Of the original 19 screened
216 primers, two loci (*Am_13*, *Am_60*) were not polymorphic, and two loci (*Am_ 33*, *Am_43*)
217 showed very little polymorphism and deviated significantly from expected heterozygosity
218 values under Hardy Weinberg equilibrium (HWE). These four loci were removed from
219 the dataset and all population genetic statistics were calculated using the remaining 15
220 loci (Supplement 2). No other loci or populations deviated from Hardy-Weinberg
221 equilibrium or were significantly linked. Overall, we had <0.5% missing data.
222
223 *Statistical summary*— For all tests, none of the population genetic parameters differed
224 significantly among life stages (bootstrap ANOVA P-value >0.05) or within ponds and
225 among life stages (permutation t-test P-value ≥0.25; Fig. 2, Tables 1–2), regardless of
226 whether or not siblings were present in the data. The proportion of samples removed due
227 to sibship was nearly significant (bootstrap P-value = 0.053, Fig. 2f), with a greater
228 proportion of field-collected samples being omitted from larvae. There was also an
229 increase in the estimated mean F_{ST} calculated in the larval and embryo data as compared
230 to adults (Fig. 2c), but we note that this increase was not significant. However, this trend

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231 was not observed when genetic distance was measured using allele frequencies (D_C, Fig.
232 2d). Values of population genetic summary statistics calculated on data sets with siblings
233 removed are given as the mean (±standard deviation). Effective population size among
234 ponds averaged 87.4 (±25.28) for adults, 82.4 (±25.58) for embryos, and 64 (±4.32) for
235 larvae. Average rarefied allelic richness of adults was 3.83 (±0.22), 3.95 (±0.23) for
236 embryos, and 4.13 (±0.20) for larvae. The average observed heterozygosity was 0.53
237 (±0.01) for adults, 0.51 (±0.03) for embryos, and 0.51 (±0.01) for larvae. On average, we
238 removed 33.2% (±0.09) of larval samples due to sibship, while only 13% (±0.084) of
239 adult and 14.98% (±0.09) of embryo samples were removed. Pairwise genetic distances
240 between ponds measured using F_{ST} averaged 0.011(±0.008) in adults, 0.021(±0.007) in
241 larvae, and 0.019 (±0.014) in embryos (Table 3).

242 When comparing each life stage at each summary metric, we found no significant
243 differences between data containing the sibling pairs and data with removed sibling pairs
244 (permutation t-test P-value ≥ 0.19; Fig. 2, Tables 1–2). We found that the mixing of life
245 stages resulted in genetic estimates of A_r, H_o, F_{ST}, and D_C that, on average, did not differ
246 from estimates made for each specific life stage (Tables 1–2). There were, however, up to
247 three pond-pair F_{ST} values that fell outside of the bootstrapped 95% confidence intervals
248 (Table 3). When genetic distance was measured using D_C, only one pond-pair fell outside
249 of the bootstrapped confidence interval. In general, there was a greater frequency of
250 pairwise genetic distance measures based on embryo samples to fall outside of the mixed
251 sample confidence interval. For both F_{ST} and D_C, the removal of siblings resulted in more
252 pairwise estimates falling outside of the mixed sample confidence interval (Table 3). Due
253 to sample size, clear inferences cannot be drawn from larvae. With regard to IBD, only

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254 tests using adult samples (with and without siblings) resulted in significant relationships
255 (Table 4), and the IBD relationship was stronger when genetic distance was measured
256 using D_C (Fig. 3). IBD tests from embryos or larvae had much lower Mantel *r*
257 correlations and were not significant. The mixing of tissue samples resulted in non-
258 significant IBD tests when using F_{ST} ($p > 0.05$; Table 4), but had little effect when using
259 D_C (Table 4).

260 Our assessment of increasing the proportion of larval or embryo tissue samples
261 clearly demonstrated that the calculated Mantel *r* decreases as the proportion of larval
262 and embryo samples increases (Fig. 3). This pattern was consistent regardless of whether
263 genetic distance was measured using F_{ST} or D_C. Correspondingly, the average P-value of
264 the Mantel test increased as the proportion of larval and embryo samples increased. In
265 this analysis, the mean bootstrapped P-value for tests using F_{ST} started at 0.057 (0.055–
266 0.60) for adult-only samples, and increased to 0.231 (0.223–0.239) for larvae/embryo-
267 only samples (Fig. 3b). In contrast, the P-value for Mantel tests with D_C started at 0.019
268 (0.018–0.020) when only adults were included and increased to 0.146 (0.140–0.152)
269 without adult samples (Fig. 3d). The 0.05 threshold is passed when the proportion of
270 larvae and embryos in the sample reaches 0.60–0.65 (P-values = 0.047–0.054,
271 respectively).
272

273 **Discussion**

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

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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_{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_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_C, and we found that the IBD relationship seen in adult-only 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_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

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300 stages may be evident in landscape genetic analyses, with different conclusions
301 potentially being drawn from adult-only samples as compared to larvae, embryo, or
302 mixed-tissue samples. Further, such relationships may be sensitive to the genetic distance
303 statistic used.

