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INFLUENCE OF ENVIRONMENTAL FEATURES ON SPERMATOPHORE PLACEMENT IN SPOTTED SALAMANDERS (AMBYSTOMA MACULATUM)

Megan A. Kuechle
Virginia Commonwealth University

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

Megan Kuechle
Master of Science Degree in Environmental Studies

Director: Rodney J. Dyer, Ph.D.
Director, Center for Environmental Studies

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ABSTRACT

Successful reproduction in salamanders is driven by behavioral, environmental, and temporal interactions among adults. While much of our understanding of salamander mating systems is based upon either courtship behavior of both sexes or aspects of female choice, the decisions made by males regarding where to place spermatophores is much less quantified. In this study, we mapped male spermatophore placement in the spotted salamander (*Ambystoma maculatum*) with respect to ecological and spatial locations within a vernal pool complex in Charles City County, Virginia. The overall goal was to use the spatial and ecological placement of spermatophores to determine if individuals deposit spermatophores randomly within the vernal pool or if males exhibited specific preferences for deposition. Using comprehensive surveys of the vernal pools and individual spermatophores within a $0.5m^2$ grid and terrestrial LIDAR, a total of 218 spermatophores were identified and mapped. We repeated these surveys for two successive breeding seasons. Placement occurred at an intermediate depth and an intermediate distance to the edge. Males also preferred to place spermatophores on leaf substrate as opposed to sticks or conifer needles. The physical placement of spermatophores

exhibited autocorrelation in space during single reproductive events as well as across breeding seasons. These results suggest that males actively select for specific locations within a pool for spermatophore placement-a proverbial Goldilocks zone-which may be consistent with increased reproductive success. This information is key to understanding salamander mating system parameters in this species and may contribute to developing more effective management strategies.

INTRODUCTION

Breeding behavior is a vital component of the life history of an organism. Species have evolved a vast array of behavioral strategies for maximizing reproductive success (Arak 1983; Emlen and Oring 1977; Wells 1977). One such behavior is decisions about oviposition, the placement of offspring, which has significant consequences for the fitness and reproductive success of individuals (Resetarits 1996). A large literature has shown direct consequences of variation in oviposition in critical processes such as predation rates (Egan and Paton 2004), egg desiccation (Figiel and Semlitsch 1995), freezing avoidance (Petranka and Petranka 1981), individual size (Crespi and Lessig 2004), and survival (Jackson et al. 1989). Despite this large literature focusing on female oviposition site choice, there are far fewer studies focusing on male behavior during mating, particularly on their placement of spermatophores (Arnold 1976; Blanchard 1930; Harris and Lucas 2002).

In salamanders, adults mate in vernal pools, temporary bodies of water formed by precipitation, and the larvae offspring inhabit the pools until they are ready to transition to land (Baldauf 1952; Blanchard 1930; Hustling 1965). During mating, males deposit several spermatophores within the vernal pool, each containing a collection of sperm in a small "cap" with a gelatinous base (Doyle et al. 2011). Females pick up these spermatophores to internally fertilize their eggs (Arnold 1976). Male reproductive success is thus dependant upon where spermatophores are placed within the physical environment. Unlike oviposition, which focuses on ensuring the

survival of eggs independent of the male, spermatophore placement is implicitly focused on attracting the female.

Pond-breeding amphibians face a number of threats including habitat loss, changes in water regimes due to climate change, pollution, and disease, all of which increase in intensity during the breeding season (Corn 2005; Gascon et al. 2005). Wetlands have seen significant declines with an estimated 53% of wetland habitat loss in the United States from the 1780s to the 1980s (Dahl 1990). The consequences of climate change are variable, some locales are predicted to become more desiccated with increased temperatures and increased drying out of vernal pools while other areas are predicted to be much wetter throughout the breeding seasons (Brooks 2004; Semlitsch and Wilbur 1988; Wake 2007). Since wetlands are vital to the life history of salamanders, the loss of wetland habitat results in the decline of amphibian populations (McMenamin et al. 2008; Morey 1998; Semlitsch and Wilbur 1988). Even within the vernal pools, salamander populations are subjected to increased vulnerability due to pollution and diseases such as the chytrid fungus (Fisher et al. 2009; Skerratt et al. 2007). While many salamander species spend the majority of the year underground in solitary burrows or under rocks and logs (Semlitsch 1983), during the breeding season adults gather in high densities which can facilitate the spread of chytrid fungus and infect larval offspring inhabiting the pool (Fisher et al. 2009; Grant et al. 2016).

Spermatophore Placement in the Spotted Salamander

This study examined the spermatophore placement in a vernal pool complex in Charles City, Virginia. The spotted salamander (*Ambystoma maculatum*) used herein is ubiquitous, has easily identified spermatophores, exhibits a temporally restricted mating season, and has a mating system broadly representative of other species in the *Ambystomatidae*. In this species, males arrive in the vernal pool before females (Arnold 1976). If a male finds a female, he will nudge her head to confirm her gender, then place his spermatophore nearby (Arnold 1976). Males have also been observed placing their spermatophore on top of another male's spermatophore to prevent that male from mating with the female. They do not guard females or act aggressively towards other males (Arnold 1976). Once the spermatophore is placed, he will waft pheromones with his tail towards the female to provide a chemical path to the spermatophore (Arnold 1976). On average, males deposit 40 spermatophores, with production varying due to the high energy requirements to produce them (Arnold 1976; Dewsbury 1982). Females pick up an average of 18 spermatophores, and the paternity of eggs is uneven with multiple spermatophore donors (Arnold 1976; Gopurenko et al. 2006). The majority of a female's eggs are fertilized by the first spermatophore she retrieves with reduced paternity for the second and remaining spermatophores (Tennessen and Zamudio 2003).

Spermatophore placement was examined in relation to the physical location, ecological features, and inter-spermatophore spatial proximity to determine if there is a subset of habitat preferred for male placement. The physical location of placement was quantified as the distance the spermatophore was placed from the edge of the pool, the depth of the pool at the deposition site, and the depth of the spermatophore. Distance to the edge of the pool may affect the likelihood

of a female picking up a spermatophore first, as a spermatophore placed close to the edge is more likely to be the first spermatophore encountered by a female as she arrives at the pool. However, males may not place spermatophores as close to the edge as possible. If the size of the pool decreases from evaporation, then the spermatophores placed too close to the edge may no longer be within the pool and will, therefore, dry out. Since depth is correlated with distance to the edge, males placing spermatophores within a certain distance from the edge are also selecting for a range of depth. Additionally, placing spermatophores in deeper regions also prevents drying out, but placement in a region that is too deep may be more difficult for the salamander to accomplish. With these factors combined, it is hypothesized that males are placing the spermatophores at intermediate depth and distance from the edge, an area referred to as a "Goldilocks zone" (Figure 1).

The ecological context was defined as the substrate upon which the male placed the spermatophore. Substrate type varies greatly depending on where the vernal pool is located, but *Ambystomatidae* generally uses forested vernal pools. These vernal pools are characterized by a mix of leaf and stick substrate type, with occasional seed pods depending on the composition of the surrounding forest. It is hypothesized that males will select a leaf type of substrate to ease spermatophore deposition, as a large flat surface provides more space and requires less precision than a stick. Leaf substrate may also confer a reproductive advantage, as it is also more likely to be at the bottom of the pool than stick substrate, and females may be more likely to encounter the spermatophore on a leaf if they are primarily crawling on the bottom of the pool.

Finally, spatial proximity is defined as non-random placement of spermatophores relative to existing spermatophores and can be clustered, random, or dispersed. Each of these landscape features may be influencing the likelihood of the pick up of a spermatophore by a female and thus males may strategically select them. It is hypothesized that spermatophores will be clustered together, rather than placed randomly with respect to other spermatophores or dispersed evenly throughout the pool. Clustering may increase the chance that a female will select the spermatophore, as a female that just picked up a spermatophore may choose to pick up any nearby spermatophores rather than travel throughout the pool in search of more spermatophores.

Spermatophore placement may be impacted by the density of spermatophores currently present within the pool. Males cannot place a spermatophore in a location already used by other males and will turn to other, possibly less suitable habitat for placement. The density of spermatophores also changes throughout the breeding season as the number of salamanders present varies for each wave, so we may be able to see this behavior by comparing placement amongst the waves. This behavior pattern is referred to as the ideal free distribution model (Tregenza 1995). The ideal free distribution model has a number of assumptions, which include the ability of the organism to assess the ideal habitat and access all available habitat. Spermatophore placement behavior meets these assumptions, as males can travel throughout the pool and place their spermatophores in ideal areas of the pool. By examining the physical, ecological, and spatial processes impacting male spermatophore placement, we will gain an

understanding of male breeding behavior and determine how spermatophore positioning influences the life history of salamanders.

MATERIALS AND METHODS

Study Site and Focal Species

Sampling occurred at a vernal pool complex in Charles City County, Virginia during the 2018 and 2019 breeding seasons. The target pool is part of a larger collection of intermittently connected pools that are classified as perched pools—formed entirely by precipitation and not groundwater due to an impermeable layer of soil (Corn 2005). The target pool had an area of 57.45 m^2 and a perimeter of 30.64 m in 2018, but the size varies depending on the amount of rainfall. This site is within the coastal Piedmont region of Virginia which is characterized by secondary mixed conifer and hardwood forests. Surrounding the vernal pool complex are stands of loblolly pine (*Pinus taeda*) with some sweetgum (*Liquidambar styraciflua*) and American holly trees (*Ilex opaca*) present in the understory. There is minimal herbaceous vegetation, and the soil is predominantly a fine clay.

A previous genetic survey of marbled salamanders (*Ambystoma opacum*) found both marbled and spotted salamanders visited this complex annually for mating (Crouch 2008). Spotted salamanders were preferred because marbled salamanders use the pond when it is dry and spermatophores can be difficult to detect. The spotted salamander breeding season occurs annually from January to March, with precise timing dependant on the amount of precipitation and when the vernal pools fill with water (Baldauf 1952; Blanchard 1930). Adults breed in several waves during the entire breeding season, arriving at the pools following an evening rain with relatively high temperatures (Arnold 1976; Woodley and Porter 2015). Males are more

likely to begin migration at lower temperatures and typically arrive a day or two before females (Sexton et al. 1990). Mating waves last around 2-3 days, and some females may inhabit the pool at the same time as the males depending on the timing of the individual (Talentino & Landre 1991). The first wave in late winter consists of 26% of the population (mainly younger, inexperienced males), while the final wave in early spring consists of the majority of the population (Sexton et al. 1990). The sex ratio in spotted salamanders is male biased with a range of 1.6-3.5 males per female (Hillis 1977; Whitford & Vinegar 1966).

