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Distinguishing Egg Masses of Unisexual and Jefferson Salamanders

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***Abstract*.—Unisexual *Ambystoma* are difficult to distinguish from the sexual salamanders with which they interact, and all of these species are of conservation concern throughout much of their range. We examined egg masses to develop an efficient, affordable method to differentiate unisexual salamanders and Jefferson Salamanders (*A. jeffersonianum*). We developed a technique based on photographs of egg masses from three populations held in the laboratory and tested it in the field. Using photographs, we first determined the long axis of the egg mass, and then drew a line perpendicular to the long axis and counted the maximum number of embryos or egg membranes that intersected with this line. The resulting Embryo Density Index was higher in egg masses of Jefferson Salamander (mean = 6.3 ± 2.0 SD) than in unisexuals (mean = 3.8 ± 1.3). Likewise, the Egg Membrane Density Index was higher in egg masses of Jefferson Salamanders (mean = 7.7 ± 2.3) than in unisexuals (mean = 4.6 ± 1.6). Although those results were statistically significant, there was considerable overlap in egg density index values between salamander biotypes, making the methods more appropriate for population-level analyses than for determining species identity of a given individual egg mass. Our scale-independent method of visually assessing the spacing of eggs within a mass can be used across developmental stages, and it enables rapid data collection with very little equipment or cost.**

Key Words.—Ambystoma jeffersonianum; egg mass surveys; density metric; image analysis

Introduction

Unisexual vertebrates present a number of challenges to taxonomists, biologists, and wildlife managers, as they are of interspecific hybrid origins, have non-traditional and often complicated reproductive mechanisms, interact with and closely resemble non-hybrid forms of species, and exhibit atypical population structures (Dawley 1989). Accordingly, scientific and legal classifications can be controversial, population assessments inordinately laborious, and conservation prescriptions fraught with uncertainty. Such challenges are exemplified by the unisexual *Ambystoma*, an ancient, nearly all-female salamander lineage whose genetically diverse forms interact with the sexual Streamside Salamander (*A. barbouri*), Jefferson Salamander (*A. jeffersonianum*), Blue-spotted Salamander (*A. laterale*), Small-mouthed Salamander (*A. texanum*), and Eastern Tiger Salamander (*A. tigrinum*; Dawley 1989; Petranka 1998; Bogart et al. 2009; Bi and Bogart 2010).

Unisexual *Ambystoma* (hereinafter unisexuals) procreate via a complex reproductive system termed kleptogenesis, which relies on the acquisition of sperm from sympatric males of the aforementioned sexual species (Bogart et al. 2007; Bi et al. 2008). Throughout much of their geographic range, unisexuals co-occur with, use sperm from, and physically resemble Jefferson Salamander and Blue-spotted Salamander (Clanton 1934; Uzzell 1964; Bogart and Klemens 1997, 2008; Bogart et al. 2009). Population studies of these salamanders have traditionally involved intensive field collections and meticulous laboratory work (Uzzell 1964; Lowcock et al. 1991; Bogart and Klemens 1997; Noël et al. 2011; Hoffmann 2017) to understand even basic demographics, because molecular analysis is often required to properly distinguish between unisexuals and the sexual species or to assign individual salamanders to a particular biotype (i.e., any of the various nuclear genomic combinations found in the unisexual forms). Of particular interest is how relative proportions of

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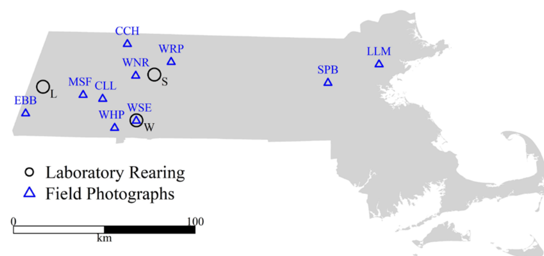

Figure 1. Locations of sampling sites (with site codes) in Massachusetts, USA, where salamander egg masses were photographed at a single moment in the field (blue triangles) and where egg masses were collected for rearing through multiple developmental stages in the laboratory (black circles). Laboratory-reared eggs were collected from the towns of Lenox (L), Sunderland (S), and West Springfield (W).

unisexuals and the sexual species change at the local scale over time, and the resulting implications for long-term population viability (Clanton 1934; Minton 1954; Wilbur 1971; Homan et al. 2007; Bogart et al. 2017). That subject has tangible applications for state wildlife agencies and other managers in regions where the salamander complex is of conservation concern, but such organizations seldom have the means to conduct resource-intensive assessments at very many sites or over wide geographic areas.