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

316 The clearest result from our study was the proportion of samples that had to be
317 removed from each life stage due to redundancy of siblings. We note that it is actually
318 quite unlikely that we sampled full sibling adults given their life history and longevity
319 (Petranka 1998), but we chose to remove these putative siblings from our data set for
320 consistency of methods among life stages. We note, however, that Goldberg & Waits
321 (2010) did not test for or remove sibling pairs from their adult samples. In our study, up
322 to 40% of larval samples collected in the field had to be removed. Although larvae are

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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 pool-breeding amphibian species, especially those that exhibit similar life history characteristics. The life history of *A. maculatum* is not unlike that *Ambytoma* spp.

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346 (Petranka 1998). It is unclear how differences in life history (e.g., life span, breeding site
347 fidelity, reproductive strategy, etc.) alter the effects of sampling different life stages, but
348 our results could be applicable to insects or fish that also exhibit Type III survivorship.
349 We found that mixed-tissue samples can lead to different conclusions when conducting
350 spatial analyses, such as IBD, and these results would likely extend to more complex
351 landscape genetic analyses as well. As such, we caution researchers to carefully consider
352 the implications of mixing samples collected from multiple life stages. Our finding that
353 population genetic parameters differed little with the removal of siblings or mixing of life
354 stages needs to be interpreted cautiously. First, we had a relatively small sample size
355 from which to draw inference, although we note that it is not much smaller than that used
356 by Goldberg & Waits (2010) who assessed eight populations of *R. luteiventris* and four
357 populations of *A. macrodactylum*. We do note that the power to infer differences is likely
358 greater in our study due to the large number of polymorphic microsatellites used. Perhaps
359 of greater importance is the fact that our populations are relatively close together
360 (maximum distance = 3,200 m) and situated within continuous forest habitat. In contrast,
361 Goldberg & Waits (2010) sampled populations separated by 2.7–18.5 km of agricultural
362 matrix. Further, the populations included in our study are very robust, with recorded
363 breeding aggregations of several hundred individuals (Semlitsch unpublished data). Like
364 Goldberg & Waits (2010), we suggest that future studies include a pilot phase to assess
365 the effects of sampling different life stages to meet the objectives of the specific project.
366 An important consideration for any population or landscape genetic study is the target
367 demographic group for which inferences are desired. If quantifying movement or
368 connectivity of populations is the main objective of a study, then sampling adult life

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369 stages may provide the most accurate inferences. If the study objectives are to quantify
370 the distribution of genetic diversity, then perhaps larvae or embryos will suffice.
371

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501 **Table 1.** Summary statistics following COLONY analysis including effective population
502 size (N_e) and the proportion of samples removed. The combined life stages sibling
503 removal follows two iterations of COLONY, the first within life stage, the second after
504 pooling life stages.

	N_e			Proportion of samples removed			
Pond	Adult	Embryo	Larvae	Adult	Embryo	Larvae	Combined life stages
1	120	92	62	0.08	0.08	0.21	0.40
2	67	114	–	0.16	0.05	–	0.25
3	100	93	–	0.00	0.12	–	0.24
4	100	55	70	0.16	0.20	0.39	0.41
5	50	58	60	0.25	0.30	0.40	0.37
Avg	87.4	82.4	64	0.13	0.15	0.33	0.33
SD	25.28	22.58	4.32	0.08	0.09	0.09	0.07

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506 **Table 2**. Rarefied allelic richness and observed heterozygosity estimates at each pond for both the full data set, and with siblings
507 removed. Mixed-tissue is the bootstrap mean and 95% confidence interval from randomly sampling all life stages together. Bolded
508 type indicates values that fall outside of the bootstrapped 95% confidence interval.
509

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Full data set

	A_r				H_o			
Pond	Adult	Embryo	Larvae	Mixed samples (95% CI)	Adult	Embryo	Larvae	Mixed samples (95% 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