Characterization of Pool Features

To map the pool and its edge, we used a terrestrial LIDAR (Light Detection And Ranging) scan (NOAA 2018). LIDAR is a surveying method that uses a pulsing laser to create a 3-D image or point cloud of an object or a landscape (NOAA 2018). This facilitated the precise mapping of spermatophore locations and our pool itself that would be unattainable with traditional GPS methods that cannot map on this small of a scale. The LIDAR laser does not penetrate the surface of water, which made the edge of the pool easily mappable. In 2019, high levels of precipitation increased the area of each pool in this complex, coalescing several previously separated pools and making it impossible to accurately map the edge of the pool to measure distance to the edge for 2019. A second LIDAR scan in the dry season allowed the quantification of the depth for the entire pool and provided a characterization of the microtopography that was previously submerged. This elevation of the base of the pool could then be compared to the elevation of the water level in 2018 at 400 randomly selected points to obtain depth measurements for the entire pool in 2018. For 2019, since the water level were

higher, we were unable to complete another LIDAR scan. However, we could estimate the depth of the pool in 2019 by comparing depth at several locations where we knew the depth for both years. The average difference in depth for these locations was 0.144m. This value was added to the base elevation for the pool in 2018 and then a new set of 400 points were randomly sampled based on the estimated boundary of the pool in 2019 to capture all available variance.

To determine if placement of spermatophores deviated from random allocation, we first characterized the composition and granularity of the pool environment. A systematic sampling of the pool determined the availability of each substrate type to establish an expected value for each if spermatophore placement was random. This sampling consisted of recording substrate type in the middle, top left, and bottom right corners of a ~0.5m^2 square as part of a grid system and resulted in a total of 543 measurements. The LIDAR map of the pool was used to measure distance to the edge for both observed spermatophores as well as to develop a null model of distances to the edge for 400 points randomly generated within the pool. These measurements provided the background environmental heterogeneity we used to test preferential placement.

Sampling

Spermatophore sampling took place during the breeding seasons of 2018 and 2019 from January to March. Initiation of migration varied across years in response to precipitation and temperature. In 2018, the first wave occurred in late February, whereas in 2019, males first arrived at the pools in early January (Figure 2; NOAA 2019). The pools in 2019 were already full due to an exceptionally wet winter prior to the start of the breeding season, which is why

breeding began earlier. Migration occurred across two waves in 2018 and three waves in 2019, the difference likely due to the late start of migration in 2018. Sampling took place the day after a rainy, warm night when migration was expected to have occurred. Temperatures remained above freezing during sampling and weather varied from a light rain to clear skies.

Sampling within the pool was standardized using a grid of ~0.5m^2 squares formed by PVC pipe frames. Using these frames to divide up the pool, a total of 177 and ~270 squares were sampled during each wave in 2018 and 2019 respectively. The increase in number of grids observed in 2019 was due to the increased size of the pool. In grids with more than 5 spermatophores, we randomly selected 5 spermatophores and measured data for each, but still recorded the total number of spermatophores observed to determine the total number of spermatophores placed within the pool. In instances of stacked spermatophores, only the top spermatophore was sampled.

For each spermatophore sampled, we recorded substrate type and two measurements of depth—the total depth of the pool at the location of the spermatophore and the depth of the spermatophore itself. The two measurements may differ if salamanders do not place their spermatophores directly on the bottom of the pool. Salamanders may also be selecting for a particular depth regardless of the total depth and place spermatophores on substrate closer to the surface. Flags placed at the location of each spermatophore allowed us to later map them using LIDAR. In the LIDAR point cloud, flags clearly stood out since the LIDAR laser does not penetrate through water. In ArcGIS Pro, points were placed at the location of each flag and thus

each spermatophore within the pool. A hand-drawn map of the pool helped connect each point with the sample number of the spermatophore collected.

To quantify the probability of detection, we completed a mock run of spermatophore collection at another pool in the complex. Utilizing the same grid system, the presence or absence of spermatophores was measured with two observers. This was necessary to ensure low detection in deeper parts of the pool was not a result of detection failure. The resulting detection probability was found to be 82%.

Analysis

To examine the impact of physical location, we analyzed distance to the edge of the pool and the depth of the pool. With these features, we expected placement at an intermediate distance and depth, which defines a Goldilocks zone. The distance to the edge for the 400 randomly selected points represented distance to the edge for the entire pool. A permutation test resampled distance for the randomly generated points 500 times to create a distribution of mean and variance in both distance and depth representing our null hypothesis of random placement with respect to the existing heterogeneity within the pool. A one-tailed t-test determined if variance in distance for 2018 spermatophores was significantly less than the distribution of variance for the random points. This quantified the difference in the range of distance for the spermatophores and the random points as having less variance means the range of values is narrower. Another one-tailed t-test determined the difference for the means for spermatophores and random points. However, the alternative hypothesis for the mean t-test was that the

spermatophore mean would be greater than the distribution of random means as the range of values for the random points was left-skewed. In order to examine depth, we compared total depth for spermatophores to the depth for the entire pool as determined by the LIDAR scans. Unlike distance to the edge, we were able to measure depth for the entire pool in 2018 and 2019 by estimating the depth in 2019. However, we still used a permutation test and a one-tailed t-test to compare variance and the mean of depth for the spermatophores to the distribution of variance and the mean for the entire pool. For both distance and depth, we chose to measure the difference in variance and the mean because a significant difference in variance would be the result of placement within a smaller range of what is actually available, and any difference in the mean would show whether the spermatophores are placed at an intermediate range or at another range. We also performed a Pearson's correlation test to determine the relationship between distance to the edge and depth, with the expectation that the two variables would be strongly correlated.

For preference of spermatophore placement on particular substrate types, we compared observed substrate preference to the measured substrate types in the pool using a Chi-squared test. Expected substrate use was determined using the recorded proportions of each substrate type available in the pool during the systematic sampling. Observed substrate use was recorded for spermatophores in both 2018 and 2019.

To examine the impact of spatial proximity, we used a global Moran's Index test. This test measured spatial autocorrelation of the spermatophores in both 2018 and 2019. A two-tailed t-test determined significance, where a z-score greater than 1.95 meant the spermatophores were clustered, a negative z-score less than -1.95 meant the spermatophores were dispersed or placed in avoidance, and any value in between -1.95 to 1.95 meant placement was random with respect to placement of other spermatophores. It was hypothesized that spermatophores would be clustered, or that we would find a positive z-score greater than 1.95.

RESULTS

We sampled 109 spermatophores during each breeding season (Figure 3) of a total of 188 and 253 spermatophores observed in 2018 and 2019 respectively. Spermatophores collected increased as the breeding season continued with 93 and 83 of the 109 spermatophores collected during the final wave of 2018 and 2019 respectively. Several grid squares had over 10 spermatophores, with the maximum number of spermatophores in a square being 30 in 2019. The highest density of spermatophores was found in the northwest corner of the pool during both 2018 and 2019. Spermatophore locations did not vary much between breeding seasons, but a slight shift of placement further outward during the 2019 season is likely due to the increased size of the vernal pool during that year.

To test our Goldilocks zone hypothesis, we examined variance and the mean for distance to the edge and depth of the pool. Distance to the edge of the pool ranged from 0.007-2.452m in 2018 with no measurements done in 2019 due to the lack of a clear edge to the pool (Figure 4). Variance for the spermatophores was 0.358, which was significantly different from a permutation of variance for the random points (random var = 0.656, p-value < 0.0001). This significantly lower variance in distance suggests males selected for specific distances relative to the edge of the pool. The mean distance to the edge was 1.311m, which was also significantly different from the distribution of distance to the edge for the randomly selected points (random mean = 1.150m, p-value= 0.048). The mean distance for spermatophores was significantly

higher, which suggests that placement was at an intermediate distance from the edge. The mean for random placement has a left skewed distribution due to the increase in potential placement area as one gets closer to the edge, and so we expect it would be lower than the mean spermatophore distance under intermediate placement. Additionally, if placement had occurred at the center of the pool, then the mean would have been much higher, and if placement occurred near the edge then the mean would not be significantly different than the mean under placement that is random with respect to the available heterogeneity.

Spermatophore depth ranged from 0.051-0.178m in 2018 and increased to 0.076-0.343m in 2019 due to the high amount of precipitation (Figure 5). The mean spermatophore depth nearly doubled, from 0.129m in 2018 to 0.247m in 2019. Total depth was slightly higher than spermatophore depth, as not every spermatophore was placed on the bottom. Total depth ranged from 0.114-0.229m with a mean of 0.170m in 2018 and again increased to 0.152-0.381m with a mean of 0.292m in 2019 (Figure 6). Variance in 2018 and 2019 for the spermatophore depth differed significantly, with a variance of 0.00054 and 0.00267 respectively which was lower than the variance for the entire pool (2018 var = 0.00373, 2019 var = 0.01158; p-value < 0.0001 for both 2018 and 2019). A difference in variance suggests that the salamanders were selecting for a specific depths and that placement was not random with respect to the existing heterogeneity. The mean depths of 0.170m for 2018 and 0.292m for 2019 were also significantly different than the random means (random mean 2018 = 0.242m, 2019= 0.341m; 2018 p-value < 0.001, 2019 p-value = 0.002; Figure 7, Figure 8). This suggests intermediate placement because the range of depth for the pool is skewed to the right with most values of

depth being rather deep on average. The average for the spermatophores was significantly lower than this but not close to zero, suggesting a preference for intermediate placement.

We also compared depth and distance to the edge together for each spermatophore collected and each random point sampled (Figure 9). In Figure 9, we see an increase in depth as distance to the edge increases. A Pearson's correlation test confirmed that distance to the edge and depth had a strong positive correlation (cor = 0.430, df- 102, $P<0.001$). The range of spermatophores is clustered in the middle of both axes, suggesting selection for an intermediate depth and distance to the edge.

Our ecological feature, substrate type, consisted of four types: broadleaves, conifer needles, sweetgum seed pods, and sticks. In 2018, eight of the collected spermatophores found free-floating had to be excluded from the analysis since it was not clear which substrate upon which they were originally placed. Substrate type varied across each breeding season, with no spermatophores placed on sweetgum seedpods during the 2019 season. Both 2018 and 2019 substrate use differed significantly from the expected substrate use ($\chi^2 = 31.6796$, df = 3, p-value < 0.0001, $\chi^2=15.5537$, df =2, p-value = 0.0004; for 2018 and 2019 respectively, Table 1). These results also suggest a preference for broadleaf substrate, which made up the largest proportion of the spermatophore substrate even though it was also the most common substrate type in the pool.

To examine spatial proximity, we calculated spatial autocorrelation for each year. Spermatophore placement initially appeared clustered together once mapped. The global Moran's Index test revealed significant spatial autocorrelation and clustering for both breeding seasons (Z-score = 23.9521, p-value = 0.0016, Z-score 9.1051, p-value <0.0001; for 2018 and 2019 respectively, Table 2). For each test, the ArcGIS pro tool calculated a distance threshold which was 0.680m for 2018 and 0.833m for 2019. A positive Z-score over 1.95 suggests that spermatophores were clustered and not randomly placed or dispersed with respect to the other spermatophores.

