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USING ENVIRONMENTAL DNA TO IMPROVE DETECTION AND SHORTEN SURVEY TIME FRAMES FOR ENDANGERED FAIRY SHRIMP

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Abstract

Introduction and Objectives. Vernal pools and associated federally listed species are common on Department of Defense lands in California that are used for active military training and other mission essential activities. On military installations in southern California, intensive surveys for vernal pool endemic federally listed fairy shrimp species must be completed prior to training and construction activities, potentially delaying these activities for years. Environmental DNA (eDNA) is a relatively new tool that can often detect target organisms in soil and water samples more efficiently and accurately than conventional survey methods. We developed eDNA methods to detect three species of fairy shrimp in soil and water samples and compared detection probabilities and costs with standard field-based methods.

Technical Approach. We developed and validated quantitative PCR (Polymerase Chain Reaction) assays for San Diego (*Branchinecta sandiegonensis*), versatile (*B. lindahli*), and Riverside (*Streptocephalus woottoni*) fairy shrimp. We collected water samples from 10 pools with simultaneous dipnet fairy shrimp surveys repeatedly during the 2017 and 2018 wet seasons and collected soil samples from the same pools during the 2017 dry season. We also sampled an additional 20 pools once during the wet seasons and sampled one additional pool during the dry season. We analyzed all samples for eDNA of the three fairy shrimp species.

Results. During the wet season, target species eDNA was detected at every location where they were seen during field surveys (N = 11) and an additional six sites where they were not observed in the field. We detected target species eDNA at six of the eight sites with dipnet surveys or eDNA evidence of target species in the previous wet season. The sites without detections in the dry season had low levels of eDNA during the wet season and no field detections. The cost of eDNA sampling one time in a single pool was higher than a single dipnet survey. Therefore, the overall annual costs of either a dipnet or eDNA sampling program depends on the number of sampling events that are needed to detect the species. If detection probabilities for eDNA sampling are higher than for dipnet surveys, the overall cost to sample a single pool for a season would be lower for eDNA surveys compared to dipnet surveys.

Benefits. We showed that eDNA sampling for fairy shrimp can reliably detect fairy shrimp at rates the same as or higher than field surveys. Environmental DNA methods are highly accurate, which can prevent misidentification for species that are difficult to identify in the field. Additionally, eDNA sampling does not require a USFWS permit, which has stringent requirements for qualifications of survey personnel and may take months to obtain. We showed that eDNA sampling can detect fairy shrimp later in the season than field surveys, and eDNA may be more likely to accurately detect them early in the season when immature fairy shrimp are often difficult to distinguish from co-occurring species. With the high eDNA detection probabilities shown in this study throughout an extended sampling season, combined with reliably accurate species identification from the validated fairy shrimp eDNA assay, eDNA sampling may shorten the overall survey time needed to verify the presence of federally endangered fairy shrimp.

Introduction

Vernal pools are seasonal ponds with high local endemism and high biodiversity (Zedler 1987). Annual hydrological extremes from inundation to complete desiccation as well as high interannual precipitation variability have resulted in life history strategies that include dormant cysts or seeds that can persist for decades (Zedler 1990). The cryptic nature of dormant stages and the potential length of dormancy makes these species, particularly crustaceans, difficult to detect. Due to high levels of landscape change in the Mediterranean type ecosystems of California, the number of vernal pools has been dramatically reduced overall, though this decline has been much lower on military facilities. As a result, Department of Defense (DoD) installations contain some of the most extensive and best examples of vernal pools remaining in California, particularly in the southern part of the state.

In San Diego County over 80% of the remaining pools occur on military land. Federally listed endangered species widely occur in the remaining pools. As a result, DoD assumes an enormous management and Endangered Species Act consultation burden associated with vernal pool species, including several species of fairy shrimp. Current U.S. Fish and Wildlife Service (USFWS) survey protocols for federally listed fairy shrimp require intensive efforts that can span up to five years. Surveys can be particularly difficult and time-consuming in pools with low numbers of shrimp, high turbidity, and high vegetation cover. Distinguishing between species can be difficult, even under a microscope, and misidentifications have resulted in expensive, unnecessary mitigation and encroachment on military training when the non-listed species was mistaken for the listed species. Because of the time frames involved, surveys can be very costly; more importantly, they can delay implementation of new training activities and military construction projects by years.

Project Description

Background

Environmental DNA (eDNA), a relatively new technique for detecting target organisms from DNA in water and soil samples, has been shown to be a highly sensitive and efficient tool for inventory and monitoring of aquatic species in a variety of freshwater and marine systems. Key advantages to the eDNA method include: (1) more efficient detection of species where individuals are difficult to capture or occur in low densities; (2) the ability to identify multiple species from a single water sample; (3) accurate identification of target species without causing harm to the organism; (4) lower impacts to the vernal pool ecosystem compared to dipnet surveys; and (5) reduced permitting requirements when traditional survey methods such as dipnetting can be replaced by collection of water samples.

