Environmental DNA: testing assumptions with experimentation

Justification

Environmental DNA (eDNA) is a burgeoning tool with important implications for conservations, including the monitoring of present and recently biodiversity. Using molecular techniques, genetic material in the environment which has been shed or otherwise deposited by living organisms can be detected in water or soil samples. eDNA methods have been used for a variety of purposes including estimating occupancy and abundance of species, monitoring endangered species, and establishing the arrival of alien invasive species. This developing technology relies on many assumptions that have yet to be empirically tested and requires a stronger empirical understanding of potential constraints and limitations.

Aquatic species, including amphibians, have been the focus of several eDNA studies, including the detection of rare species using eDNA (Pilliod et al. 2013), and surveillance for pathogens (Kirshtein et al. 2009). Estimating population abundance, in addition to presence/absence, is another advancement that improves management opportunities of this research (Pilliod et al. 2013, Rees et al. 2014, and Wheat et al. 2016). Estimating population density is possible via a linear regression model that relates the concentration of eDNA molecules in a water sample to biomass. This method assumes that the size of individuals does not confound abundance estimates, and that all life stages produce DNA at rates proportional to their biomass.

It's important to identify any confounding variables in abundance estimates, namely the size of individuals, and the life stages of the individuals present. Several large individuals may shed equivalent amounts of eDNA as many small individuals. As such, size and density may be interacting to complicate estimate results. Further, species with complex life histories may result in variation of amount of genetic material that is shed into the environment. As such, we aim to empirically test how body size, life stage, and decay state influences eDNA detection in aquatic systems.

Objectives

This study used a factorial mesocosm design to quantify American bullfrog (*Lithobates castebeianus)* eDNA to **assess the relationship between body size, density, and eDNA shedding rates.**

This study will yield important results that have implications for all eDNA studies done on aquatic systems. Further, we will be testing American bullfrogs, a pervasive invasive species in the Pacific Northwest. Early detection is essential for control and eradication, and information on population size and life stages present would be incredibly helpful in designing an appropriate management strategy. If we detect differences across life stages in genetic material shedding rates, it could mean that some life stages of amphibians are more easily detected by eDNA sampling techniques. Further, if body size and number of individuals is not confounded, eDNA will be an appropriate tool for estimating densities of invading populations.

Methods/Procedures

Experimental Set-Up

Prior to handling animals, experimental tanks were washed and scrubbed, then filled with water in the experimental room, located in Nash Hall. Water was allowed to sit overnight. Experimental tanks were not used for more than one treatment. Each tank was labeled according to the treatment type and number. Lights in the experimental room were set on a 13-hour timer, to mimic natural lighting conditions, reducing stress on the animals. Animals were weighted and measured individual, then transferred to a temporary bucket before deployment in tanks. 3-hour treatment groups were weighed and measured the night before placement in tanks the following day, and 36-hour treatment groups were weighed and measured the same day as deployment. All animals were housed in the Garcia Lab in Nash Hall when not placed in experimental tanks.

Nets used to place animals in the containers were pre-washed with soap, and subjected to 2 treatments of 1200 joule UVB light, one per each side of the net. UVB treatment was accomplished in the Levi Lab of Nash Hall and destroys any potential DNA contamination. Sterile gloves were worn during this procedure to limit contamination, and no nets were used without this method of sterilization. Animals were transferred from the temporary housing bucket to the net, rinsed with water from the experimental room to remove any residual water from their temporary container, and placed into treatment tanks. Sampling containers were washed and scrubbed with water, then soaked in 50% bleach solution for 1 minute before being rinsed 3 times and dried. They were then subjected to 1200 joules of UVB light, and sterile gloves were used to handle the container.

Animal Capture and Care

Animals were collected from Dunawi Creek, in Corvallis, Oregon. Animals were collected with nets, and transferred to 5-gallon buckets for transportation back to the Garcia Lab. Once in the lab, animals were kept in multiple 5-gallon buckets using an oxygen pump to keep oxygen levels sufficient in the containers. Animals were weighed and measured before being transferred to experimental units. Care was taken not to select animals that had obvious injuries. After the conclusion of the experiment, animals were euthanized with MS222 following IACUC protocol, and are being stored in the Garcia lab.

Experimental Design

Objective: assess the relationship between individual body size and group density on the amount of genetic material detected by eDNA techniques.