	A_r				H_o			
Pond	Adult	Embryo	Larvae	Mixed samples (95% CI)	Adult	Embryo	Larvae	Mixed samples (95% 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	–

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511 **Table 3.** Pairwise genetic distances (F_{ST} and D_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

	F_{ST}				D_C			
Pond-pair	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

	F_{ST}				D_C			
Pond-pair	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)

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1_4	**0.024**	**0.021**	**0.024**	0.008 (0.000–0.017)	0.243	**0.258**	0.234	0.229 (0.200–0.256)
1_5	0.020	**0.036**	0.028	0.017 (0.006–0.030)	0.242	**0.273**	0.249	0.224 (0.195–0.251)
2_3	**0.005**	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	**0.259**	–	0.213 (0.189–0.235)
2_5	0.015	**0.040**	–	0.019 (0.007–0.031)	0.226	**0.276**	–	0.203 (0.178–0.228)
3_4	**0.000**	0.006	–	0.016 (0.007–0.028)	0.179	0.191	–	0.173 (0.146–0.203)
3_5	0.013	**0.037**	–	0.007 (-0.001–0.017)	**0.262**	**0.256**	–	0.212 (0.189–0.237)
4_5	0.004	0.020	0.012	0.008 (-0.002–0.020)	0.232	**0.240**	0.227	0.213 (0.187–0.239)
Avg	0.011	0.019	0.021	0.011 (0.002–0.022)	0.226	**0.240**	**0.237**	0.21 (0.185–0.235)
SD	0.008	0.014	0.007	–	0.025	0.030	0.011	–

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517 **Table 4.** Results of simple Mantel tests assessing the correlation between genetic distance and geographic distance. Mixed life stage
518 represents 1,000 bootstrap iterations, and the corresponding Mantel *r* and P-value estimates are the mean and 95% confidence
519 intervals of the bootstrap iterations. Mantel P-values were estimated from 100,000 permutations.

Full data set

	F_{ST}		D_C	
Life stage	**Mantel *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_{ST}		D_C	
Life stage	**Mantel *r***	**P-value**	**Mantel *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

520

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521 **Figure 1.** Map of Daniel Boone Conservation Area depicting the locations of the five
522 ponds used in this study. Wildlife ponds are ponds readily used by amphibians, such as
523 *Ambystoma maculatum*, for reproduction.
524
525 **Figure 2.** Bar plots representing mean values of a) observed heterozygosity, b) rarefied
526 allelic richness (A_r), c) genetic distance (F_{ST}), d) genetic distance (D_C), e) effective
527 population size (N_e), and f) proportion of samples removed due to sibship. Solid bars
528 represent values containing full siblings, patterned bars represent values after sibling
529 removal, and error bars represent standard deviations.
530
531 **Figure 3.** Change in Mantel *r* when using F_{ST} (a) and D_C (b), and the corresponding
532 change in the P-value (c = F_{ST}; d = D_C) with increasing proportion of tissue samples
533 coming from larvae and embryos. The dashed line in c and d is drawn at 0.05 to indicate
534 the traditional threshold for significance. Mean (solid black line) and 95% confidence
535 intervals (gray shading) were estimated at 0.05 increments between from 0 to 1. A
536 proportion of 0 represents an adult-only sample, while a proportion of 1 represents a
537 larvae/embryo-only sample. At each 0.05 increment, 1000 bootstrap samples were
538 conducted and Mantel P-values were estimated from 100,000 permutations.
539