Given the large cluster in the Northwest corner of the pond (Figure 3), we also examined the impact of the immediate surroundings of the pool to determine if slope or flow accumulation had any impact on where spermatophores were placed. We used ArcGIS to determine the slope and elevation for the surrounding area and then performed a flow accumulation analysis to identify any drainages the males may be using to access the pool. Elevation in the perimeter surrounding the pool did not vary much (Figure 10), and the flow accumulation showed a small drainage in the southwest corner of the pool (Figure 11). Therefore, the clustering in the Northwest corner is not a result of a drainage or difference in elevation.

DISCUSSION

The objective of this study was to examine how physical, ecological, and spatial proximity features impact placement of spermatophores by male spotted salamanders. Our results suggest that males select for particular locations within the pool that meet a set of criteria. Spermatophore placement is not random and males strategically place their spermatophores within the vernal pool, which may increase reproductive success.

When examining the physical location associated with placement, the salamanders placed spermatophores at an intermediate distance to the edge and at an intermediate depth in the vernal pool, as predicted by our Goldilocks zone hypothesis. Distance to the edge was significantly different to random placement according to the permutation test for both variance and the mean. The significantly different variance means that the spermatophores were placed within a subset of the available range of distance, and the significantly higher mean suggests intermediate placement. Random placement had a left skew and a smaller mean, but the difference was not so great as to suggest placement within the area furthest from the edge. We also found a significant difference in variance in depth for both years, again suggesting a selection for a subset of the full range of depth available. Mean depth was also significantly different, with spermatophore depth being lower on average than the range in the pool itself for both years. A lower mean depth suggests a preference for intermediate placement, since the range of depth for the random points skewed right and the average was higher than it would be if placement was normally distributed and the mean placement was intermediate. Distance to

the edge and depth are strongly correlated, and thus we cannot determine the degree to which each feature individually influences placement. However, since distance to the edge impacts the time of encounter for a female, it is likely that males are selecting for distance to the edge. Depth is likely less important, as placement in 2019 still occurred in a similar location despite depth changing drastically between the two years.

Placement within the Goldilocks zone likely increases the chance that females will encounter the spermatophore first upon arriving at the pool but reduces the chance of drying out when placed in shallow areas. Spermatophores encountered and picked up first by females will likely fertilize more eggs, resulting in increased reproductive success for males who place their spermatophores within the Goldilocks zone (Tennessen and Zamudio 2003). There is a similar phenomenon in egg placement of marbled salamanders, where females place egg masses in low to intermediate areas before the pools fill up with water (Croshaw and Scott 2006). This prevents eggs from hatching too early before water levels are high enough or too late reducing larvae survivorship (Croshaw and Scott 2006).

The impact of the Goldilocks zone on placement likely decreases with increasing density, as preferred locations are taken by other males. This pattern follows the ideal distribution model, where males began to use less-than ideal habitat as the Goldilocks zone became saturated. Salamanders within the first few waves were able to use the ideal habitat since the density of males was low, but those in the final wave had to use other habitat as the density became higher. Placement during the final wave began to stray away from the Goldilocks zone.

Our results suggest that our ecological feature, substrate type, also influences spermatophore placement. Spermatophore substrate type was significantly different from the available proportions for both 2018 and 2019. Additionally, the majority of spermatophores were placed on the broadleaf substrate. This preference for leaves due to their presence at the bottom of the pool, as sticks were more likely to be higher up in the water column. Leaves also provide a broad, flat surface which could make it easier to place and cement the spermatophore.

Spatial proximity was also found to be significant, with a Global Moran's Index test revealing that males clustered their spermatophores or showed spatial autocorrelation. Clustering of spermatophores has been found in other studies, as males place spermatophores near any present females in the pool during breeding, and will place their spermatophores on top of another male's spermatophore (Arnold 1976). This autocorrelation may also increase the odds of the spermatophore being picked up, since the females are less likely to encounter a lone spermatophore. Other studies have also found similar levels of temporal autocorrelation, which is likely due to salamanders entering the vernal pool from the same location and orientation each year (Arnold 1976; Phillips and Sexton 1989). Our ecological and spatial proximity results suggest the importance of substrate type and presence of other spermatophores in spermatophore placement, and that salamanders are not only considering the physical features of potential locations.

There is little information available about spermatophore placement, and so there are still a number of questions to be addressed regarding placement behavior. The first of which is to

determine if this prime spermatophore placement translates to reproductive success. It is possible that males are selecting for these environmental features but there is no resulting difference in reproductive success. This could be due to selection of the spermatophores by females based on a currently unknown criteria, such as size and quality of the spermatophore itself. Future studies will also want to examine the individual preferences of male salamanders. Each individual may only place their spermatophores in a certain area of the pool to increase their odds with one female, or may place them randomly throughout to increase their odds of siring eggs from different clutches. Additionally, they may also specialize or generalize, such as only placing spermatophores on sticks. These individual strategies are not apparent in this study of population behavior. Placement may also be influenced by a number of other factors, including factors that influence oviposition such as UV exposure and predation (Egan and Paton 2004). This study will also need to be replicated in different types of breeding pools used by salamanders. Woodward (1982) found that female egg laying behavior differed between females using permanent and temporary pools, so male spermatophore behavior may change as well. This pool did not have any exposed soil, so preference for placement on a leaf may change depending on the substrate available, as not all vernal pools are within forests. The presence of other salamander species may also impact spermatophore placement such as segregating by species, and other salamander species may have different strategies for placement depending on differences in their breeding behavior such as timing of breeding migration or courtship during mating. There are still a number of additional factors and questions that need to be addressed to complete our understanding of spermatophore placement behavior.

The future of salamanders and other amphibians faces a number of challenges, the most significant of which is climate change. Climate change is predicted to cause reductions in home ranges, survivorship, and reproductive ability (Corn 2005). Vernal pools are also threatened by climate change, as more episodic rainfall and higher temperatures will cause vernal pools to decrease in size and remain dry for longer periods of time. Increased drying periods will impact the timing of breeding migration and may decrease survivorship of larvae (Brooks 2004). Decreased vernal pool sizes may also impact spermatophore placement since males place spermatophores at similar locations each year. If the vernal pool decreases in size, the Goldilocks zone will shrink as well, meaning males will have to compete more for spermatophore placement and may not be able to successfully mate at all. This may then impact the population with decreased reproduction and genetic diversity, however as egg mortality does not strongly impact amphibian decline, any decline may be minor (Vonesh and De la Cruz 2002). Land managers and conservationists will want to examine their wetland habitat to identify the goldilocks zone and prevent disturbance like trampling through the pool when possible. They will need to monitor the pools each year and ensure the goldilocks zone remains at a sufficient size and depth. Spermatophore placement should also be considered when modifying or creating a wetland habitat. A number of man-made wetlands are created in order to replace those lost to human development, and these wetlands may not be optimized for spermatophore placement. This will become especially important for endangered or threatened species, where maximizing reproductive success is key. Species like the endangered California tiger salamander (*Ambystoma californiense*) have a similar method of breeding and our study of

spermatophore placement could be applied for them as well (U.S. Fish and Wildlife Service 2005; Loredo and Van Vuren 1996).

Spermatophore placement is an often-overlooked component of breeding behavior. The results of this study suggest that spermatophore placement is dependent on the conditions at the breeding pool. We found that males strategically place their spermatophore within an intermediate zone on leaf substrate and near other spermatophores. This behavior likely increases the reproductive success of the male and the likelihood that their spermatophore is picked up by a female. Applying this knowledge to management practices is vital to conserving spotted salamanders and other salamander species. Spermatophore placement is a critical component of salamander breeding behavior that we need to further examine in order to better study salamander populations and their dynamics.

LITERATURE CITED

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TABLES

Table 1. Substrate type for each sampling year and Chi-squared test results. Eight spermatophores in 2018 were free-floating and do not have an associated substrate. Systematic sampling of the pool determined the proportion of each substrate available which would be the expected use under random placement. 2018 and 2019 results were then compared to the expected proportions in a Chi-squared test.

	2018	2019	Expected
Broad leaf	88	86	326
Conifer Needle	7	11	127
Stick	4	12	78
Sweetgum seed pod	2	0	12
Total	101	109	543
Degrees of Freedom	3	2	
χ^2 value	31.6796	15.5537	
P-value	<0.0001	0.0004	

Table 2. Spatial autocorrelation in 2018 and 2019. Precise locations of spermatophores were measured in 2018 using flagging and a LIDAR image of the vernal pool. 2019 locations were estimated using a grid system during sampling. Spatial autocorrelation was calculated using a Global Moran's Index test in ArcGIS Pro.

	2018	2019
Moran's Index	0.9574	0.3678
Variance	0.0016	0.0017
Z-score	23.9521	9.1051
P-value	<0.0001	<0.0001
Distance Threshold	0.680m	0.833m