We have previously demonstrated that eDNA can be used to successfully detect amphibians on military installations, including Chiricahua leopard frogs, Sonora tiger salamanders, reticulated flatwoods salamanders, ornate chorus frogs, and American bullfrogs (Goldberg et al. 2018), as well as fish (bull trout, brook trout, and Chinook salmon), under funding from the Department of Defense's Environmental Security Technology Certification Program (ESTCP). In separate studies, we have also used this technique to detect invertebrates (New Zealand mudsnails,

Goldberg et al. 2013), and amphibian pathogens (amphibian pathogenic chytrid fungus, ranavirus). Additionally, we successfully used eDNA to detect Arizona treefrogs and northern Mexican gartersnakes at Fort Huachuca, AZ, under Legacy project 12-616.

The occurrence of fairy shrimp in vernal pools on DoD installations in California creates a widespread challenge for military managers from all four services as they strive to balance mission readiness training with natural resources protection obligations. The current USFWS survey protocol to determine the presence or absence of federally listed fairy shrimp in vernal pools requires intensive effort over a one to five-year time frame. Surveys to establish absence entail either: (1) two wet seasons of attempting to net fairy shrimp, which are often only millimeters long, in pools that fill during the rainy season and dry in the summer; or (2) one wet season of surveys followed by one dry season, in which soil samples are collected from pools, and fairy shrimp cysts are separated from the soil and hatched out to identify shrimp to species. This extensive time frame can delay decisions about training, construction, and management activities, sometimes for years. Environmental DNA offers a possible alternative by increasing sensitivity, improving efficiency, and eliminating species misidentification. Although this demonstration was conducted at MCAS Miramar, the sampling methods developed under this project are applicable to other DoD installations in the region that support the three target species, including Marine Corps Base Camp Pendleton, Detachment Fallbrook, Naval Auxiliary Landing Field San Clemente Island, and Silver Strand Training Complex South.

Objective

The objective of this project was to develop eDNA methods to detect three species of fairy shrimp in water and soil samples and compare this approach to standard field-based surveys for these species. Two of these species, San Diego fairy shrimp (*Branchinecta sandiegonensis*) and Riverside fairy shrimp (*Streptocephalus woottoni*) are listed as endangered under the Endangered Species Act. Versatile fairy shrimp (*B. lindahli)* are common throughout the range of the other species and are very similar in morphology to San Diego fairy shrimp. We paired eDNA sampling with standardized surveys for fairy shrimp, analyzed the field and eDNA samples in parallel, and compared detection probabilities and costs through the season for each approach.

Methodology

Study location

Marine Corps Air Station (MCAS) Miramar contains an extensive network of vernal pool habitat, where three different fairy shrimp species and one hybrid have been documented. Ten vernal pools were chosen for repeat sampling throughout the rainy season. The ten pools were selected to represent a range of fairy shrimp inhabitancy and included pools where: (1) no shrimp were ever documented; (2) only *B. sandiegonensis* adults and cysts were previously documented; (3) only *B. sandiegonensis* cysts were previously documented; and (4) *B. sandiegonensis* and *S. wootoni* were both previously documented. The ten selected pools were sampled 4 – 6 times during the 2017 rainy season, depending on when the pool dried. Eight of the 10 pools were also

sampled once after initial ponding in January 2018 (the remaining two pools never ponded in the 2018 rainy season and therefore were not sampled). Soil samples were collected at each of the ten selected pools during the dry season. An additional 21 pools were sampled one time during the 2017 rainy season to demonstrate the applicability of sampling method across additional sites. One of these sites was sampled during the dry season because *B. lindahli* had been detected during the wet season during this project.

Field Sampling: Wet season sampling

Water sample collection: The pools began ponding after precipitation events in late November 2016. We collected water samples between 30 December 2016 and 18 April 2017 from 10 pools. Each of the pools was sampled 4 – 6 times during the 2017 rainy season, depending on when the pool dried. Water samples were collected from an additional 2 pools one time during the same time period. In addition, 8 of the 10 pools were sampled again on 10 January 2018 within a week of the first ponding event to evaluate whether eDNA could be detected before shrimp had hatched and matured to the stage that they were identifiable. This was done because the sampling in 2017 began approximately 4-5 weeks after initial ponding when the shrimp had already become identifiable.

Sampling was planned to be done weekly but sometimes occurred at two- and three-week intervals, due to either heavy rain or logistic difficulties that prevented field work. In each pool, water was sampled at four locations. The sample locations were positioned to capture variability. For pools that were at least 3 m wide and long, sample locations were positioned on the north, south, east and west edges of the pool. For long and skinny pools, such as a pool in a road, sample locations were spaced evenly along the length of the pool.