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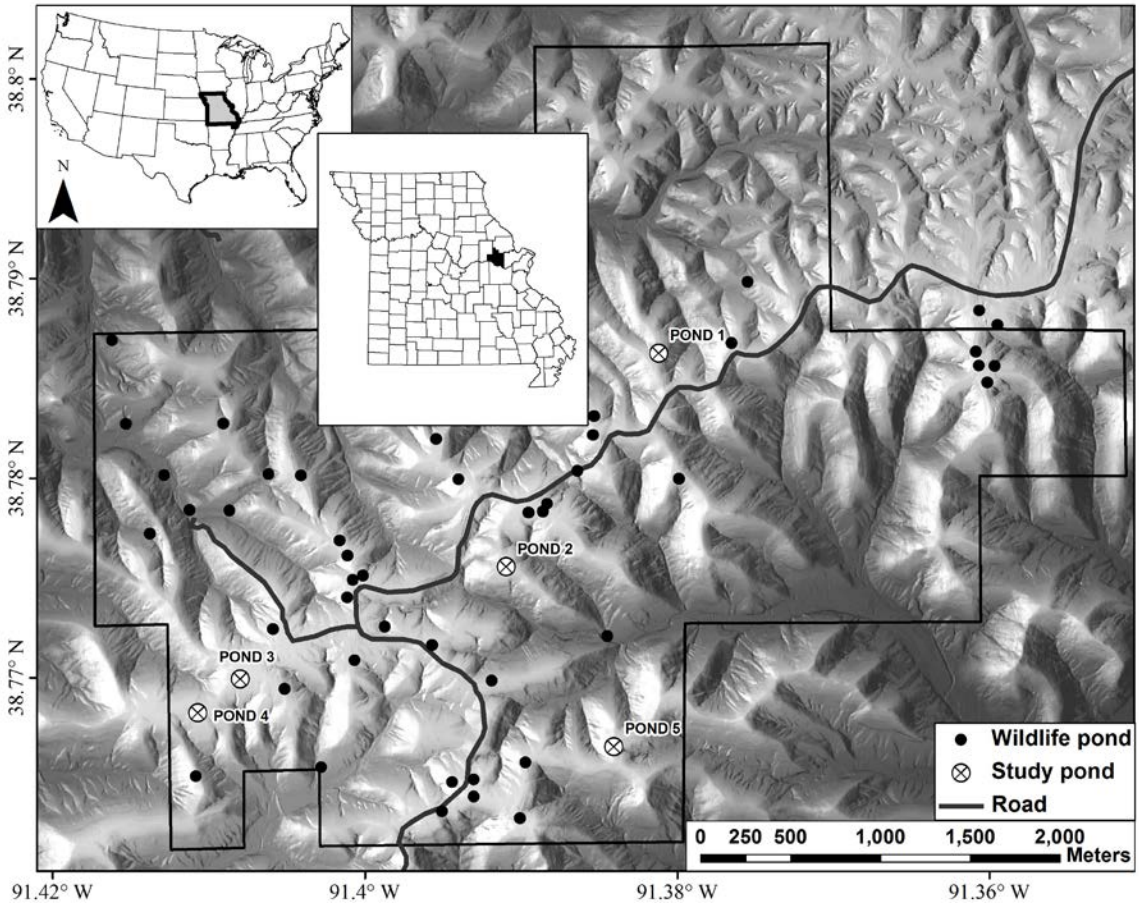

Fig. 1

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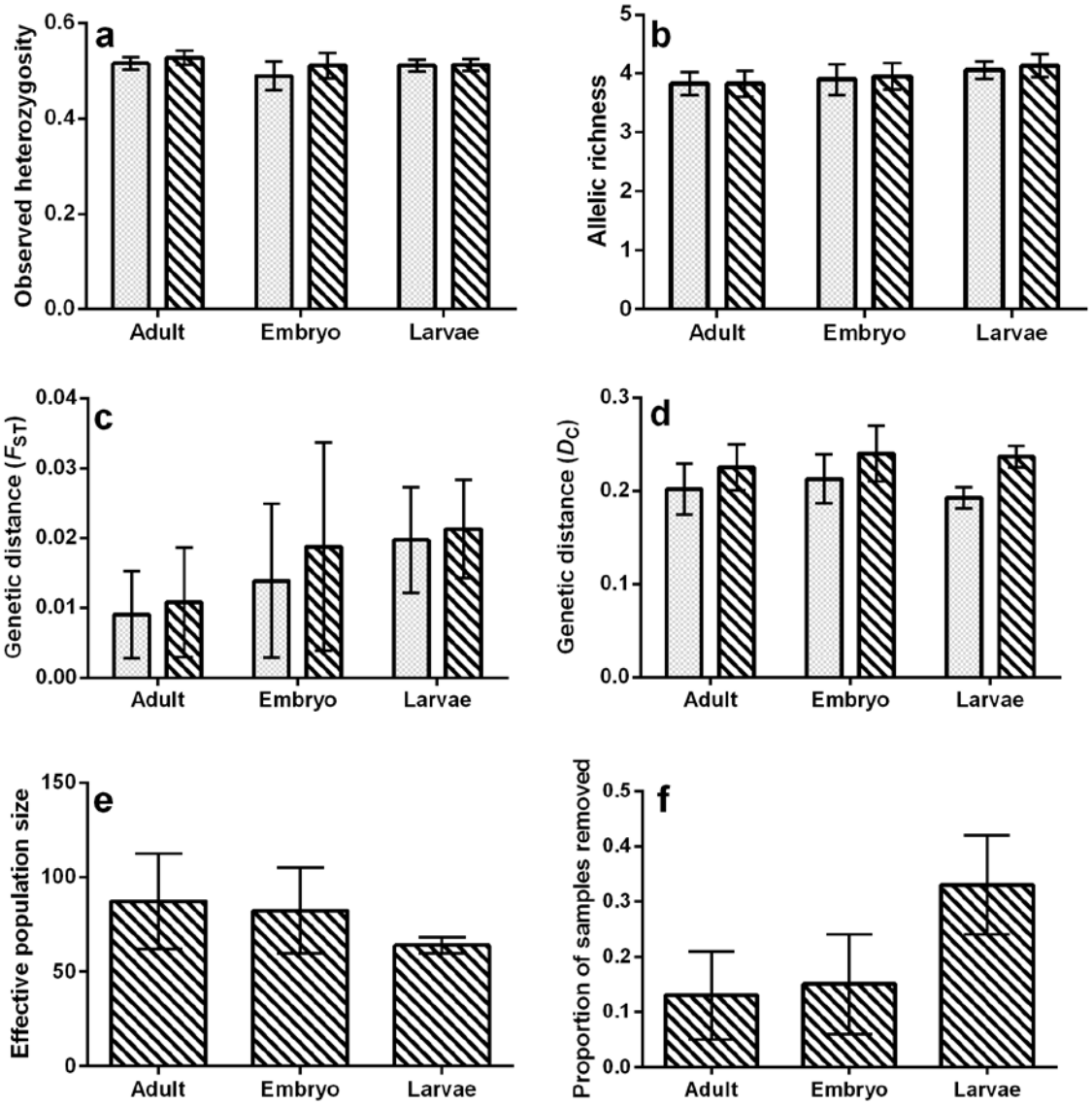