FIGURES

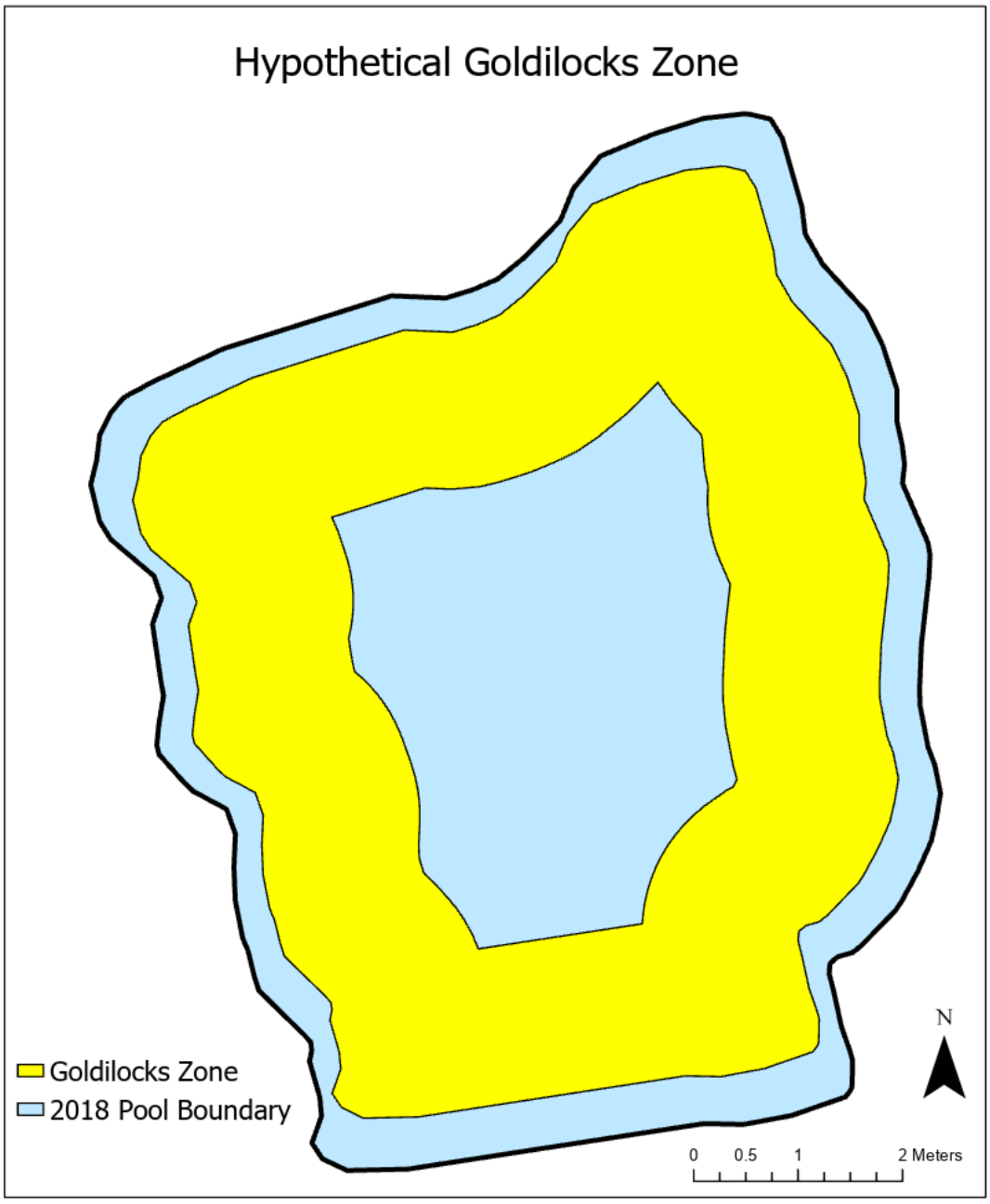

Figure 1. Illustration of the hypothetical Goldilocks zone. The zone begins about 0.5 meters from the edge of the vernal pool and is one meter thick. The boundary is the perimeter of the study pool as mapped using LIDAR during the 2018 breeding season. This region is hypothesized to be the optimal location for spermatophore placement, where depth and distance to the edge of the pool are intermediate.

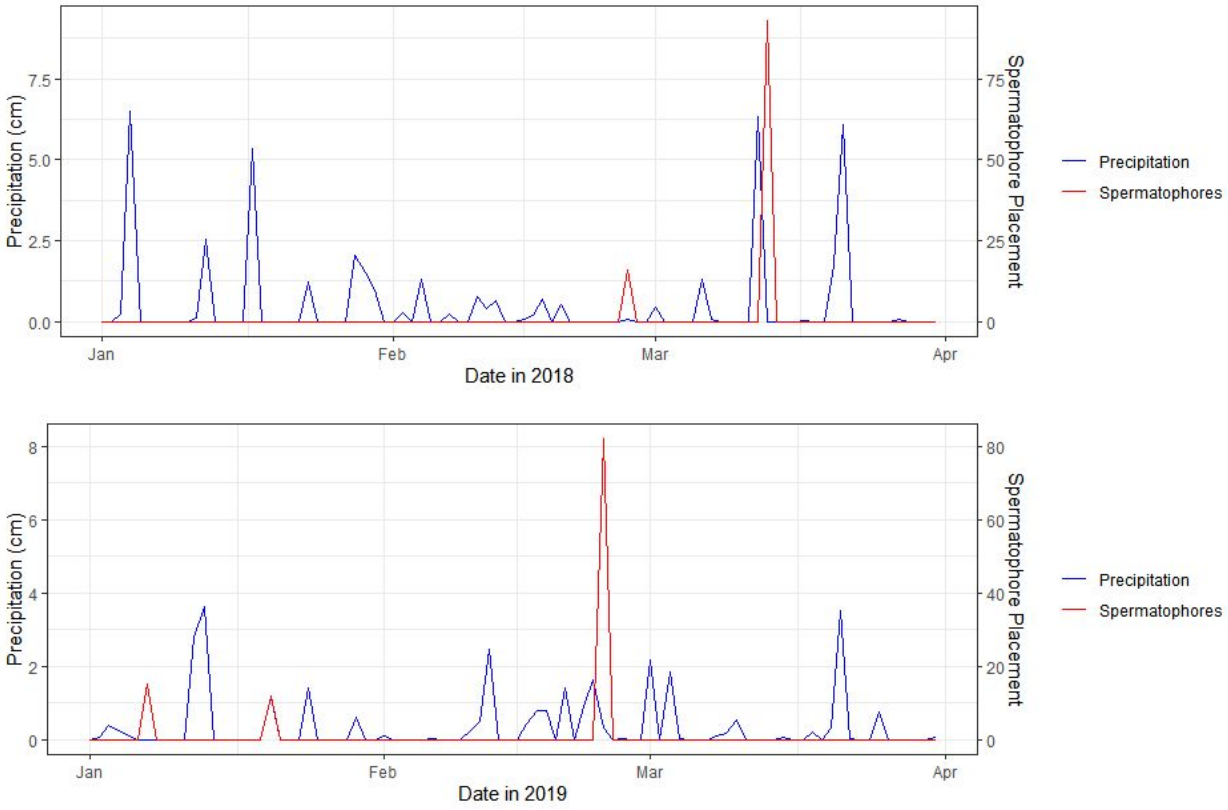

Figure 2. Precipitation rate and spermatophore placement throughout the 2018 and 2019 breeding seasons. Precipitation values were obtained from the NOAA Climate Database. Spermatophores were placed during 2 waves in 2018 and 3 waves in 2019. Males arrive and place spermatophores during or immediately following an evening rain.

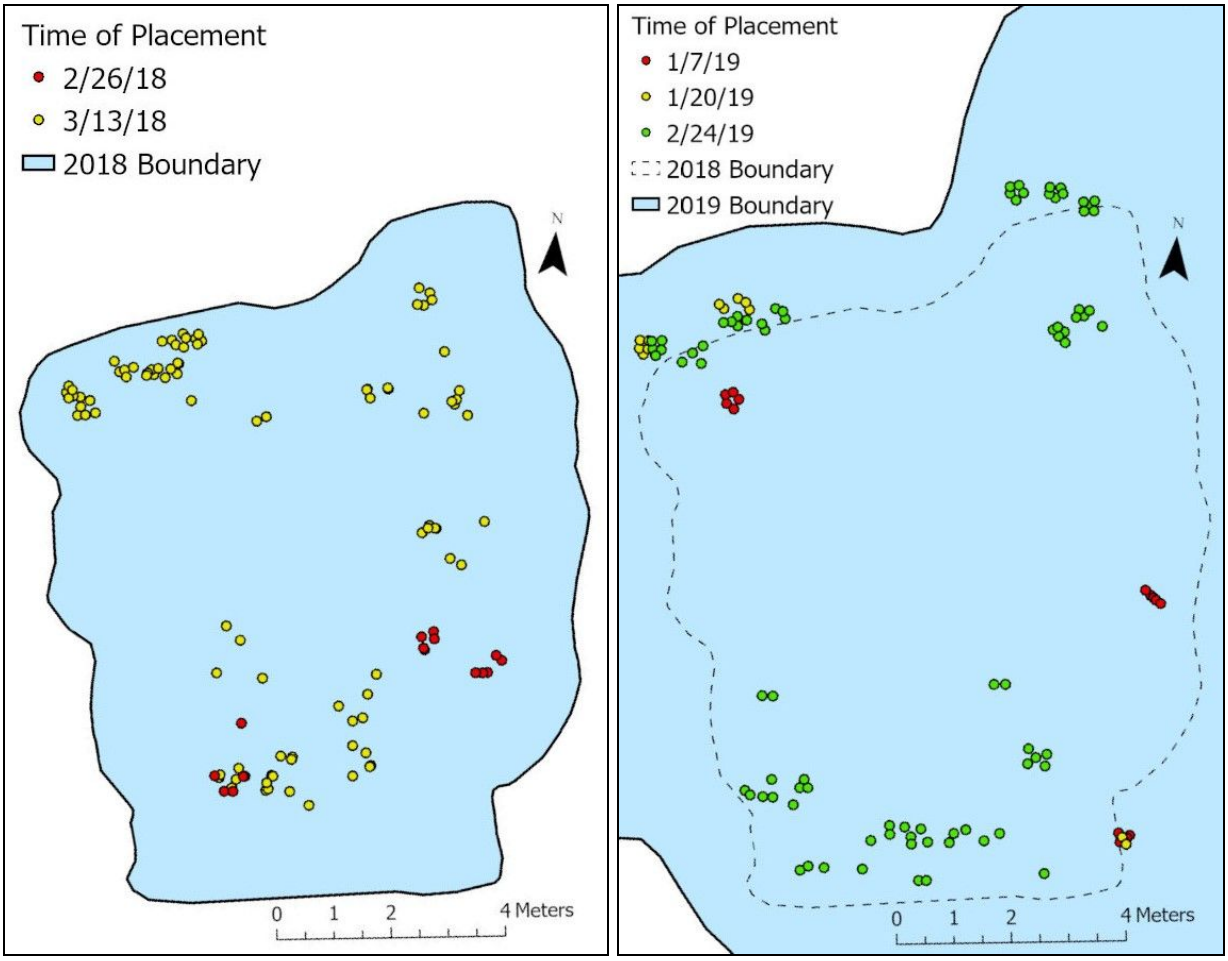

Figure 3. Location of each spermatophore sampled during the 2018 and 2019 breeding season. Sampling of the vernal pool involved a ~0.5 m^2 grid where up to 5 spermatophores were sampled. Spermatophores were collected during multiple days throughout the season as salamanders breed in several waves, with the majority of salamanders breeding during the final wave. We mapped 2018 locations using LIDAR and approximated 2019 locations using the grid system and a hand-drawn map of spermatophore locations within the grid. The 2018 boundary is the perimeter of the study pool as mapped using LIDAR. The 2019 boundary is the estimated perimeter of the pool as observed. The boundary extends past the map frame as the high amount of precipitation resulted in a large pool with no clear edge.