Several studies (Uzzell 1964; Lowcock et al. 1992; Charney et al. 2014) have made progress in identifying field methods to distinguish between Blue-spotted Salamander and its unisexual associates on the basis of adult morphology, which may help to reduce costs and labor associated with assessments of intermediate to long-term trends in local population structure. Those studies, however, were not able to distinguish between Jefferson Salamander and unisexuals. Furthermore, assessments that rely on direct observations of adults remain labor intensive, due to the sampling methods involved (e.g., installation and operation of drift-fence and pitfall arrays and multi-day deployment of aquatic funnel traps). Indirect indicators (e.g., eggs) may be better subjects to evaluate when attempting to distinguish between salamanders that are difficult to sample and have similar adult morphologies (e.g., Kraus and Petranka 1989). Conceivably, egg-mass observations could be an effective way to obtain, at the very least, crude demographic data for evaluating population trends, provided that field evidence (1) can be collected rapidly, (2) can be analyzed inexpensively, and (3) will allow for differentiation between salamander types. We sought to identify such a method with respect to egg masses of Jefferson Salamander and its unisexual associates. In this study, we used laboratory-reared embryos to develop a simple metric to distinguish the morphology of Jefferson Salamander and unisexual egg masses, and then tested this metric on egg masses photographed in the field.

Materials and Methods

Laboratory rearing.—We obtained 11 egg masses from three Massachusetts, USA, populations to rear in the laboratory (Fig. 1). We collected two egg masses from Sunderland on 11 March 2016, five egg masses from West Springfield on 12 March 2016, and four egg masses from Lenox on 15 March 2016. We collected 356 individual eggs in total, with egg masses containing between 13 and 75 eggs each (mean = 32).

We transferred egg masses to 20.8 L (5.5-gallon) glass tanks, with each tank containing eggs from a single population. To maintain water levels, we added water collected from the source ponds. Tanks were housed in a basement level space in Northfield, Massachusetts. Water temperatures in the tanks over the duration of the study ranged from 11° C to 16° C. We aerated water and maintained tanks with separate equipment to minimize potential for disease transfer between source populations. We maintained natural light cycles using 6,500K full spectrum fluorescent lights on natural daylight timers. We transferred hatchling larvae to clean containers and released them back into the appropriate source ponds. We retained one embryo per egg mass for DNA analysis.

Field surveys.—We photographed and collected genetic data from 94 egg masses observed among 10 ponds (Fig. 1) during field surveys conducted mid-April through early May 2017. In each survey, a single observer (JEK) approached a pond and visually scanned the water column for presence of egg masses from the bank or from within the pond. The observer selected directions of travel haphazardly, and sampled the first 10 egg masses encountered; however, the observer skipped an egg mass if it (1) was not accessible for clear photos, (2) did not have a viable embryo for collection of DNA, or (3) appeared to be too damaged (e.g., by predators) to allow for a reliable assessment of morphology (e.g., number or position of embryos). When the observer encountered a group of masses together on the same stem, he sampled only one mass from the group, selecting the first one that was determined to meet the aforementioned criteria (i.e., was accessible for clear photos, had at least one viable embryo, and was sufficiently intact). At one site, the observer returned to a group and sampled additional masses because of an insufficient number of accessible masses elsewhere in the pond to meet the target sample size during the survey.

When the observer identified an egg mass for sampling, he photographed it *in situ* from multiple angles and then processed it to collect a DNA sample. Processing consisted of excising an embryo (including its encapsulating membranes) from an outer portion of the mass by hand and transferring the intact embryo to

a temporary holding container (individually marked plastic bag with some pond water). At completion of the egg-mass survey, the observer finished processing each of the collected embryos by puncturing its encapsulating membranes with a sterilized scissor tip, excising embryonic material, and transferring the material to an individually marked ethanol vial.

To minimize chances of sampling multiple egg masses deposited by a particular salamander, the observer usually exited the pond and re-entered it at a different point during the survey, or he moved briskly to another area within the pond at least 5 m away before resuming a careful search for the next egg mass to sample. Several ponds, however, were too small or their egg mass distributions too clustered to exercise that approach effectively. In those cases, the observer was forced to sample egg masses occurring < 5 m apart. We surveyed each pond only once.

Density metric.—Using our laboratory-reared egg masses, we explored potential ways to quantify what we perceived (visually) to be differences in the relative spacing of eggs within a mass between Jefferson Salamander and unisexuals. We tentatively identified a possible metric that could be used both in a standardized fashion and independent of scale. We then tested the efficacy of the metric by analyzing the collection of photographs of egg masses from the field surveys. To minimize bias, we applied the metric and calculated values without prior knowledge of the mass type (i.e., Jefferson Salamander vs. the different unisexual biotypes). We conducted ANOVAs ($\alpha = 0.05$) using the nlme package in R to test for significant differences in metric among types, holding population as a random variable.