Comprehensive efforts were made to avoid cross-contamination between pools. Rubber boots covered with plastic garbage bags were worn when sampling a pool and only used on a single pool. The garbage bags prevented mud from caking on boots, which would require more lengthy decontamination between pool sampling. Efforts were also made to avoid entering the pool at all when sampling. Using new gloves at each pool, an 8 oz plastic deli container was used to scoop 250 mL water from each sample location. A new, single-use deli container was used for each pool. The water sample in the deli container was first inspected for immature fairy shrimp, or nauplii, and mature fairy shrimp. If either were found, the sample was returned to the pool and a new sample was taken. Then, the 250 mL water sample was poured through a square of white, flour sack cloth covering the opening of a 1 L new or disinfected Nalgene bottle and affixed with a rubber band. This pre-filtering ensured that any shrimp or plant debris that was not removed by re-sampling could be returned to the pool via rinsing the flour sack filter in the pool after use. This process was repeated at all four sample locations, each time adding the sample to the same Nalgene bottle, and therefore, mixing all four samples from a single pool in one bottle. When all four samples were taken, the cloth filter was removed and rinsed in the pool to return captured material and then was discarded. The Nalgene bottle was tightly sealed, labeled, and stored in a cooler for off-site processing.

Rubber boots were disinfected between pools using a 10% bleach solution and then rinsed thoroughly with tap water. New gloves and new garbage bags to cover boots were used at each

pool. Used gloves and garbage bags were immediately bagged for disposal after each use. Before reuse, Nalgene bottles were disinfected in a 50% bleach solution and rinsed three times with tap water. When a disinfected Nalgene bottle was used for a new sample, the bottle was rinsed three time in the pool itself to ensure that any bleach residue was removed prior to taking the sample.

Water samples were transported in a cooler to a laboratory for vacuum filtration within 6 or less hours of collection. One pool water sample was filtered at a time to reduce cross-contamination. 250 mL of the 1 L sample was poured into four separate filtration apparatuses. The filtration apparatus consisted of a 48-mm single use filter funnel containing a 5µm polyethersulfone (PES) membrane filter (Sterlitech, Kent, WA), attached with a rubber stopper to a 1 L filter flask. All four filtration apparatuses were attached with tubing to a manifold with six valves that was attached to a vacuum pump (3.5 CFM Single-stage 5 Pa Rotary Vane Economy Vacuum Pump, Zeny). After each pool sample was filtered, using clean gloves and disinfected filter forceps, the filter was removed from the filter funnel and placed in a 2 mL O-ring tube with 1 mL 95% ethanol. To serve as a negative control, distilled water was processed the same as the field water samples (i.e. poured through flour cloth sack filter into a 1 L Nalgene bottle) and filtered in the laboratory for each day of sampling.

Dipnetting Surveys: Dipnet surveys followed USFWS survey guidelines for federally listed large branchiopods (USFWS 2015).

Field Sampling: Dry season sampling

All pools were dried by May 2017 and between 30 August and 6 October 2017, we collected soil samples once from each of the same 10 repeatedly sampled vernal pools, plus one of the additional 20 pools sampled, totaling 11 pools. Soil samples were collected between 30 August and 6 October 2017 and in May 2018. We sampled ten evenly spaced points along two transects in each pool. Transects ran the length and width of the pool, crossing perpendicularly in the center of the pool. At each sample location, we used a disinfected, stainless steel spoon to dig approximately 2-5 mL from the top 1 cm of soil. The soil was then sieved into a paper coffee filter using a plastic filter frame (8" non-metallic sieve frame, Gilson Company, Inc., Lewis Center, OH) with a disposable, polyester #70 mesh filter (0.223mm aperture, Gilson Company, Inc., Lewis Center, OH). Approximately 1 g sieved soil was added to a 15 mL tube with 3 mL of Longmire's buffer (Longmire et al. 1997). Soil captured by the mesh filter was returned to the sampling point. Unlike water samples, the ten soil samples from each pool were kept separate for eDNA extraction.

To prevent cross-contamination, field shoes were covered with plastic garbage bags prior to entering a dried pool and disposed of after each pool. Care was taken not to step in or place sampling equipment on the sampling point. However, because sampling points were identified prior to sampling, based on the sum of the length and width (measured by pacing) the pool was walked in by one person prior to collecting soil samples. New gloves were used at each of the ten sampling points in one pool. Used gloves, mesh and coffee filters, and garbage bags were immediately bagged for disposal after each use. Before reuse, plastic filter frames and stainless, steel spoons were disinfected in a 50% bleach solution, rinsed thoroughly with tap water, and either air dried or dried with clean paper towels.