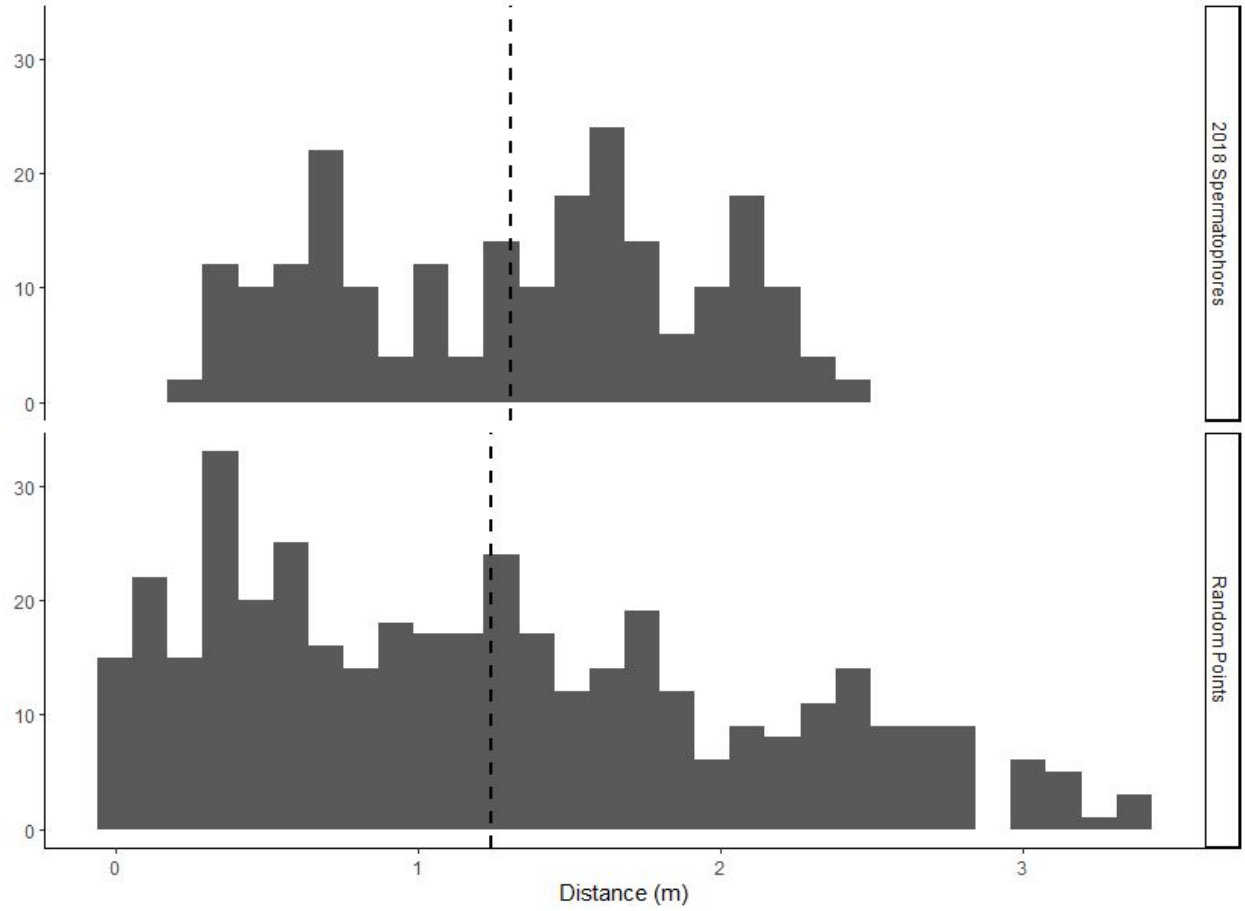

Figure 4. Distribution of distance to the edge (m) for 2018 spermatophores and random points. The dashed lines represents the means of 1.311m and 1.150m for the 2018 spermatophores and random points respectively. We mapped 109 spermatophore locations and the boundary of the pool using flagging and LIDAR. Distance to the edge was calculated using the Near tool in ArcGIS Pro to measure euclidean distance to the perimeter of the pool. 400 points were randomly selected within the boundary of the pool to establish available distance to the edge for the entire pool.

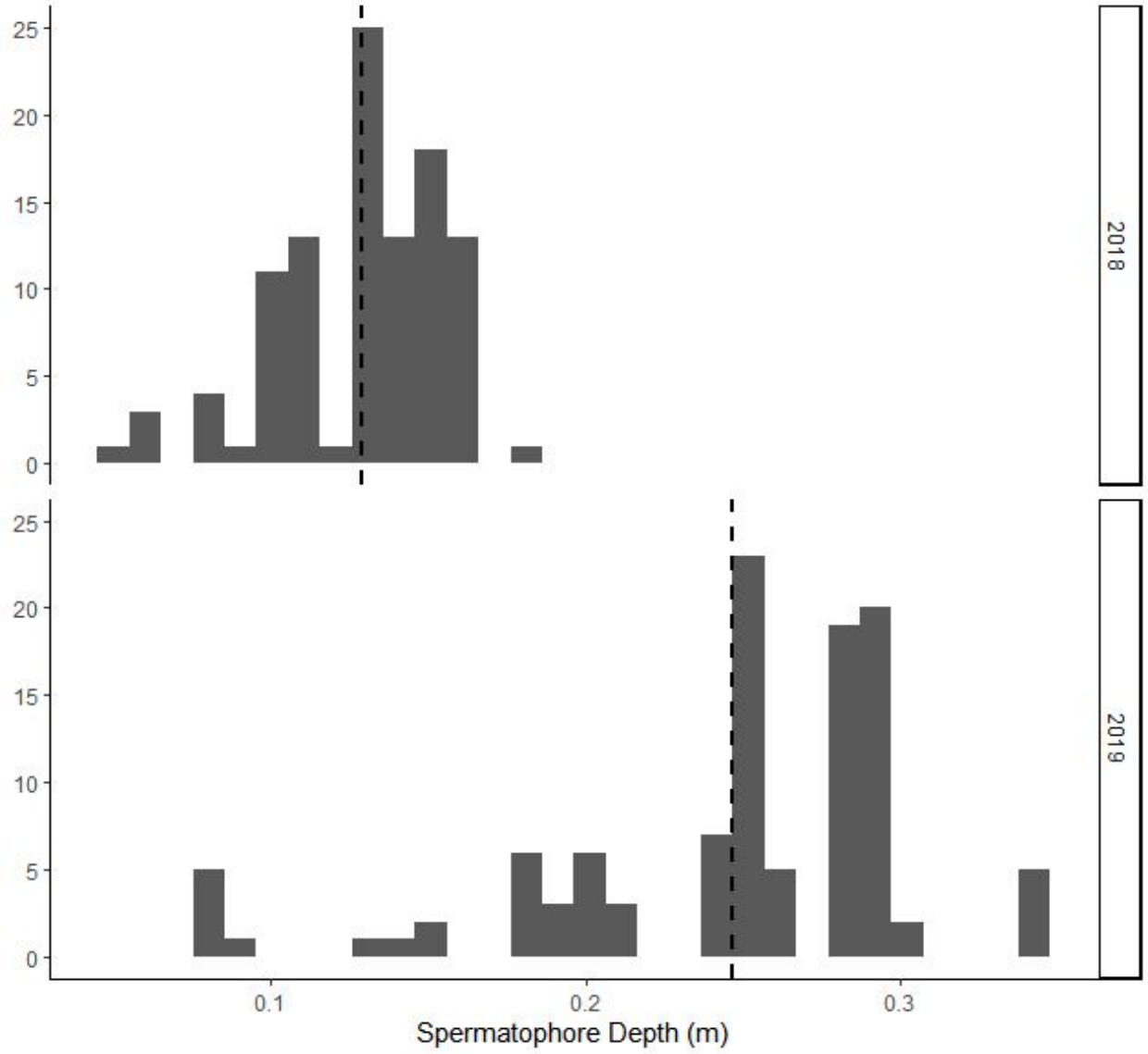

Figure 5. Distribution of depth of the spermatophore itself (m) during the 2018 and 2019 breeding seasons. Dashed lines represent the means of 0.129 m for 2018 and 0.247 m for 2019. We sampled 109 spermatophores in 2018 and 2019. Depth was measured manually while sampling the spermatophores, but depth could not be determined for 5 spermatophores in 2018.

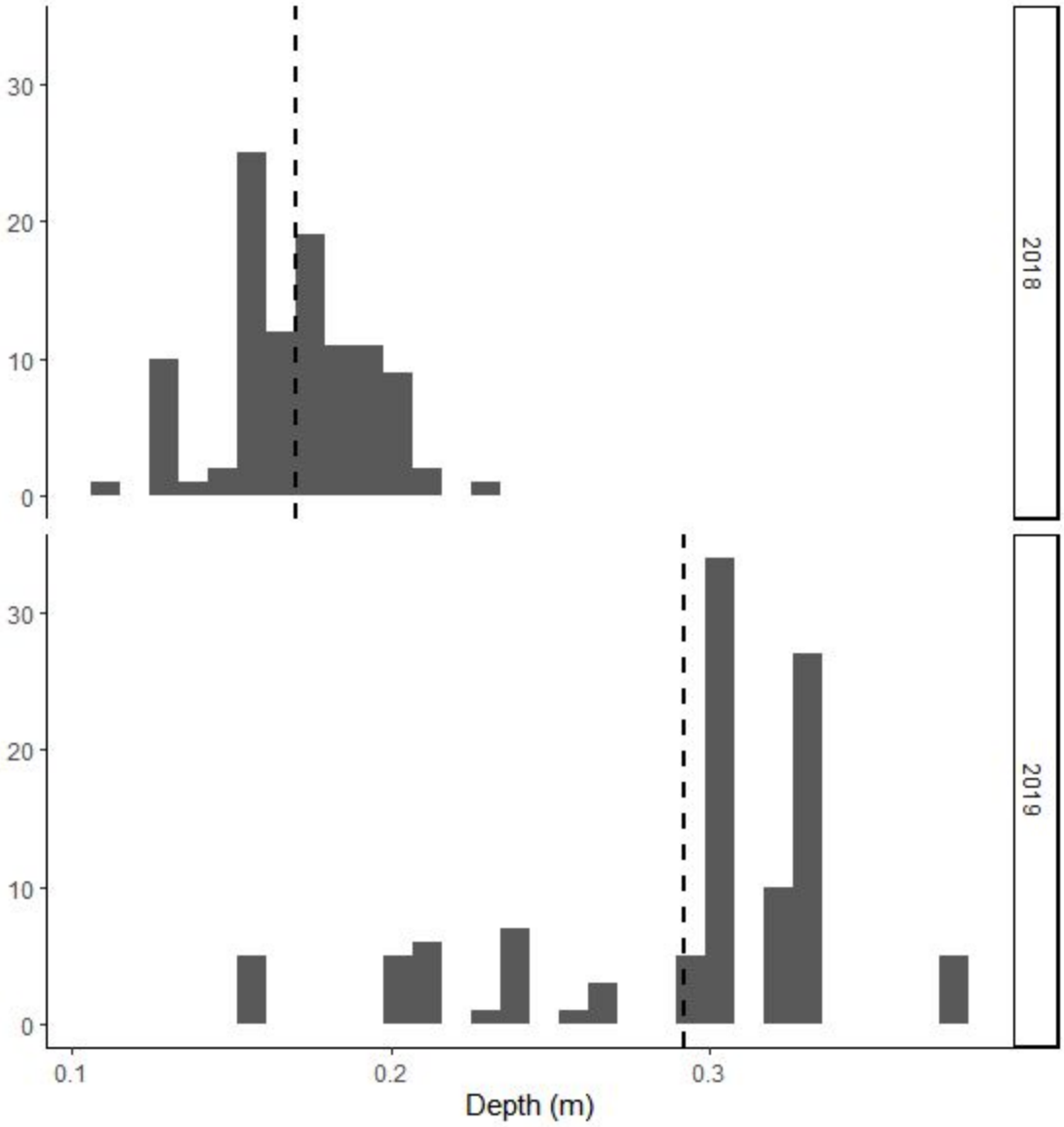

Figure 6. Distribution of the total depth of the pool (m) at the location of each spermatophore sampled during 2018 and 2019. Dashed lines represent the means of 0.170 m for 2018 and 0.292 m for 2019. We sampled 109 spermatophores in 2018 and 2019. Depth was measured manually while sampling the spermatophores, but depth could not be determined for 5 spermatophores in 2018.