DNA extraction, D-loop sequencing, and analysis.—We extracted DNA from the 11 lab-reared egg samples following the protocol outlined by Fetzner (1999). For the 94 field samples, we extracted DNA using a DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) following manufacturer protocols with slight initial preparation modifications (see Appendix). To identify species type (i.e., Jefferson Salamander vs. unisexual), we amplified the mitochondrial D-loop from each of the 105 DNA samples using polymerase chain reaction (PCR) and then sequenced the resulting PCR products. We sequenced lab-reared samples from the DL1 primer and the field samples via THR and 651 primers (Schaffer and McKnight 1996; see Appendix). Sequences were accessioned at NCBI GenBank under accession numbers MK185107–MK185211. We downloaded D-loop sequences for *Ambystoma jeffersonianum*, *A. laterale*, and an LLJ unisexual from GenBank (GenBank IDs: EF184166, EF184163, and EF184209) and used these sequences as references to map D-loop reads from each sample using the map to reference algorithm in Geneious 9.0 (Biomatters Limited, Auckland, New Zealand). We removed reference sequences from each assembly, examined contigs to correct sequencing errors, and created a consensus D-loop sequence for each individual. We aligned consensus sequences using CLUSTALW (Thompson et al. 1994).

Microsatellite amplification and analysis.—We attempted to identify nuclear genotype and ploidy of the 94 field samples using six previously published nuclear microsatellite markers (AjeD94, AjeD75, AjeD346, AjeD422, AjeD283, and AjeD378; Julian et al. 2003). Microsatellite loci AjeD94, AjeD75, and AjeD346 are reported to amplify alleles that differ in size between *A. jeffersonianum* and *A. laterale* with little to no overlap (Julian et al. 2003, Ramsden et al. 2006). AjeD422 and AjeD283 amplify alleles that overlap in size between the two species (Julian et al. 2003), but they may provide additional evidence to determine ploidy for each sample. AjeD378 only amplifies alleles from *A. jeffersonianum* (Julian et al. 2003).

We used the microsatellite analysis plugin tool in Geneious 9.0 to analyze microsat data. We imported each .fsa file into Geneious and calibrated the LIZ labeled size standard to identify known fragment sizes. We identified each microsat fragment size by interpolation against LIZ size standards. To distinguish between J and L alleles, we binned fragment sizes according to reported J and L allele sizes (Julian et al. 2003; Ramsden et al. 2006; Charney et al. 2014). When fragment sizes overlapped, we identified those alleles as ambiguous between J and L. We examined all six loci to make a determination of nuclear genotype and ploidy, using the majority of evidence to call a genotype. In several cases of equivocal genotype calls, we assigned genotype to the higher ploidy state. Ultimately, 82 samples amplified sufficiently for assignment of a nuclear genotype.

Results

Density metric.—We successfully devised a simple, scale-independent metric to distinguish the unisexual and Jefferson Salamander egg masses in the laboratory (Fig. 2). The singular metric is intended to capture an overall morphology controlled by several factors that may vary both within and across species. Such controlling factors include the number of eggs laid per egg mass, the geometry of an egg mass (round versus elongated), and the thickness of the gelatinous matrix separating the eggs from each other within a mass. The metric is performed on a side- or top-view image of an egg mass. Typically, these egg masses are laid on a stick or leaf petiole, and we first identify this line

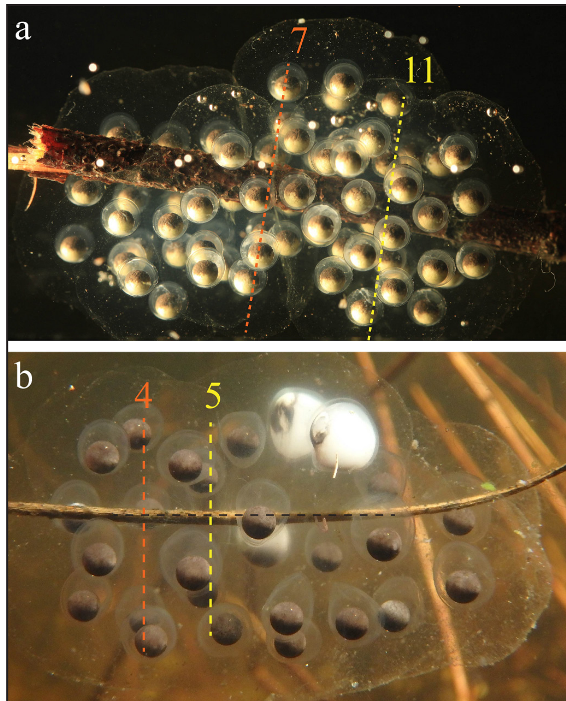

Figure 2. Example egg masses of (a) Jefferson Salamander (*Ambystoma jeffersonianum*) and (b) unisexual Ambystoma at two different stages of development. Dotted lines and accompanying numbers represent density index estimates based on intersecting the maximum number of embryos (orange) or egg membranes (yellow). (a Photographed by Noah Charney and b by Jacob Kubel)

as the long axis of the egg mass. If no such stick is present, we draw a line through the long axis, following the natural symmetry of the egg mass. Conceptually, we then evaluate the density of eggs at any point along this axis by constructing a perpendicular plane and counting the number of eggs intersected by that plane. In practice, we define the edge of this plane, which is parallel to our viewing angle in our image, by drawing a line perpendicular to the long axis in the image. We then slide this perpendicular line along the mass until it intersects the maximum number of embryos. The maximum number of intersected embryos is what we define as the embryo density index characteristic of the egg mass. Alternatively, one could also maximize the number of egg membranes intersected.