Assay Development and Validation

We designed a multiplex quantitative PCR (qPCR) assay to detect *Branchinecta sandiegonensis*, *B. lindahli*, and *Streptocephalus woottoni* mitochondrial DNA (mtDNA) in eDNA samples based on cytochrome oxidase I sequence data (Lahti et al. 2010, Vandergast et al. 2009). Previous work by Vandergast et al. (2009) developed and validated a similar test for encysted embryos of *Branchinecta* using conventional PCR and longer fragments. Because eDNA is highly degraded and target DNA rare within environmental samples, we took a probe-based qPCR approach. To ensure that false positives would not be created for co-occurring fairy shrimp species, we designed each set of primers and probe to have at least five base pair (bp) changes total from the co-occurring species, including *B. lynchi*, with at least unique base within 5 bases of 3' end of the primers and in the center of the probe. We ran reactions using 1X QuantiNova Pathogen Master Mix (Qiagen Inc., Hilden, Germany), 0.2 µM of each primer, and 0.2 µM of each probe on a Bio-rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each qPCR included 3 µL of DNA extract in a total volume of 15 µL. We also included an internal positive control (QuantiNova IC; Qiagen) in each well to test for inhibition of the qPCR reaction. The cycling protocol started with 2 minutes at 95°C then ran for 50 cycles of 95°C for 5s followed by 60°C for 30s. We validated the assay using tissue samples collected from 10 each of *B. sandiegonensis*, *B. lindahli*, and 6 *S. woottoni*. Tissue samples were provided by Dr. Charles Black (MCAS Miramar) and Dr. Andrew Bohonak (San Diego State University). The closest population of *B. lynchi* is in Riverside County; samples from this area would have to be used to further validate this assay before it could be applied there. After each individual assay had been validated, we tested and confirmed that a multiplex of the three assays would not reduce sensitivity or reaction efficiency.

Sample Analysis

We extracted filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). In 2017, soil samples were extracted with the DNeasy Blood & Tissue Kit (Qiagen, Inc.) with a subset also extracted with the MoBio Power Soil Kit (MoBio, Inc.). In 2018, all soil samples were extracted with the latter method. All filter and soil sample extractions and qPCR set up were conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no tissue samples have been handled in this room. A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of samples. All samples in 2017 were analyzed with the multiplex validated as described above. In 2018, only *B. sandiegonensis* and *B. lindahli* were included in the multiplex. Soil samples testing as inhibited in 2017 were cleaned with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research) and reanalyzed. No other samples tested as inhibited.

Table 1. Primer and probe sequences for multiplex of fairy shrimp qPCR species detection.

Species	**Primer/probe**	**Sequence**
Branchinecta sandiegonensis 145 bp	BASA_F	GGATCAATAGTAGAAAGTGGRGCTGG
	BASA_R	TAACTGCACYRCTAATAGATGAAAC
	BASA-Probe	ABY-TCCACTGAAGGCCCTGCATGAGC-QSY
B. lindahli 130 bp	BALI_F	GGATTTGGAAAYTGACTAGTTCCS
	BALI_R	TTTCTACTATTGATCCTGCTAGGAGTAAAGT
	BALI-Probe	FAM-ATACTACCTCCCGCTTTAA-MGB
Streptocephalus woottoni 79 bp	STWO F	TCTCTTACATTACTTGTTGCTAGCTCAA
	STWO R	CAGAGAGCGGTGGGTAAACTG
	STWO-Probe	Cy5-TCCACCCCG/TAO/TTCCCGCTCCGCTTT-IowaBlackRQ

Cost Analysis

Cost elements for eDNA sampling: Operational costs of implementing an eDNA-based monitoring program include both front-end and per-sample costs. Front-end costs are required for developing and validating qPCR assays for target species. Per-sample costs represent the ongoing costs of collecting and analyzing water samples for a monitoring program. In the cost model detailed below and in Table 2, cost element 1 represents one-time, front-end costs, while cost elements 5 through 7 represent per-sample costs that would be required in an ongoing monitoring program for federally listed fairy shrimp. Cost element 8 involves permitting and reporting activities that are required to comply with USFWS survey guidelines for either eDNA or dipnet sampling.

Cost element 1 represents front-end costs that must be completed for each species. Cost drivers for these elements include the availability of existing (1) primer/probe sets or sequence data for target species in the installation area, (2) tissue samples for target and closely related non-target species, and (3) adequate qPCR protocols for target species. Per-species costs could be substantially reduced if existing species-specific primer/probe sets could be used and would be substantially increased if new sequence data need to be developed. Costs would be lower if DNA

tissue samples were already owned by or easily obtained by the operating laboratory and would increase with the number of samples that need to be collected in the field. If an adequate qPCR test is available for a target species, costs for element 1 would be significantly reduced, although for each new laboratory there are some costs involved in validating assay performance.