Fig. 2

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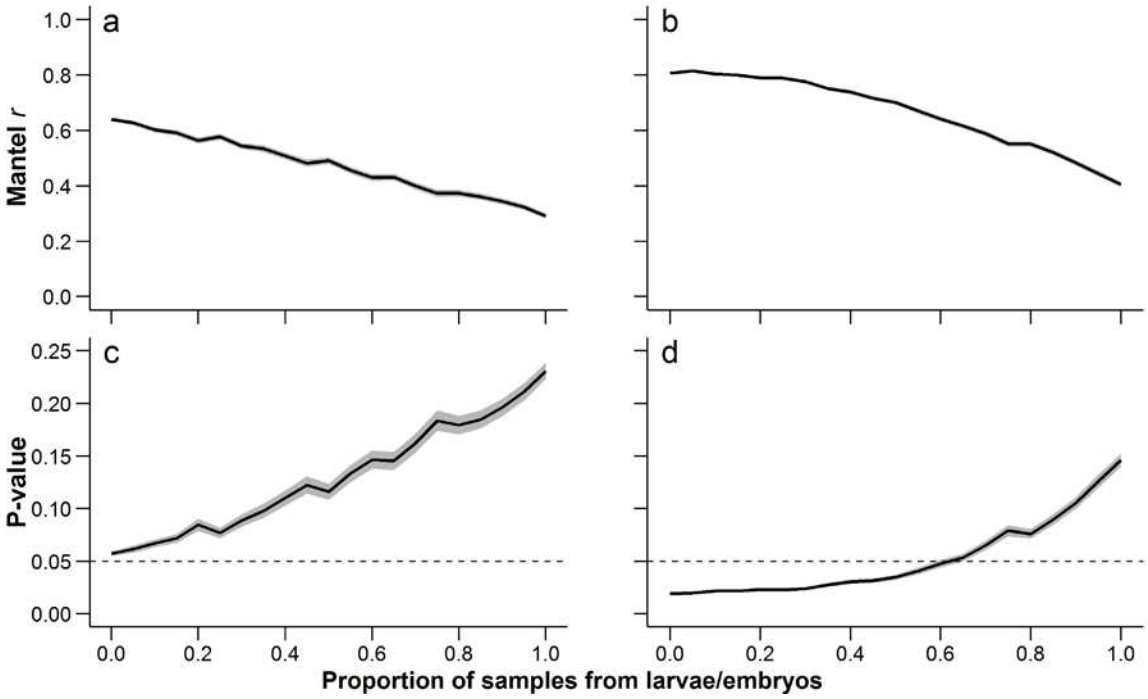

Fig. 3

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