We applied our metric for each laboratory-reared egg mass, spreading across developmental stages, except when the mass became too cloudy or indistinct for reliable counting of embryos. For all stages of the three Jefferson Salamander egg masses, the embryo density index values were 6 or higher (Fig. 3a). For all stages of the eight unisexual salamander egg masses, the embryo density index values were 5 or lower.

Field samples.—Jefferson Salamanders were the host sexual species in eight of the 10 ponds sampled, based on either direct genetic detection (i.e., mtDNA sequencing of sexual species) or indirect genetic inference (i.e., nuclear microsatellite analysis of unisexuals). Blue-spotted Salamanders were the presumed sexual species at two ponds, based on either genetic confirmation from an individual salamander captured < 5 m from a pond (LLM) or geographic location (LLM, SPB). The two species are not known to share ponds anywhere in Massachusetts (Bogart and Klemens 1997, 2008; Charney et al. 2014), and based on habitat characteristics of the sites we sampled, we do not believe both species occupied a given site.

Among ponds with Jefferson Salamander hosts (n = 8), Embryo Density Index values were higher ($F_{1,61}$ = 32.0, $P < 0.001$) in egg masses of Jefferson Salamander (mean = 6.3 ± 2.1 SD) than in egg masses of unisexuals (mean = 4.1 ± 1.1; Fig. 3b and 4). In those same ponds, 89% of the unisexual egg masses exhibited an Embryo Density Index ≤ 5. In contrast, 74% of the Jefferson Salamander egg masses had an Embryo Density Index

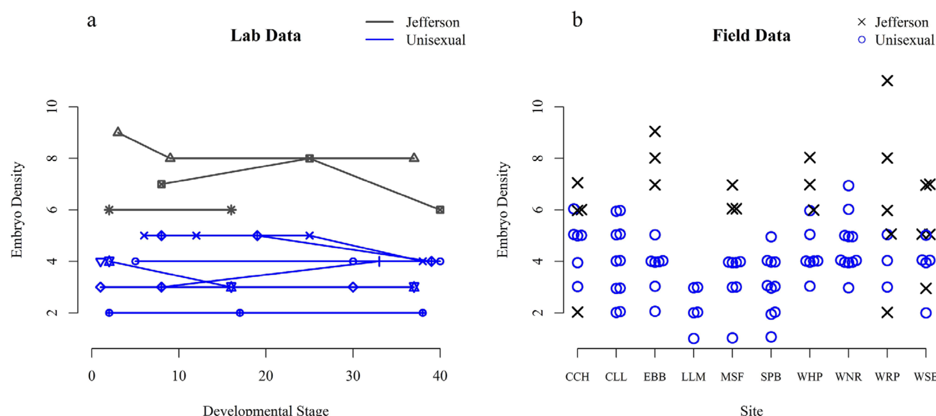

Figure 3. Embryo density index estimates for Jefferson Salamander (*Ambystoma jeffersonianum*) and unisexual Ambystoma salamander egg masses measured across developmental stages, based on the most advanced embryo in each mass (Schreckenberg and Jacobson 1975) of 11 egg masses reared in the laboratory (a), and of 94 egg masses observed at 10 sites in the field (b), using site codes shown in Figure 1.

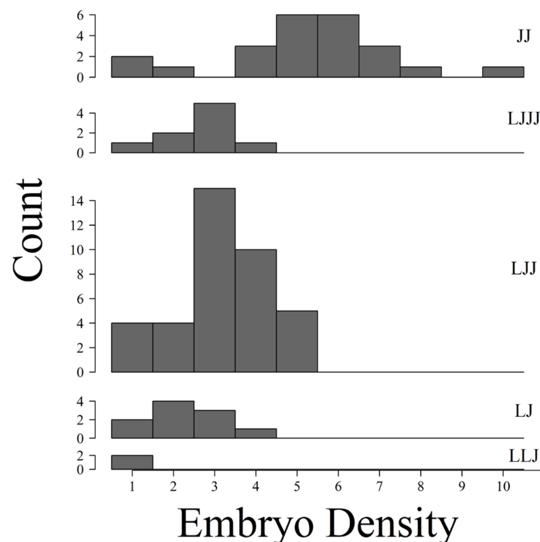

Figure 4. Histograms of embryo density index values in 82 egg masses observed in the field and for which a nuclear genotype was obtained from a single embryo. Genotypes are based on microsatellites of the parent genomes, L for the Blue-spotted Salamander (*Ambystoma laterale*), and J for the Jefferson Salamander (*A. jeffersonianum*). All but JJ individuals are members of the unisexual lineage. An egg mass from a unisexual lineage occasionally contains multiple genotypes (Bogart et al. 2007, 2009); consequently, the one embryo sampled from each such mass may not necessarily represent the parental genotype or all other genotypes in the mass.