Table 2. Cost elements included in implementing an eDNA sampling program for fairy shrimp.

Cost Element	Description
1. Assay development and validation	Labor costs to analyze available sequence data and create sets of diagnostic primer/probe sets for 3 species; obtain samples from researchers; and optimize species-specific qPCR tests.
2. Water sampling equipment procurement	Cost of periodic (estimated 2 years) replacement of vacuum pumps, manifolds, flasks, stoppers, tubing, forceps, boots, and sample bottles. Equipment for decontamination of equipment and boots between sampling sites. Annual labor costs for procuring equipment.
3. Water filter sample collection	Average labor and travel costs required for sample collection.
4. Water sampling per-sample consumable cost	Rate of consumables used for collecting and filtering each sample, including filters, single-use filter funnels, ethanol tubes, gloves, trash bags, plastic containers, towels, rubber bands, and bleach.
5. Water filter sample analysis	Per-sample cost of analyzing samples using qPCR tests, including laboratory technician and PI labor and lab consumables.
6. Soil sampling equipment procurement	Cost of periodic (estimated 2 years) replacement of sieve frames, screens, spoons, and pin flags. Equipment for decontamination of equipment and boots between sampling sites. Annual labor costs for procuring equipment.
7. Soil filter sample collection	Labor and on-site travel costs required for sample collection
8. Soil sampling per-sample consumable cost	Rate of consumables used for collecting and filtering each sample, including sieve mesh squares, gloves, coffee filters, trash bags, bleach, and tubes with preservative buffer.
9. Soil sample analysis	Per-sample cost of analyzing samples using qPCR tests, including laboratory technician and PI labor and lab consumables.
10. Permitting and reporting	Labor for preparing permit applications, reporting, and coordinating with USFWS.

Cost elements for dipnet surveys (wet season) and soil sampling (dry season): Cost elements for fairy shrimp following the USFWS protocol both wet season and dry season sampling (Table 3). Permitting and reporting, cost element 1, cover both sampling methods, assuming that separate permits and reports are not required for the different methods.

Table 3. Cost elements included in surveys for fairy shrimp following USFWS protocol (USFWS 2015).

Cost Element	Description
1. Permitting and reporting	Labor for preparing permit applications, reporting, and coordinating with USFWS.
2. Dip net equipment procurement	Periodic replacement of nets, waders, and buckets. Annual labor costs for procuring equipment.
3. Water filter sample collection	Average labor and travel costs.
4. Dip netting per-survey consumable cost	Quaternary ammonia used for disinfecting boots and equipment.
5. Soil sampling equipment procurement	Annual labor costs for procuring equipment.
6. Soil filter sample collection	Average labor and travel costs.
7. Soil sampling per-sample consumable cost	Rate of consumables used for collecting and filtering each sample, including sieve mesh squares, gloves, coffee filters, trash bags, bleach, and tubes with preservative buffer.
8. Soil sample analysis	Per-sample cost of analyzing samples using qPCR tests, including laboratory technician and PI labor and lab consumables.

Cost comparison: For this analysis, we used the actual costs of the water filter sampling we conducted to calculate the average cost of conducting 1 eDNA survey for 1 pool. We compared this metric, termed a "survey pool," with the average cost of 1 dipnet survey for 1 pool. Detection of the target species in a single survey can determine the total number of surveys required to determine presence of the target species. For example, USFWS may approve suspension of wet season surveys for a pool if one or more of the listed fairy shrimp species are positively identified in that pool (USFWS 2015). Therefore, we used the estimated survey pool costs to create a matrix of the comparative costs of eDNA and dipnet sampling according to the number of surveys required to detect target species.

We recorded the actual costs of developing and validating an assay for three fairy shrimp species (*Branchinecta sandiegonensis*, *B. lindahli*, and *Streptocephalus woottoni*) to describe front-end costs for this project. Permitting and recording costs were estimated based on anticipated labor needed to meet these required elements of fairy shrimp surveys.

Results and Discussion

Assay Development and Validation

Each assay passed validation, amplifying for all samples of the respective species and none of the samples for the other species. Assays multiplexed without reducing specificity, sensitivity, or efficiency. No sites were able to be sampled with current populations of *Streptocephalos woottoni,* leaving that assay needing to be validated *in situ.*

Wet Season Sampling

We used eDNA methods to survey 32 pools for fairy shrimp in the 2017 wet season and conducted supplemental sampling of 8 sites in early 2018. We found eDNA of *B. lindahli* and/or *B. sandiegonensis* at 15 sites. Ten sites were sampled multiple times throughout the season, typically with concurrent dipnet sampling (Figure 1).