In order to see if there was a quantifiable difference between the amount of detectable eDNA depending on body size, I utilized a 3X2 factorial design, with three size/density treatments (1^{st} year tadpoles, 2^{nd} year tadpoles, 1^{st} + 2^{nd} year tadpoles) with a biomass constant of 50 grams (+/- 1.5 grams), and two sampling treatment groups, one being sampled after 3 hours and the other after 36 hours. Within treatments, total mass of bullfrog tadpoles was constant at 50 grams (for

example, in the 1st year treatment, there were add approximately 12 1st year tadpoles, 3 2nd year tadpoles, or 8 1st year tadpoles + 1 2nd year tadpole) (fig.1). Each treatment combination was replicated 4 times, resulting in 24 total experimental units. 4 treatment tanks were created using the same protocol, but did not have animals placed in them, and they were used as negative control tanks, sampled after 24 hours.

Figure 1 - Experimental Treatment Groups

eDNA Sampling Protocol

Water samples (250 mL) from experimental units were collected using sampling containers that had been sterilized prior to use. Water samples were collected from the center of the experimental tanks, and care was taken to not disturb the water while sampling as much as possible. 3 samples were collected from each experimental tank. Samples were immediately taken to the Garcia Lab, where a peristaltic pump was used to filter the water through single-use filter funnels containing a 0.20-micron cellulose nitrate filter. Filters were taken upstairs to the Levi Lab, where they were processed in groups of 12. Filters were removed from the funnels with forceps, rolled and placed into a 2 ml test tube filled with ethanol. Gloves were changed between every filter to avoid contamination. 50% bleach and sterile water were used to sterilize and rinse forceps before each use. Sample vials were stored at room temperature or colder and away from light until lab processing.

Data analysis

DNA was extracted from the filter papers with the Qiagen DNeasy Tissue and Blood Kit, using the method described by Pilliod et al. (2013). A primer published by Ficetola et al. (2008) was used to amplify bullfrog DNA, as it was designed to be species specific. Prior to ordering, I checked both the forward and reverse primer against other published sequences from other frog species and confirmed that it didn't match any sequence besides bullfrogs. After arriving, the primer was checked with a positive and negative sample using a standard PCR protocol and 3% agarose gels. The samples were ran using digital droplet PCR technology, using Evagreen super-mix, which negates the need for DNA probes. After amplification, results were analyzed in Microsoft Excel and R.

Results

The analysis revealed surprising results from the experiment. The first stage of analysis looked at concentration differences within each age group (Fig. 2). The highest amount of observed DNA was in the 2nd year 3-hour treatment group, and the lowest was in the 2nd year 36-hour group. The two groups were found to have significantly different values (p-

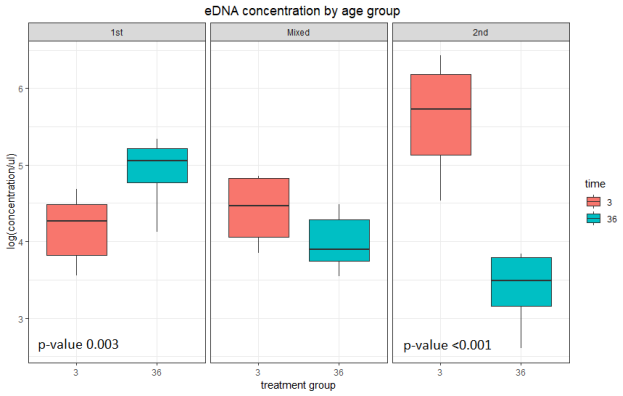

Figure 2 - eDNA concentrations by age groups

value <0.001, Welch's 2 sample t-test). While the amount of DNA in the 36-hour treatment group was significantly less than the 3-hour group for the 2nd year individuals, the opposite was true in the treatment groups with the 1st year individuals. The amount of DNA detected in the 36-hour group for the 1st years was significantly higher than the 1st year 3-hour group (p-value 0.003, Welch's 2 sample t-test). The 3-hour mixed group and the 36-hour mixed groups were not found to be significantly different.

When just the treatment groups within the different sampling time groups were compared, there were interesting results (Fig. 3). The 1st and 2nd year 3-hour groups were significantly different (p-value 0.00016, pairwise t-test), as were the 2nd year and mixed 3-hour groups (p-value 0.00372, pairwise t-test). The 1st year and mixed 36-hour groups were significantly different (p-value 0.039, pairwise t-test), and the 1st year and 2nd year 36-hour groups were also significantly different (p-value <0.001, pairwise t-test).