≥ 6, and 48% had an Embryo Density Index ≥ 7. We obtained similar results when we based the density metric on egg membranes rather than embryos in ponds with Jefferson Salamander hosts (Appendix Fig. 1 and 2). Egg Membrane Density Index values were higher ($F_{1,61}$ = 31.0, P < 0.001) in Jefferson Salamander (mean = 7.7 ± 2.4) than in unisexuals (mean = 4.9 ± 1.4), with 96% of unisexual egg masses having a Membrane Density Index ≤ 7, and 65% of Jefferson Salamander egg masses having a density index ≥ 8.

In general, the Embryo Density Index in unisexual egg masses appeared to be negatively related to the proportion of L genomes present in the nuclear DNA (i.e., relative to J genomes; Fig. 4). Unisexuals in the two Blue-spotted Salamander ponds consisted of LJ and LLJ genotype individuals and had lower ($F_{1,8}$ = 4.10, P = 0.008) embryo density index values (mean = 2.8 ± 1.1) and lower ($F_{1,8}$ = 10.3, P = 0.010) Egg Membrane Density Index values (mean = 3.2 ± 1.3) than the aforementioned unisexuals in Jefferson Salamander ponds, which consisted primarily of LJJ and LJJJ individuals; however, we caution interpretation of those comparisons, as the genotype of only one unisexual embryo is not necessarily indicative of the genotype of the parental unisexual salamander or of the predominant genotype among embryos in the egg mass (Bogart et al. 2007, 2009). Full data tables and photographs of all egg masses will be submitted to Dryad Digital Repository (https://datadryad.org/).

Discussion

Previous studies have characterized the egg morphology of Jefferson Salamander as a cylindrical or oblong, gelatinous mass averaging 14–35 eggs, with upper limits of approximately 60 eggs (Smith 1911; Bishop 1941; Seibert and Brandon 1960; Smith 1983; Brodman 2002). Unisexuals, however, may be represented in some of those samples, as egg morphology of unisexuals has been characterized as generally similar to Jefferson Salamander (Uzzell 1964; Morris and Brandon 1984), and some of the aforementioned study sites (Smith 1911; Bishop 1941; Brodman 2002) occurred within the geographic range of the unisexual lineage. We are aware of anecdotal accounts of perceived differences in the physical appearances of Jefferson and unisexual salamander egg masses (e.g., cylindrical vs. oval shape, high vs. low percentage of viable eggs), and we have suspected some additional differences (e.g., number and spatial density of eggs within the mass) during casual observation. Our study is the first to develop a quantitative method for distinguishing between egg masses of Jefferson Salamander and unisexuals based on an analysis of field-observation information alone, and the method is supported by genetic data.

Panek (1978) claimed that the two salamander types could be distinguished in the field based on direct comparisons of egg size, but that study did not disclose details of actual egg sizes and did not confirm type assignments with ploidy or genetic analyses. Licht and Bogart (1989) observed two distinct size classes of eggs in their laboratory study of a Blue-spotted Salamander and Small-mouthed Salamander system, and they noted that egg-size alone was not a sufficient basis for differentiating the sexual species from unisexuals; eggs of diploid unisexuals were of similar size to those of pure Blue-spotted Salamanders, and both size classes were observed in eggs of triploid unisexuals. We suggest further that comparison of egg size may not be a practical method for differentiating Jefferson Salamander and unisexuals in the field, as measuring absolute sizes directly or even obtaining photographs with an accurate scale-bar in a non-destructive way is difficult. Egg masses are surrounded by gelatinous envelopes which can be several centimeters thick and exclude rulers or calipers from the proximity of the individual eggs.

Our visual assessment of egg density within an egg mass met the criteria we set forth regarding rapid collection of field evidence and ease of analysis. Before

applying our method, one must assume that the field observer or image analyst is sufficiently trained in visual identification to exclude from the analysis egg masses of unrelated species, such as *Ambystoma maculatum*, which co-occur in the ponds. Our method then requires only that the observer obtain clear photographs of egg masses in the field and that the analyst superimpose lines on the collected images to calculate a density index value. The method is scale-independent (i.e., does not require use of a scale bar), thus providing consistent results not only among photographs with varying scales, but also across developmental stages of egg masses. Masses tend to expand as they mature, such that egg centers become spaced farther apart; however, our density metric is relatively unaffected because the embryos and egg membranes themselves also expand, compensating for the overall mass expansion. In addition to the aforementioned benefits, our method has the advantage of being nondestructive, which is an important consideration where Jefferson Salamander is either legally protected or of conservation concern.