Pool ID	Survey type	1st Survey 12/30/2016-1/13/2017	2nd Survey 1/13/2017-1/26/2017	3rd Survey 1/26/2017-2/20/2017	4th Survey 2/3/2017-3/3/2017	5th Survey 2/20/2017-3/3/2017	6th Survey 2/22/2017-3/17/2017	7th Survey 3/17/2017-4/6/2017	8th Survey 4/6/2017-4/18/2017	Supplemental Survey January 2018
19	Dipnet									
	eDNA									
57	Dipnet									
	eDNA									
100	Dipnet									
	eDNA									
329	Dipnet									
	eDNA									
330	Dipnet									
	eDNA									
399	Dipnet									
	eDNA									
732	Dipnet									
	eDNA									
829	Dipnet									
	eDNA									
Riverside	Dipnet									
	eDNA									
Village	Dipnet									
	eDNA									

Dipnet detection | eDNA detection | eDNA - 1 of 4 samples | No detection | No survey

Figure 1. Survey timing and results for eDNA from water and dipnet sampling for fairy shrimp. All detections were *Branchinecta sandiegonensis* except Village, at which *Branchinecta lindahli* was detected. eDNA detections were out of four filter samples of 250 mL each collected and combined from different parts of the pool. We note that eDNA was detected in only one of four samples in pool 329 on two occasions and given a lapse in field decontamination practices at the site is strongly suspected to be field contamination. Dipnet surveys for Village were conducted approximately two weeks after water sampling.

In 2017, we detected eDNA of *B. lindahli* and *B. sandiegonensis* at every location where they were seen during field surveys (N = 11), plus six sites where they were not observed directly (Figure 1). Of those six additional sites, four had documentation of the species being present in previous years and two were new observations (854, 59/77). Of the four sites with previous observations, three were single visits without concurrent dipnet sampling. In one sampling event for pool 732 (1/11/2017), San Diego fairy shrimp were visually detected in the water sample and were not found in concurrent dipnet or eDNA sampling. However, the species was detected in eDNA samples in March 2017. Replicate filter samples were highly consistent, with a detection rate of 1.0 every time there was a field survey (dipnet) detection. Sampling events where there was no field detection had rates of 0.5-1.0, except for one site (329) with two observations at 0.25 that were suspected to be from field contamination and one site (Village) where eDNA detection became inconsistent 1 month after the last field detection.

Environmental DNA detections were more consistent through time than field surveys. At three of the six sites that were sampled through the season that had field detections of the species, eDNA

lengthened the season of detection by 1-4 visits (1 week – 2 months; Figure 2). At one of these sites, eDNA detected the species a month prior to field detection, but then not for the next two surveys until both field and eDNA surveys had detections. At the remaining three sites, eDNA detections exactly matched field detections (N = 1), filled in at a middle time when there was no field detection (N = 1), and tested positive at one time point two months after the single field detection at the site (N = 1).

In 2018, early season sampling (10 January) detected fairy shrimp at 2 sites with eDNA and no sites with field sampling. Of those two sites, one was dry by 26 January and the other was confirmed with field methods to have fairy shrimp at that time.