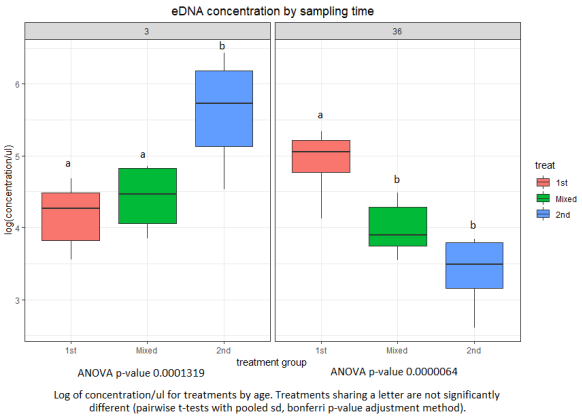

Figure 3 - eDNA concentration by sampling time

The relationship of time and eDNA amplification were different between ages, with the 2nd year group showing the most extreme values and highest amount of variability. It's unknown if older animals that are closer to metamorphose react differently to stressors, and if this helps to explain the results. It's also possible that their microbiota is different than younger individuals, and if these communities increase after time in experimental tanks, eventually exhibiting DNA shredding behavior, consuming shed skin cells, or otherwise impact DNA shed rates. The results were unexpected, and the slight decrease (although not statistically significant) seen in the mixed 3-hour and 36-hour groups could support this. The results call into questions assumptions behind eDNA abundance estimates, and warrant further investigation.

Impact

eDNA sampling is emerging as the future of wildlife and fisheries monitoring, due to its efficiency, non-invasiveness, ease in standardization, sensitivity, and cost efficiency (Thomsen et al. 2015). Its use has been shown to have improved detection results of invasive species relative to traditional methods, and its application is becoming more widespread. As eDNA sampling becomes more commonplace among federal, state, and private organizations, there is a need to test some of the assumptions associated with conclusions that it draws. This project serves to challenge assumptions used for abundance estimates, to better understand the effects of age and size of individuals on eDNA deposition. As such this project has the potential to impact a multitude of studies and management efforts of invasive, rare, and cryptic species.

This project is relevant to Oregon's natural resources, as the findings have the potential to assist with detection of rare or invasive species, including the invasive American bullfrog. A voracious predator, bullfrogs have negatively impacted species native to Oregon (Pearl et al. 2004). Understanding the effect that body size and life stage can have on detectability will aid in the

development of control and mitigation efforts. Further, the relationship between individual size and abundance estimates would help to correctly estimate population densities, allowing for better management decisions. This type of study has not been done before and has the potential to also aid in further research involving not only amphibians, but also other taxa.

This project should be considered a continuation of previous research experience with Dr. Tiffany Garcia (Department of Fisheries and Wildlife) and her lab. In my 2017 summer internship, I assisted in a project looking at minimum bullfrog reproduction size, finding that adults from Willamette Valley populations become sexually mature at smaller sizes relative to adults from the bullfrog native range. Dr. Garcia has been investigating the impacts of American bullfrogs on Oregon aquatic communities since 2006 and has publications on predator-prey relationships, competitive interactions, disease transfer, and resilience to abiotic factors such as pond permanence and UV-B radiation.

The results of this study have immediate impacts on the assumptions behind eDNA abundance estimates, as it suggests that a larger number of individuals does not automatically result in higher amounts of amplifiable eDNA. The 2nd year 3-hour groups that contained 3-4 individuals resulted in significantly higher amounts of DNA than the 1st year 3-hour groups that contained an average of 11 individuals, in some cases 3 times as many individuals as the 1st year groups. This could indicate that abundance estimates relying on equal shedding rates per individual would result in inaccurate estimates, particularly when animals have very recently entered sampling areas. The opposite pattern was observed with the 36-hour groups, which seems to suggest that these relationships change over time, and further investigation should look at time intervals to quantify this. The results of this research suggest that the relationship between individual biomass, density, and DNA shedding rates are not as clear cut as originally anticipated and commonly assumed.

Further research will attempt to take a closer look at the temporal change in the relationship and increase the intervals in sampling times. While this study used different treatment units for each sampling time, a further study will sample the same unit at multiple intervals, which will allow for understanding of how the relationship changes over time. The next step of this interval will be conducted in the John L Fryer aquatic animal health lab. This well help reduce the amount of contamination. While the control tanks had almost no amplification, there was some, which makes the importance for locating a cleaner facility for the following research. The follow-up research will closely resemble this study, with some differences. Animals will be collected from the same location, and 50-gram treatment units will be used. However, this research will have 2 water samples taken at 3, 12, 18, 24, and 36-hour intervals, which will allow for quantification of the change in DNA over time. As a response in the communities of the microbiomes of the tadpoles could be altering the amount of amplifiable DNA molecules, bacteria colonies will be grown on agar plates from water sampled at each interval, to attempt to detect a change. Additionally, disturbance of animals will be altered, to see if stress events alter the amount of DNA shed.

Communications

The results have been shared with faculty at OSU, and with USFS employees at the Corvallis research station. As the results are unexpected, they've generated some interest, and further investigation seems highly warranted. The data was presented at the agricultural sciences experiential expo. The poster will also be submitted to the Society for Northwest Vertebrate Biology conference which takes place February 2019. This will help increase the exposure of the research, and will be a great way to get feedback and ideas for how to proceed with the follow up this spring.

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