Although the egg density index distributions of unisexual and Jefferson Salamander egg masses in our study clearly represent distinct statistical populations, there is considerable overlap at lower egg density index values. That may be explained in part by individual salamanders occasionally depositing masses with unusually low numbers of eggs, such as could happen if a female terminates egg deposition prematurely. For example, premature termination could result from lack of stability at the deposition site (Bishop 1941), physical disturbance by other animals (e.g., other salamanders or breeding amphibians), or a decrease in temperature below a physiological threshold. Thus, one could expect to encounter lower-than-expected egg density index values in Jefferson Salamander egg masses with some regularity, as the small masses (e.g., fewer than 10 eggs) will inherently have low density index values when evaluated by our metric. Conversely, it would seem less plausible for a salamander to deposit masses with much greater-than-expected egg density index values. Our data support that contention, as the minimum egg density index values between unisexuals and Jefferson Salamanders overlapped, but the observed maximum, mean, and median density index values diverged. In our sample, two of three Jefferson Salamander egg masses with a low density index value had very few total embryos (n = 4 and n = 5). Application of our metric might be improved by identifying a minimum threshold for egg-mass size (e.g., number of eggs) when sampling, though we caution that such an adjustment could discriminate disproportionately against egg masses of unisexuals.

The best application of our density metric is probably for basic population-level assessments, rather than for definitive identification of a given egg mass at a pond. By photographing a random sample of egg masses within a population, one should be able to estimate crude proportions of Jefferson versus unisexual salamanders present, which could help inform management decisions. Some populations of unisexuals, however, rely on Blue-spotted Salamanders for their sexual hosts and others rely on Jefferson Salamanders; our metric should also enable discrimination between those two population types whenever the higher-density Jefferson Salamander egg masses are detected incidentally. In that sense, the metric can aid in species identification of novel populations.

Our metric is not intended for identifying populations of Blue-spotted Salamander specifically, nor is it suitable for estimating the relative proportion of unisexuals in a Blue-spotted Salamander population. Blue-spotted Salamanders typically deposit eggs singly or, sometimes, side-by-side in a string or clump of several eggs without a unifying gelatinous matrix (Clanton 1934; Stille 1954; Bleakney 1957; Uzzell 1964; Gilhen 1974); hence, there is no egg mass per se to which our metric could be applied. Although unisexuals in Blue-spotted Salamander populations do deposit actual masses, they also deposit eggs singly (Clanton 1934; Uzzell 1964; Wilbur 1971). Thus, any attempt to estimate relative or proportional abundance of those salamanders on the basis of egg counts would have to rely on genetic sampling to identify the taxa, as single eggs of unisexuals are not readily distinguishable from those of Blue-spotted Salamanders in the field. Clanton (1934) noted that both diameter and pigmentation of freshly dissected ovarian eggs differed between Blue-spotted Salamanders and unisexuals, but how that relationship holds after eggs are deposited in a pond and begin to develop is not known. Regardless, assessments of population structure in Blue-spotted Salamander systems should rely on sampling of adult individuals rather than eggs, as independent egg samples would be difficult to obtain. A single female can conceivably scatter hundreds of eggs or dozens of small masses throughout a pond, based on published clutch sizes of 50–538 mature ova (Clanton 1934; Gilhen 1974).

One possible challenge in the use of our density metric for Jefferson Salamander systems is potential observer bias in application of the metric. For example, eggs within a mass are often obscured by other eggs, by the stick upon which the mass is deposited, by algae, or by mold within the egg-mass matrix, all of which could conceivably lead to differences among observers in perceived intersections. In our study, the same observer (NDC) applied the metric to all egg masses within a single, short time frame. Looking back, one may find reason to assign a slightly higher or slightly lower value for the density index of a given egg mass.

To help maximize egg visibility for our analysis of field samples, we took several photographs of each mass from multiple angles, which added considerable time to field sampling. Such an approach may not be practical under some circumstances (e.g., assessments conducted at large ponds with many egg masses or very cold water).