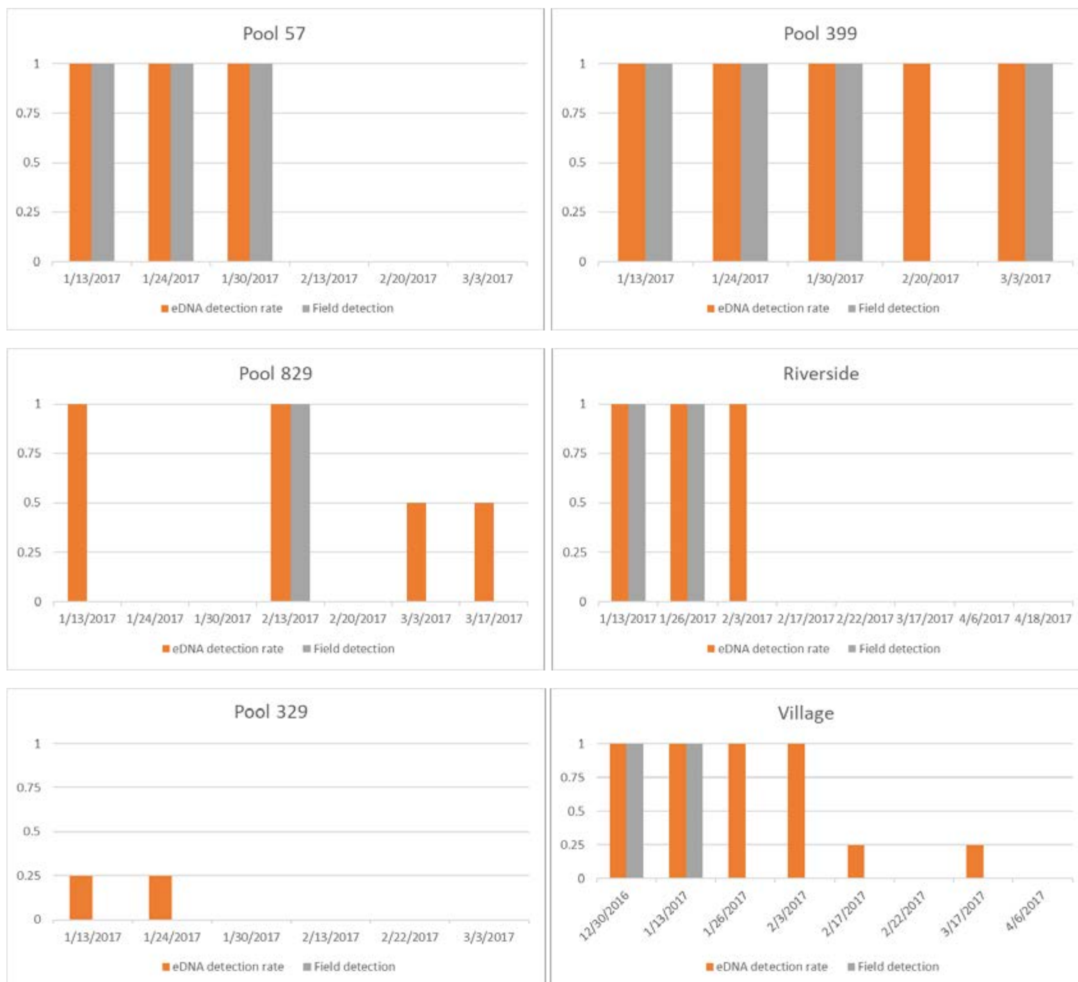

Figure 2. Comparative detection of fairy shrimp using eDNA and field methods (visual and dipnetting). All sites were *Branchinecta sandiegonensis* except for Village, which was *Branchinecta lindahli.* eDNA detections were out of four filter samples of 250 mL each collected and combined from different parts of the pool. We note that eDNA was detected in only one of four samples in pool 329 on two occasions and given a lapse in field decontamination practices at the site is strongly suspected to be field contamination.

Dry Season Sampling

In 2017, we detected target species at six of the eight sites with field or eDNA evidence of target species in the previous wet season, with rates 0.1-0.3 per sample. Both sites that were undetected had low levels of eDNA during the wet season and no field detections (N = 1) or field detection only in early January (N = 1).

In 2018, we detected target species at four sites (rates 0.1-0.5), including the two where the species was detected in the wet season. Of the two other sites, both had detections in 2017, one was not sampled in 2018, and the other was sampled in January without fairy shrimp detected.

Cost Analysis

Table 4 lists the actual costs of assay development and validation, estimated costs of permitting and reporting requirements, and estimated cost of surveying one pool one time (survey pool) for both eDNA and dipnet sampling. Environmental DNA sampling costs assume 4 water samples collected per survey and includes vehicle, equipment, consumables, laboratory analysis, and labor for field preparation, water sample collection, sample processing by a biologist without specialized fairy shrimp survey training. Dipnet survey costs include equipment, vehicle, and labor for a biologist trained to conduct fairy shrimp surveys per USFWS guidelines.

Table 4. Costs for dipnet and eDNA water sampling for three fairy shrimp species at MCAS Miramar, 2016-2018.

Cost Element	Estimated Costs for Dipnet Surveys	Estimated Costs for eDNA Sampling
Assay development and validation (front-end cost)	NA	$**2,474**
Permitting and reporting (life of project)	$1,440	$1,440
Estimated cost of 1 survey of 1 pool	$167	$324

If any of the listed fairy shrimp species are detected in a pool or other water feature, USFWS may agree to suspend further surveys in that feature for the remaining wet season (USFWS 2017). Therefore, the overall annual cost of either a dipnet or eDNA sampling program depends on the number of surveys that are needed to detect the species. Using the estimated survey pool costs for both sampling methods, we compared the difference in costs based on the number of surveys needed to detect the species with each sampling method (Table 5).

For example, if both methods detect target species in the first survey, the eDNA survey would cost an estimated $157 more than the dipnet survey because of the higher survey pool cost for eDNA ($324 minus $167). If detection probability is higher with eDNA surveys and target species can be detected in fewer surveys than dipnet surveys, the cost to sample that pool would be lower than with dipnet sampling. Conversely, survey costs would be higher for eDNA sampling if target species can be detected easily with one or more dipnet surveys and more than one eDNA survey is needed to detect the species.