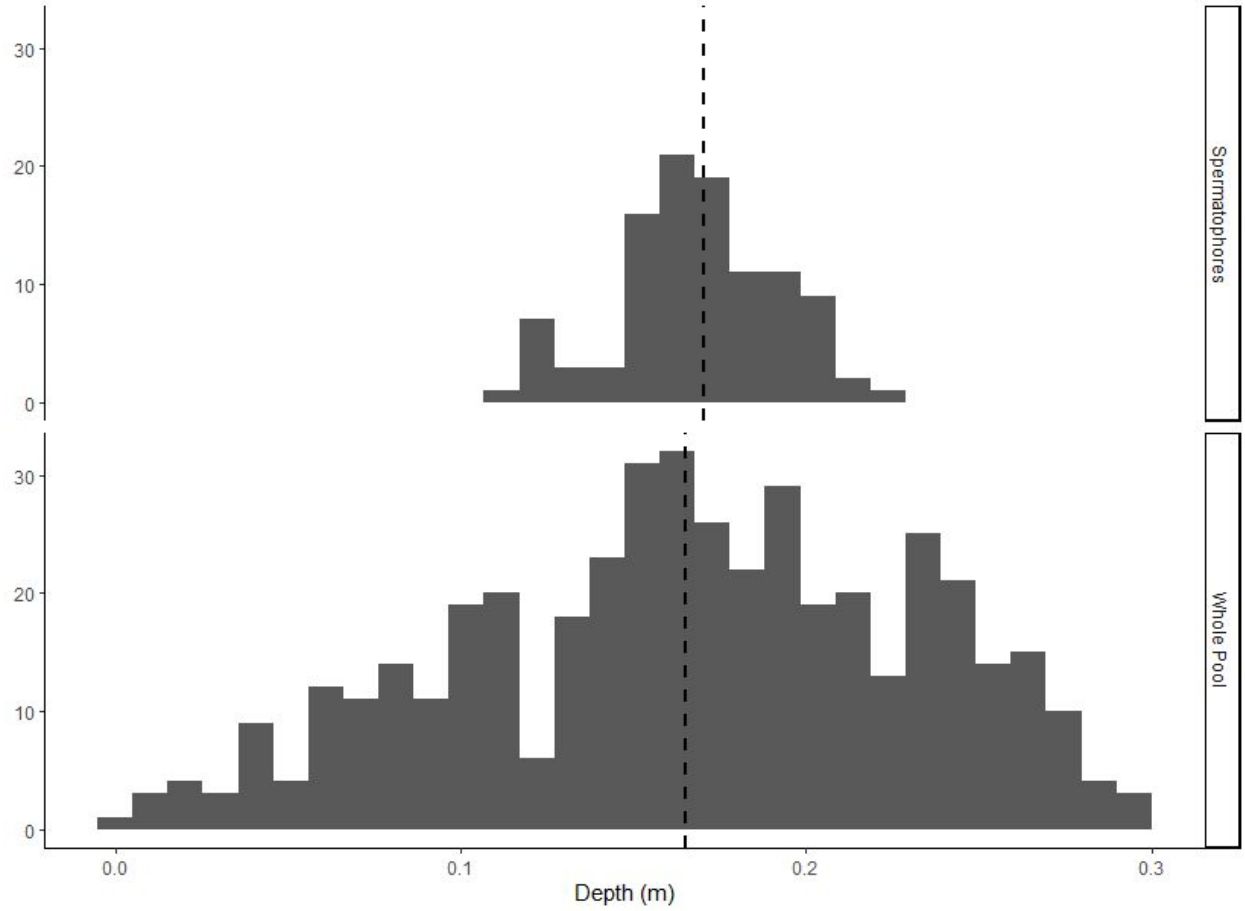

Figure 7. Distribution of depth for spermatophores and for the pool in 2018. Dashed lines represent the means of 0.170 m for 2018 and 0.165 m for the whole pool. We sampled 109 spermatophores in 2018. Depth was measured manually while sampling the spermatophores, but depth could not be determined for 5 spermatophores. To determine depth for the entire pool, we measured the difference in elevation between the base of the pool in a LIDAR scan and the water level as recorded in another LIDAR scan.

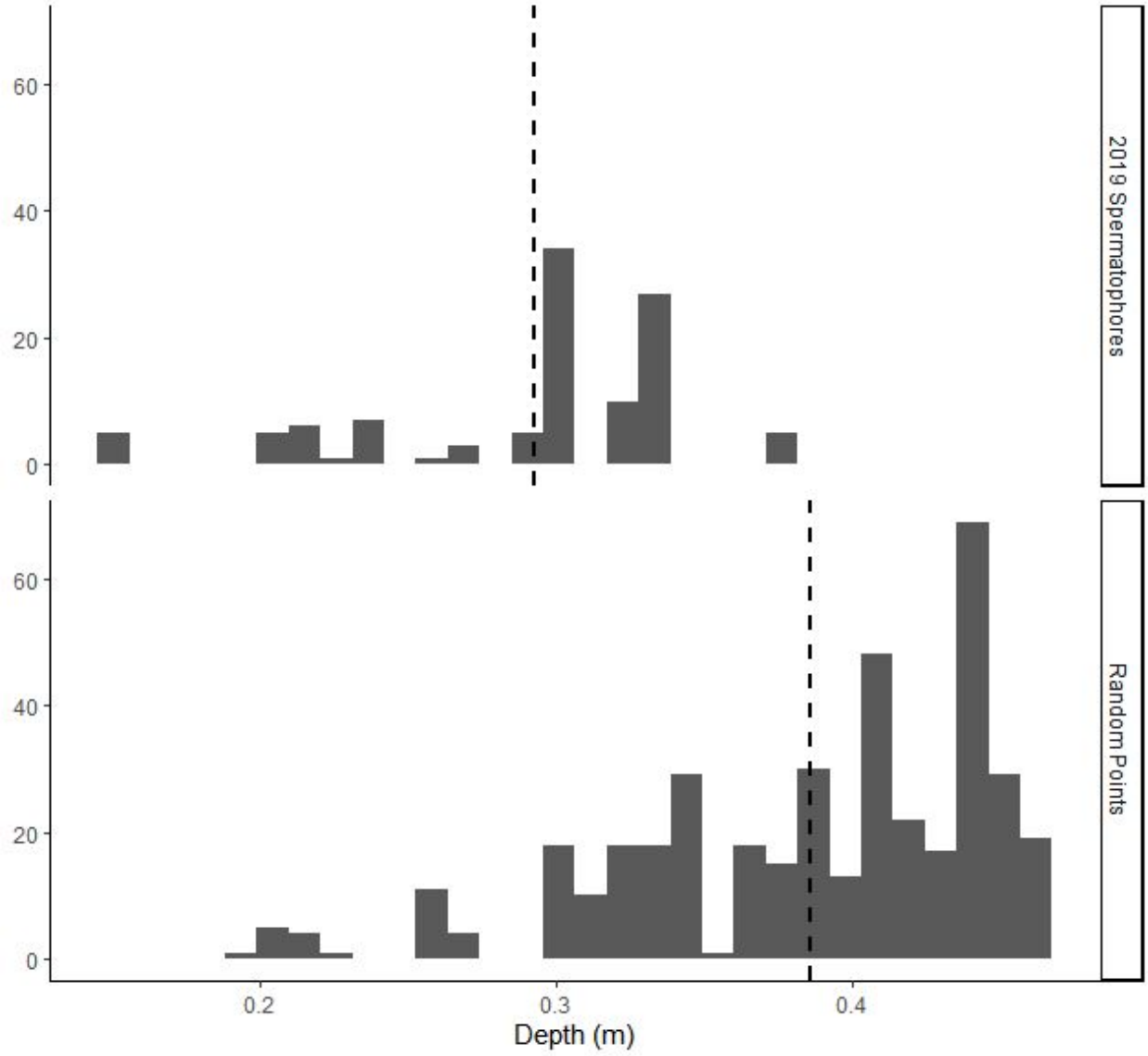

Figure 8. Distribution of depth for spermatophores and for the pool in 2019. Dashed lines represent the means of 0.292 m for 2018 and 0.386 m for the random points. We sampled 109 spermatophores in 2019. Depth was measured manually while sampling the spermatophores. To determine depth for the entire pool, we measured the difference in elevation between the base of the pool in a LIDAR scan and the water level as recorded in 2018 in another LIDAR scan. We then estimated the difference in depth between 2018 and 2019 by comparing depth for each year at several locations, resulting in a difference of 0.144 m. This difference was added to the 2018 random points to measure depth for the pool in 2019.

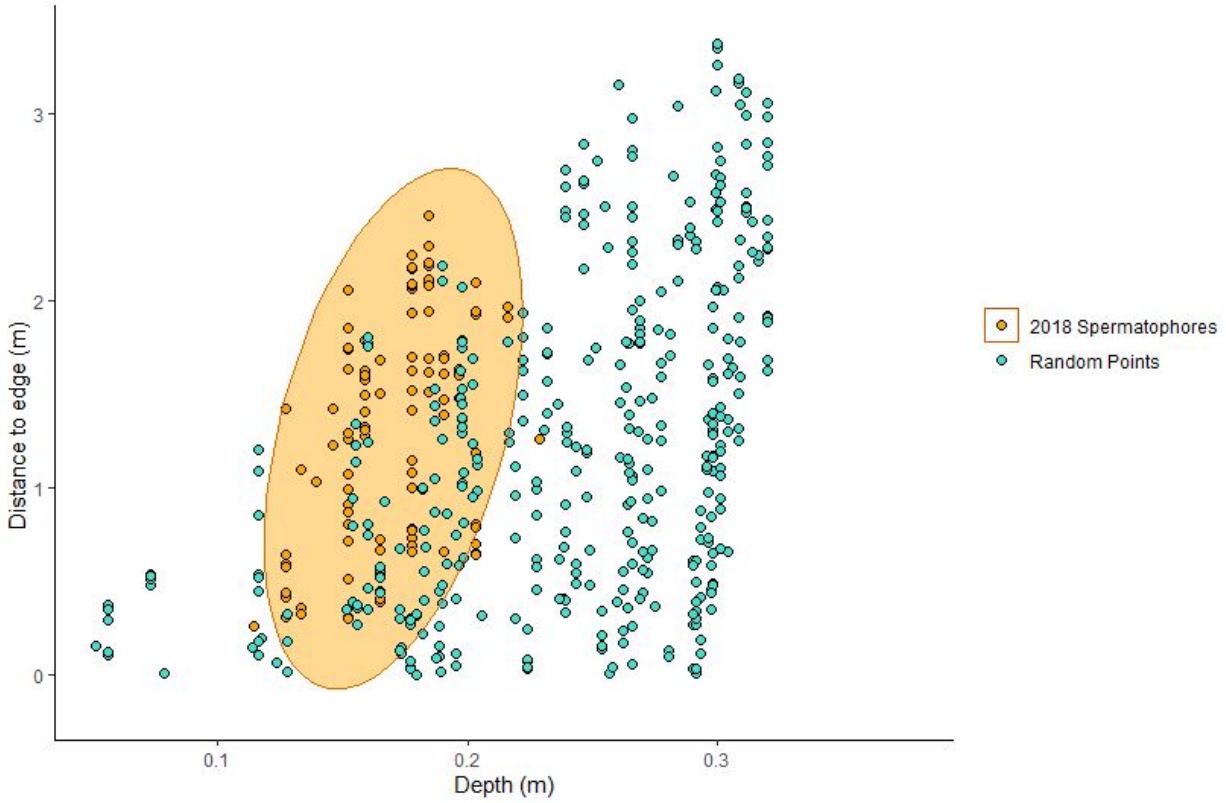

Figure 9. Distance to the edge and Depth for each 2018 spermatophore and random point. 2018 spermatophores were precisely mapped using LIDAR and distance to the edge was calculated using the near tool in ArcGIS Pro. The random points were created in ArcGIS Pro and distance to the edge was also calculated with the near tool. Depth was calculated for the random points using a second LIDAR scan of the pool while dry and the Near 3D tool.