Although our approach for distinguishing egg masses of Jefferson and unisexual salamanders may allow for cursory characterizations of local population structures and tracking their trends over time, we note some general caveats about the use of salamander egg mass data for those purposes. First, egg mass abundance is primarily a measure of female reproductive effort and may not be indicative of the abundance of males in a population (though small numbers of masses can be caused by a scarcity of males; Bogart et al. 2017). Second, because male Jefferson Salamanders can discriminate against unisexuals (Dawley and Dawley 1986) and mate preferentially with conspecific females (Uzzell and Goldblatt 1967), numbers of observed egg masses may underestimate the actual proportion of adult unisexuals in a local population disproportionately (assuming a greater proportion of unisexuals fail to obtain sperm compared to female Jefferson Salamanders). Third, not all females within a local population necessarily arrive at a pond and deposit eggs at the same time (Williams 1973; Jackson 1990; Lowcock et al. 1991, 1992) or in the same year (Williams 1973; Homan et al. 2007), and they most certainly do not deposit eggs in equally accessible parts of the pond or with equal visibility. Lastly, a given female in a Jefferson Salamander population may deposit multiple egg masses, potentially reducing the independence of field samples (though not likely to the degree we described previously for Blue-spotted Salamander systems). All of the aforementioned factors may cause samples of egg masses to be a biased segment of the local population of salamanders, and so we recommend to users of our density metric that they implement repeated applications over multiple years to enable an assessment of variation before forming conclusions about local population structure at a given site.

Despite those limitations, our study does provide a basis for expanding the capacity of field biologists to make reasonably confident distinctions between egg masses of Jefferson Salamander and its unisexual associates. Our density metric is a quantitative and statistically defensible validation of previously suspected differences in the physical appearances of egg masses in the field. Given sufficient sample sizes, the metric provides biologists with an affordable tool to evaluate local population structures, which may aid in prioritization of sites for certain management actions (e.g., land protection, habitat restoration, regulatory classification) where the Jefferson Salamander is a species of conservation concern. Ultimately, this approach could be expanded to include additional species and/or metrics to bolster assessments of *Ambystoma* salamanders where the unisexual lineage is a confounding component of the local population.

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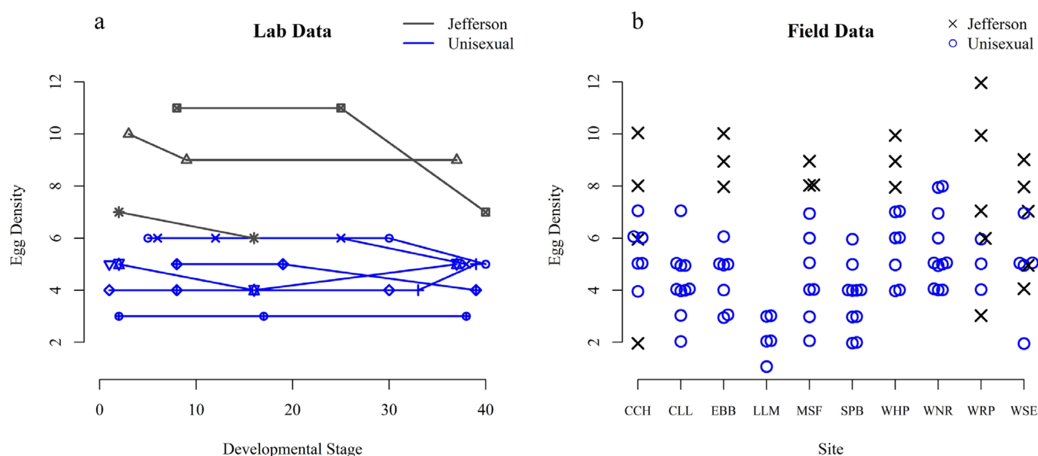
Appendix

HDNA preparation.—Prior to DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany), we lacerated samples into smaller pieces with a sterile razor blade, placed them into lysis solution along with proteinase K, and digested them overnight at 56° C in a shaker incubator. We inactivated digested reactions with buffer, treated them with 4 µl (100 mg/ml) RNase A, and purified and ethanol-washed them using silica column purification. We eluted DNA from the column two times using 100 µl hot elution buffer. We then assessed DNA quality and concentration by gel electrophoresis and QubitTM fluorometry (Life Technologies, Carlsbad, California, USA).

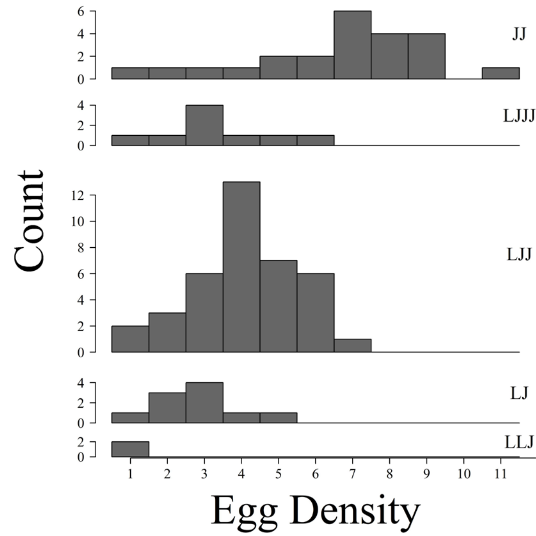
Specific PCR conditions.—For the lab-reared egg samples, we amplified the D-loop from 1 µl of extracted egg DNA using Phusion DNA polymerase (Thermo Scientific, Waltham, Massachusetts, USA) and 2 µl of mixed 10 µM DL1/007 primer pair (Shaffer and McKnight 1996) in 25 µl total reaction volumes. We cycled each reaction on a Perkin Elmer 9600 thermocycler as follows: initial denaturation at 98° C for 120 s, followed by 35 cycles of 98° C:10 s; 55° C:15 s; and 72° C:30 s. We confirmed clean amplification by agarose gel electrophoresis and purified the PCR products by Promega (Madison, Wisconsin, USA) Wizard SV Gel and PCR Cleanup System according to manufacturer's protocol. We submitted samples to the University of Massachusetts Amherst Genomics and Bioinformatics Facility for sequencing from the DL1 primer.