Table 5. Comparison of estimated costs for fairy shrimp surveys using eDNA and dipnet sampling methods. Each cell represents the difference in cost depending on the number of surveys needed to detect target species in one pool. Negative numbers (in blue) are the estimated cost savings for eDNA surveys compared to dipnet survey costs for the respective number of surveys for that cell. Positive numbers (in red) represent the estimated higher costs of eDNA surveys for those numbers of surveys.

Number of eDNA surveys \ Number of dipnet surveys	1	2	3	4	5	6	7	8
1	$157	-$11	-$178	-$345	-$513	-$680	-$847	-$1,015
2	$480	$313	$146	-$22	-$189	-$356	-$523	-$691
3	$804	$637	$470	$302	$135	-$32	-$200	-$367
4	$1,128	$961	$793	$626	$459	$292	$124	-$43
5	$1,452	$1,285	$1,117	$950	$783	$615	$448	$281
6	$1,776	$1,608	$1,441	$1,274	$1,106	$939	$772	$605
7	$2,100	$1,932	$1,765	$1,598	$1,430	$1,263	$1,096	$928
8	$2,423	$2,256	$2,089	$1,921	$1,754	$1,587	$1,420	$1,252

Discussion

Fairy Shrimp Detection

The potential benefits of eDNA sampling for detection of fairy shrimp include improved detection, extending the time window for sampling, reduced environmental impact, and improved accuracy of species identification. This study demonstrates eDNA methods are highly sensitive and accurate, with detection at more time points than field surveys in the wet season and some detection in the dry season. We found no inaccuracies in species identification, but eDNA analysis has the advantage of accurate detection without the requirements for specialized survey qualifications for fairy shrimp (which include training/testing in practical branchiopod identification and a period of supervised field experience; USFWS 2017). Environmental DNA sampling also makes it possible to accurately identify fairy shrimp early in the season, when they may not be mature enough to identify through dipnetting if morphological differences aren't yet apparent. However, survey-for-survey, dipnet sampling is less expensive than eDNA sampling. Depending on available expertise, permitting, and timeframe, installations may benefit from trying dipnetting first, applying a combination of both methods, or choosing to use eDNA only.

We found that wet season sampling extended the time window for detection of species by a month or more and detected species during surveys where field detection was not successful. Environmental DNA is known to persist after animals have perished or left the system, and the ability to detect them beyond the time when they are present can provide additional flexibility for planning and applying sampling, or eDNA can be added if dipnet sampling does not detect target species. For maximum cost-efficiency, if permitting and trained personnel are available, eDNA may be used as a supplement if dipnetting does not indicate detection.

Dry season sampling could be used to test for the presence of these species, but non-detection can only be used to infer the lack of later-season activity in the previous spring. Detection does not go back to early season activity or to previous seasons with this protocol, and therefore this protocol can never be used to infer absence. Potentially deeper sampling in the soil could yield inference to a longer temporal window. Although we found eDNA detection probabilities in soil to be low in this study, eDNA sampling may extend the survey season compared to standard survey protocols.

Cost Analysis

Development and validation of species-specific assays are a one-time cost that are not incurred again during a project. For this project, we developed and validated an assay to detect *Branchinecta sandiegonensis*, *B. lindahli*, and *Streptocephalus woottoni*, and this assay can continue to be used to detect these species without additional front-end cost.

When considering the cost of a single survey for one pool during the wet season, eDNA sampling is more expensive than dipnet sampling. Potential cost savings may occur where eDNA sampling has higher detection probability than dipnet sampling and requires fewer surveys to detect target species.

Conclusions

We showed that eDNA sampling for fairy shrimp can reliably detect fairy shrimp at rates the same or higher than field surveys. Given the stringent requirements for qualification to obtain a permit to conduct field surveys for federally listed fairy shrimp, eDNA may be an effective approach for surveying for fairy shrimp when permitted survey personnel are not available. Additionally, a significant benefit of eDNA sampling is its accuracy, which can prevent potential misidentification for species that are difficult to identify in the field. We found a low-level eDNA detection in one of three samples at one pool for which we had evidence of contamination in the field. This underscores the need for assiduous prevention of field contamination to avoid low-level positives that might be interpreted as evidence of species presence.

In this study, we found that eDNA methods detected fairy shrimp in every pool in which they were detected with dipnet surveys and may extend the sampling season because fairy shrimp eDNA was detected later in the season than dipnet surveys found, with both water and, to a lesser extent, soil sampling. Environmental DNA methods may result in cost savings and lower survey effort when they have higher detection probabilities and require fewer surveys to determine presence of fairy shrimp in a pool. With the high eDNA detection probabilities shown in this study throughout an extended sampling season, combined with the reliably accurate species identification from the validated fairy shrimp eDNA assay, eDNA sampling may shorten the overall survey time needed to verify the presence of federally endangered fairy shrimp.

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