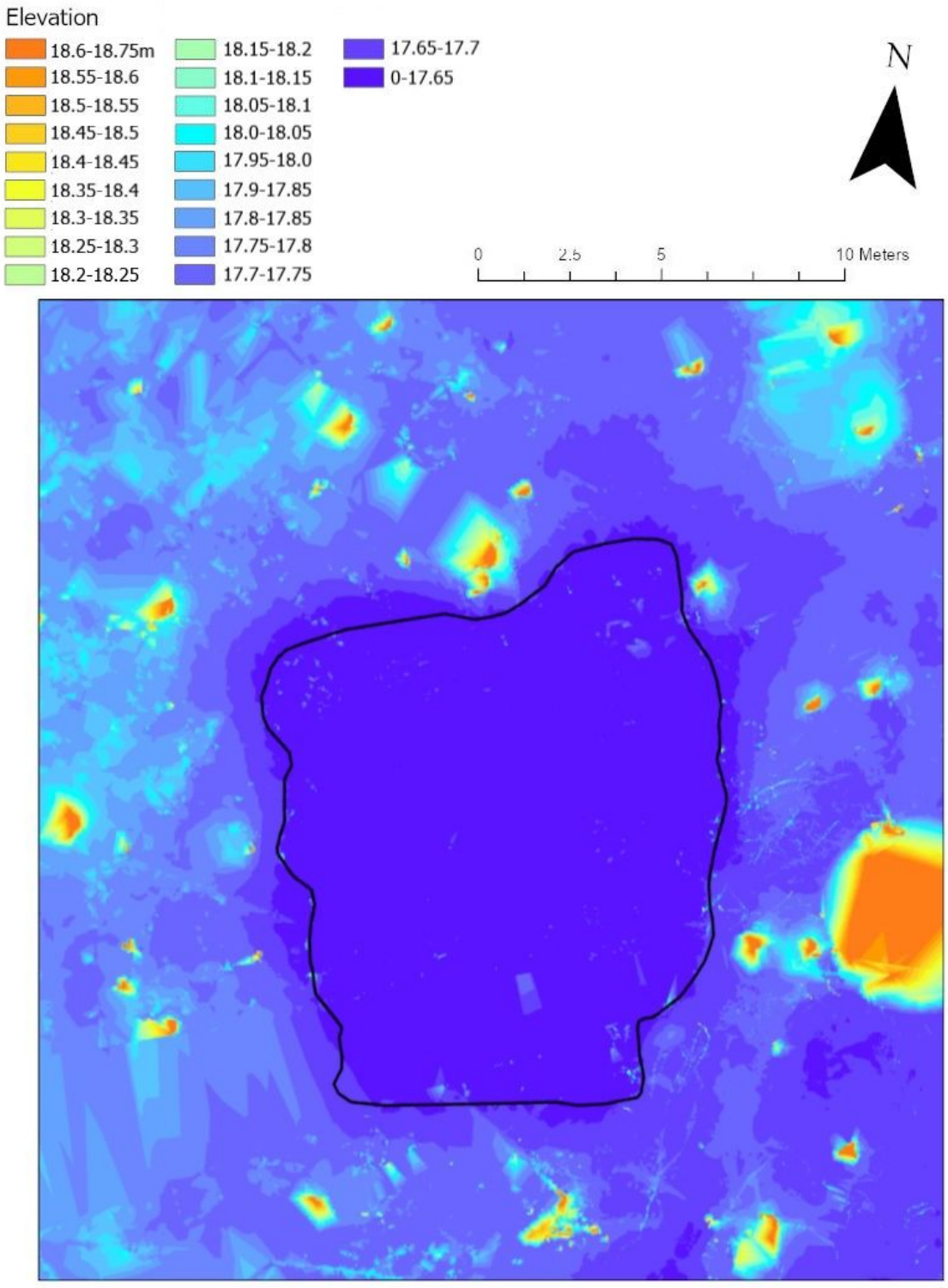

Figure 10. Elevation of the study site. The black line represents the pool boundary during 2018. Elevation was determined using a LIDAR scan.

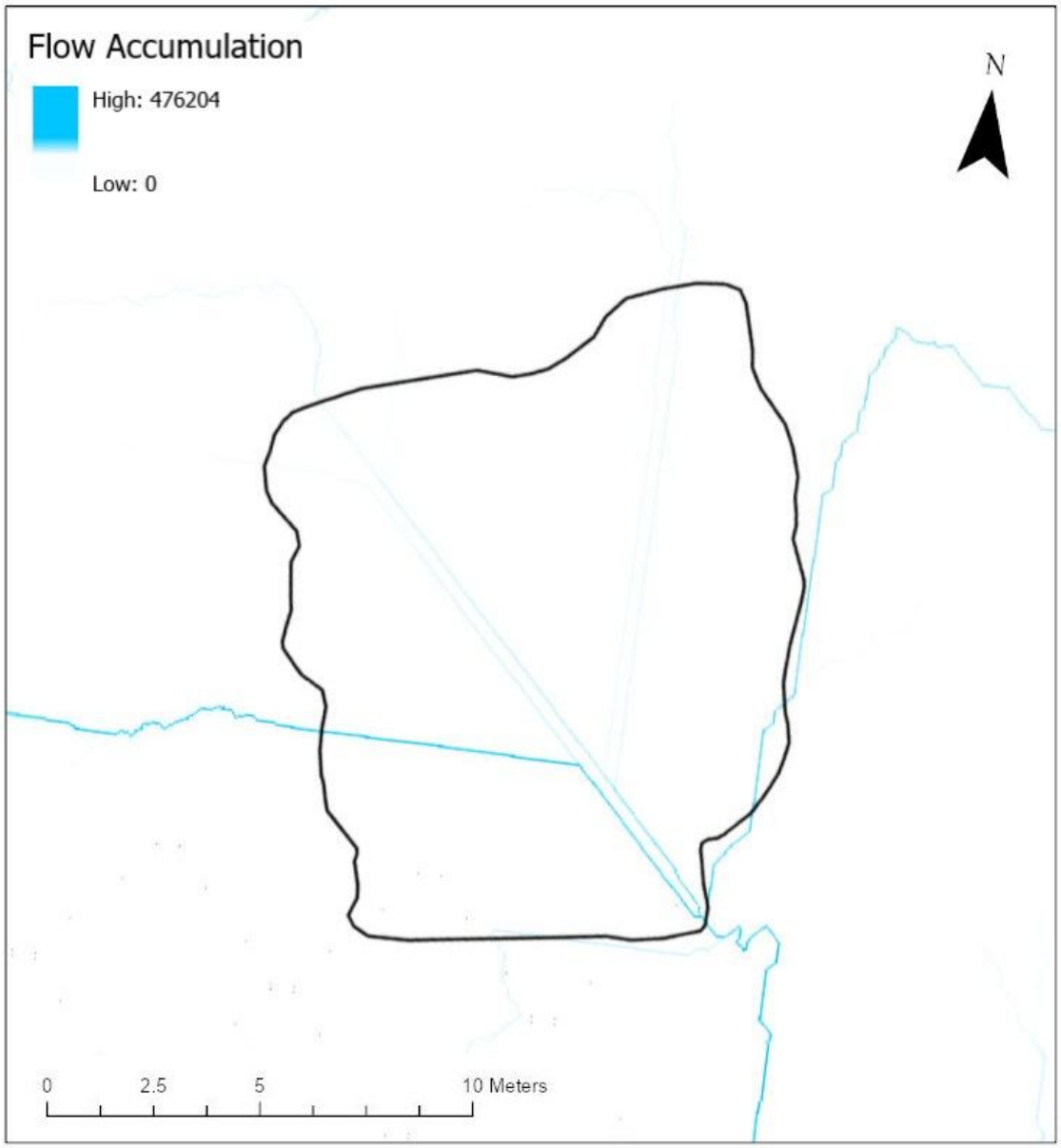

Figure 11. Flow accumulation at the study site. Flow accumulation was calculated using the flow accumulation tool in ArcGIS Pro. Elevation and fill for each raster square was calculated based on the point could from the LIDAR scan. We examined flow accumulation to determine if it affected the orientation from which the salamanders entered the pool and thus where they placed spermatophores. However since the surrounding area was largely flat there was little difference in the microtopography or drainage into the pool.