For the field samples, we performed each reaction in 10 µl volumes using the following protocol: 2 µl GoTaq 5X Buffer; 0.8 µl 1 mM dNTP Mix; 0.4 µl of mixed 10 µM primers THR and 651 (Shaffer and McKnight 1996); 0.08 µl GoTaq® Polymerase (Promega, Madison, Wisconsin, USA); 5.72 µl water, and 1 µl of DNA. We cycled each reaction on a BioRad T100 thermocycler (BioRad, Hercules, California, USA) as follows: initial denaturation at 94°C for 120 s, followed by 24 cycles of 94° C:60 s; 48° C:60 s; and 72° C:60 s. We used a ramp transition rate of 0.5° C/s for the first five cycles and did not ramp subsequent cycles. We held reactions for a final elongation step at 72° C for 600 s. To confirm amplification, we loaded PCR reactions into a 1% agarose gel, ran them at 100 V for 25 min in 1X TAE buffer, stained them with SYBR® Safe (Life Technologies, Carlsbad, California, USA), and visualized them under UV fluorescence. We removed unincorporated dNTPs and primers using ExoSapIT (Affymetrix, Santa Clara, California, USA). We conducted single-strand terminator sequencing reactions in 5 µl volumes using: 1 µl GoTaq 5X Buffer; 0.5 µl BigDye v3.1 (Life Technologies, Carlsbad, California, USA); 0.25 µl THR or 651 10 µM primer; 2.25 µl water; and 1.0 µl clean D-loop PCR product. We cycled reactions on a BioRad T100 thermocycler at 96°C:120 s, followed by 30 cycles of 96 °C:10 s; 50° C:0:05 s, and 60° C:240 s. We cleaned sequencing reactions with Sephadex size exclusion media (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA), suspended them in 15 µl HiDi formamide, and electrophoresed them on an ABI 3130 (Life Technologies, Carlsbad, California, USA) capillary sequencer to generate sequence reads.

Microsatellite amplification.—We generated two 10X primer mixes for microsatellite experiments. Mix 1 contained AjeD94, AjeD75, AjeD346, and AjeD422, and Mix 2 contained AjeD283 and AjeD378. Each primer was 2 µM concentration. We performed each multiplex PCR reaction in 10 µl volumes using 5 µl QIAGEN multiplex solution (QIAGEN, Hilden, Germany); 1 µl Q-solution; 1 µl 10X primer Mix; 1 µl water, and 2 µl of DNA. We cycled each reaction on a BioRad T100 thermocycler using the following conditions: initial denaturation at 95° C for 900 s, followed by 35 cycles of 94° C:30 s; 57° C (Mix1) or 58° C (Mix2):90 s; and 72° C:60 s, then a final elongation step at 72° C for 1,800 s. We diluted each microsatellite reaction five-fold and combined 1 µl diluted PCR product with 10.65 µl HiDi formamide and 0.35 µl GeneScan 500-LIZTM size standard (Life Technologies, Carlsbad, California, USA). We ran each reaction on an ABI 3500 automated sequencer to generate .fsa files for subsequent analysis.



Appendix Figure 1.—Egg density index estimates for Jefferson Salamander (*Ambystoma jeffersonianum*) and unisexual *Ambystoma* salamander egg masses measured across developmental stages, based on the most advanced embryo in each mass (Schreckenberg and Jacobson 1975) of 11 egg masses reared in the laboratory (a), and of 94 egg masses observed at 10 sites in the field (b), using site codes shown in Fig. 1.



Appendix Figure 2.—Histograms of embryo density index values in 82 egg masses observed in the field and for which a nuclear genotype was obtained from a single embryo. Genotypes are based on microsatellites of the parent genomes, L for the Blue-spotted Salamander (*Ambystoma laterale*), and J for the Jefferson Salamander (*A. jeffersonianum*). All but JJ individuals are members of the unisexual lineage. An egg mass from a unisexual lineage occasionally contains multiple genotypes (Bogart et al. 2007, 2009); consequently, the one embryo sampled from each such mass may not necessarily represent the parental genotype or all other genotypes in the mass.