SUPPLEMENTAL MATERIAL

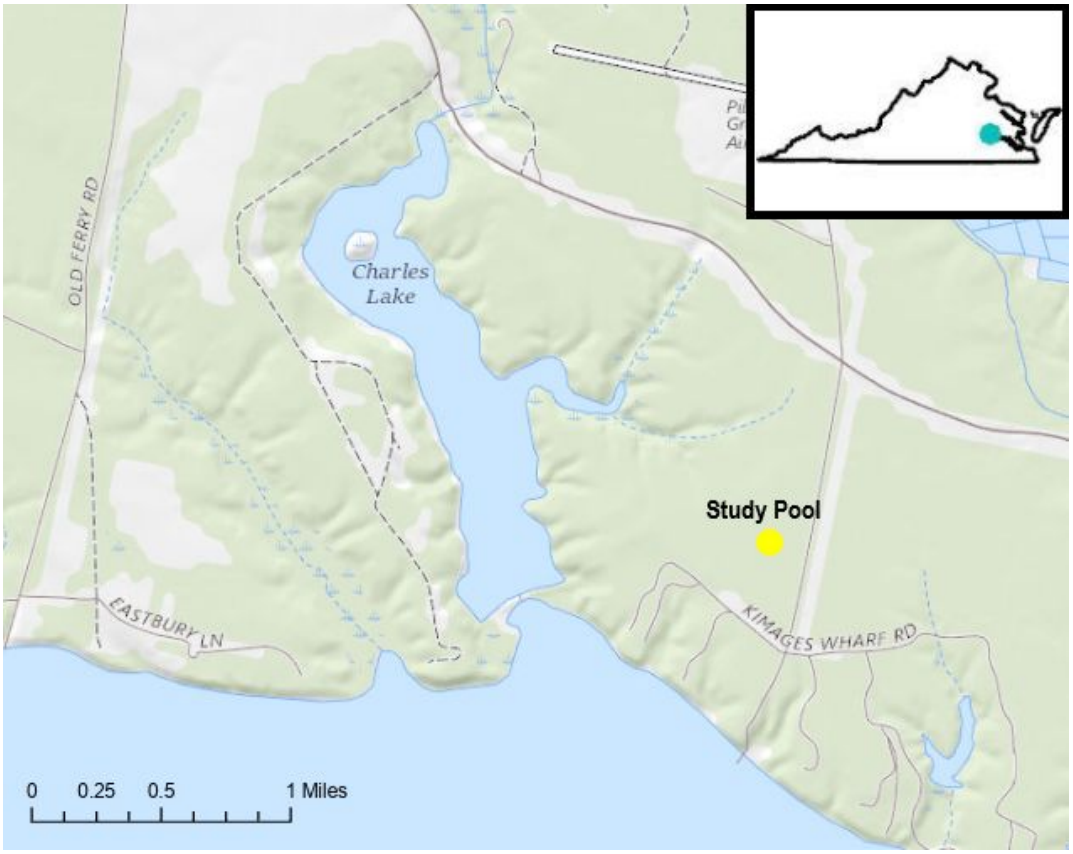

Figure S1. Map of study location. The study pool is located at the Rice River Center in Charles City, Virginia and is part of a complex of multiple vernal pools. The pool had an area of 57.45 m^2 and a perimeter of 30.64 m in 2018. It is surrounded by a mixed conifer and hardwood forest.

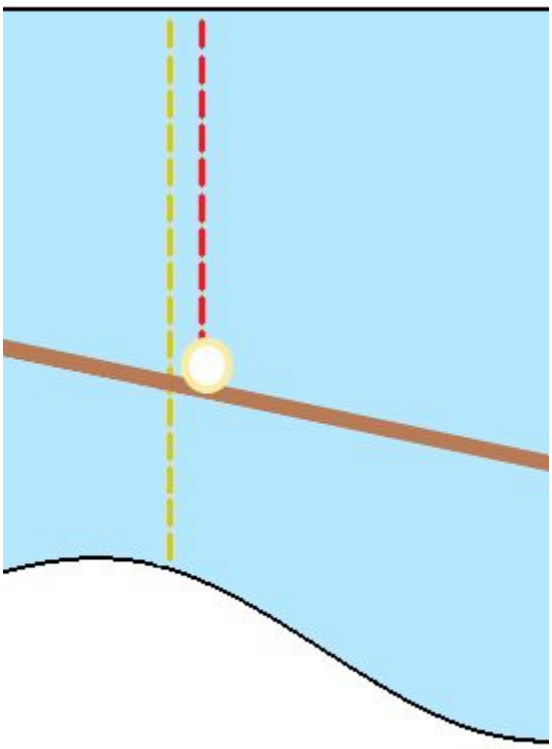

Figure S2. Diagram of the two measurements of depth for each spermatophore. The red line represents the depth of the spermatophore in the pool and the yellow line represents the total depth of the pool at the location of the spermatophore.

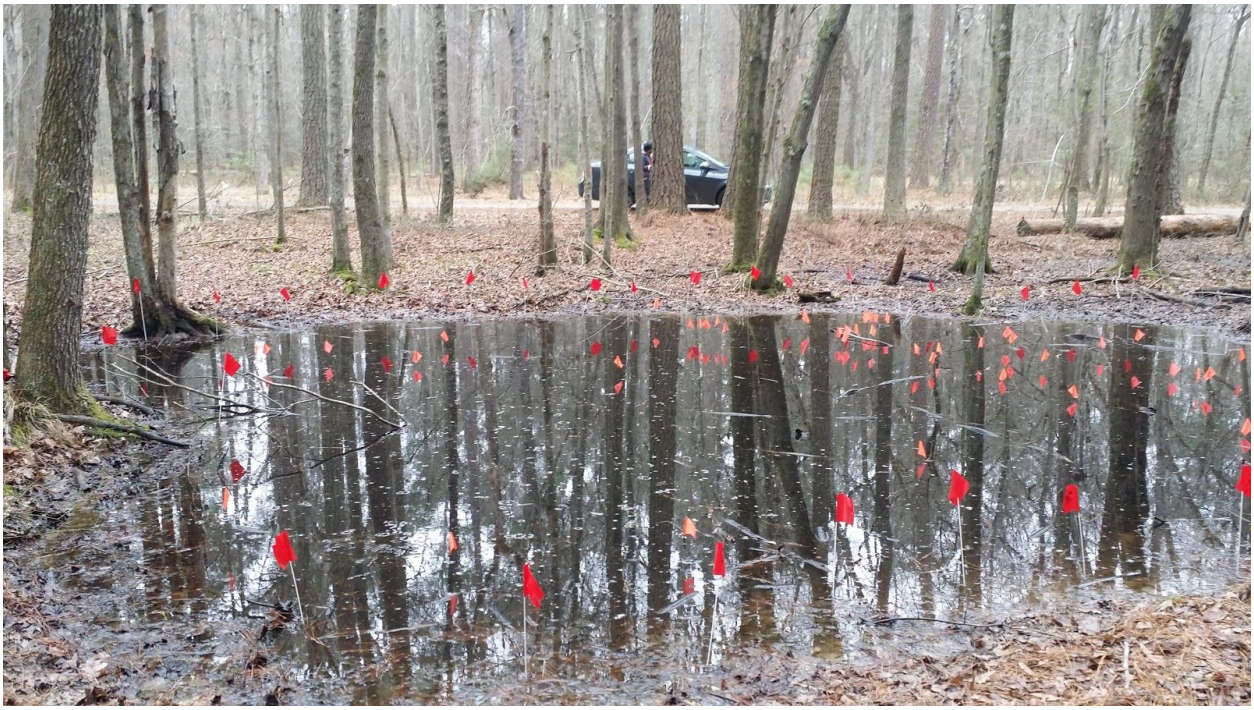

Figure S3. Study site during sampling in 2018. Red flags mark the boundary of the pool and orange flags mark spermatophore locations.

Figure S4. Study site during sampling in 2019. Red flags mark the boundary of the pool in 2018 and orange flags mark spermatophore locations.

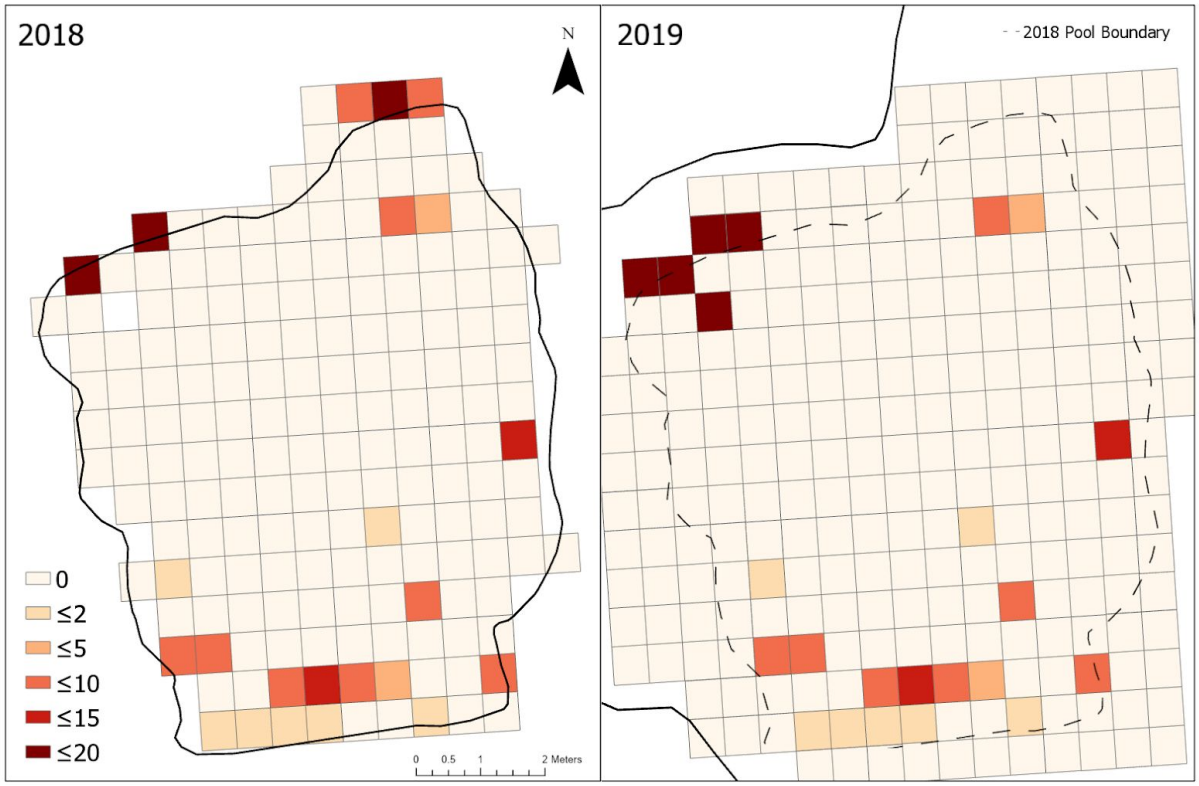

Figure S5. Total number of spermatophores observed during the 2018 breeding season. We sampled the vernal pool using a ~0.5 m^2 grid and we counted the number of spermatophores in each grid. The 2018 boundary is the perimeter of the study pool as mapped using LIDAR during the 2018 breeding season. The 2019 boundary is an estimate based on personal observations, and extends past the map frame as the high amount of precipitation resulted in a large pool with no clear edge.

Vita

Megan Kuechle was born on August 13, 1994 in Dyer, Indiana. She received a Bachelor's of Science in Wildlife from Purdue University in 2015. She graduated with honors, and then spent a year working with the William's lab on extension and outreach projects for hellbender conservation. From 2016 to 2017 she worked for the Indiana Department of Natural Resources as a Naturalist Aide, which included responsibilities such as managing the river otter trapping season and manning deer check stations. She taught introductory biology and ecology courses during her graduate studies. She also presented her thesis work at the Rice River Center Symposium in 2019 and at the VA Herpetological Society meeting in Fall 2019.