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ARTICLE

Environmental DNA Sampling of Small-Bodied Minnows: Performance Relative to Location, Species, and Traditional Sampling

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Abstract

We performed experiments in southwestern USA streams to evaluate the efficacy of environmental DNA (eDNA) sampling for two rare small-bodied minnows: Spikedace *Meda fulgida* and Loach Minnow *Rhinichthys cobitis*. We collected eDNA by filtering 5-L samples and compared detection sensitivity of eDNA assays to traditional sampling methods (electrofishing and seining) by using both techniques at 33 sites in seven streams. We used caged-fish experiments to estimate eDNA production rates, persistence, and travel distances and to estimate relationships between fish density, biomass, and eDNA quantity. Loach Minnows were detected at 22 sites by both eDNA and traditional sampling, were not detected by either technique at 7 sites, and were detected only by eDNA at 4 sites. Spikedace were detected with both techniques at 15 sites, were not detected by either technique at 8 sites, and were detected only by eDNA at 7 sites. In the Verde River and Wet Beaver Creek, both species' eDNA was detected downstream of caged fish out to our maximum sampling distance of 500 m. Estimated eDNA production rates were greater for Spikedace than for Loach Minnows, although more Spikedace were used. Production rates for both species were greater in the Verde River than in Wet Beaver Creek. Persistence of eDNA did not differ among species but was greater in Wet Beaver Creek than in the Verde River. In density experiments, the amount of Spikedace eDNA was positively related to the density and biomass of caged Spikedace, but the relationship differed between streams. We conclude that eDNA surveys are more sensitive than traditional methods for detecting rare minnows in southwestern streams. With the sensitivity to detect even a single fish in a 100-m reach, managers will be able to more effectively identify reaches occupied by threatened or endangered fish, even if a population is in decline.

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Environmental DNA (eDNA) sampling has become a useful tool for detecting aquatic species, often outperforming traditional sampling techniques, such as visual surveys, trapping, and electrofishing (Dejean et al. 2012; Smart et al. 2015; Wilcox et al. 2016). Because capture of even a single copy of DNA can permit detection of a species (Mason et al. 2018), eDNA sampling may be preferred and more cost effective (Evans et al. 2017) for occupancy estimates of species that are rare, sensitive to handling, or have low capture probabilities. If sampling effort is spatially distributed across the range of a species or at different times, this information can be used to infer its distribution or patterns of movement (McKelvey et al. 2016; Balasingham et al. 2018; Itakura et al. 2019). Likewise, an increasing number of studies have shown that eDNA concentrations can be positively correlated with abundance (Takahara et al. 2012; Doi et al. 2015; Wilcox et al. 2016; Itakura et al. 2019), but in others the relationship is unclear (Hinlo et al. 2017), likely because of sampling techniques (Wilcox et al. 2018), environmental conditions, and physiological processes of the study organisms. Understanding how eDNA-based estimates of occupancy and abundance vary among species and locations is crucial to interpreting the results of eDNA sampling (Carraro et al. 2018; Pont et al. 2018). Although eDNA sampling has been extensively trialed in many aquatic ecosystems, streams present unique challenges for this technique. For example, at a given sampling location, the amount of eDNA collected depends on the upstream biomass of fish, transport distance from the source organisms, DNA degradation rates, fish behavior, and environmental conditions, such as discharge, velocity, and stream morphology (Wilcox et al. 2016; Itakura et al. 2019), all of which can vary greatly among reaches of the same river and between rivers.

Spikedace *Meda fulgida* and Loach Minnow *Rhinichthys cobitis* are two stream-dwelling cyprinid fishes listed as endangered under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2012). Both species are endemic to the Gila River basin in Arizona and New Mexico (Minckley and Marsh 2009). Spikedace inhabit the middle of the water column in edges of fast-moving water (Rinne 1991), whereas Loach Minnows are bottom dwellers in riffles, often hiding under rocks and interstitial spaces (Propst and Bestgen 1991). Historically, Spikedace was documented in at least 20 streams and Loach Minnows in 35 streams, but both species likely inhabited additional streams with similar thermal and geomorphic characteristics that were rarely or never sampled. Sampling since 2005 indicated that the range of each species had contracted because Spikedace were detected in only three and Loach Minnows only in seven of the historically occupied streams (U.S. Fish and Wildlife Service 2012). Adequate sampling still remains an issue because many potential habitats are remote or otherwise difficult to access, and traditional sampling is costly and labor intensive. More efficient, sensitive, and noninvasive methods for detecting these fish would help biologists better understand the current distribution of the species and direct conservation efforts.

Given its advantages, eDNA sampling may be effective for documenting the presence and distribution of species like Loach Minnow and Spikedace. To support this sampling, Dysthe et al. (2016) developed quantitative PCR (qPCR) assays for detecting Loach Minnows and Spikedace. The authors did not, however, test the efficacy of eDNA sampling compared with traditional sampling or how detection rates varied between these taxa in different streams and at different densities. Consequently, our objectives were to (1) determine whether Spikedace and Loach Minnow eDNA could be consistently detected in locations where they were also detected with traditional sampling, (2) estimate eDNA production and persistence rates for low densities of Spikedace and Loach Minnows, and (3) determine whether there was a relationship between the density of Spikedace at a location in a stream and the amount of eDNA in samples collected downstream. For the third objective, we made an a priori prediction that there would be a positive relationship between the density of caged fish and the amount of eDNA collected downstream. The results of this study will assist managers in developing strategies to cost-effectively survey for rare, small-bodied fish in streams and to interpret results of eDNA surveys.

METHODS

Environmental DNA sampling versus traditional sampling.— To compare the performance of eDNA sampling to traditional methods for detecting Spikedace and Loach Minnows, we surveyed 33 locations across seven streams in Arizona and New Mexico using eDNA sampling and a combination of electrofishing and seining (Figure 1; Table 1). The locations ranged in elevation from 1,147 to 1,949 m and were distributed from mixed conifer forest to desert scrub biomes. These streams contained remnant or recently repatriated populations of these species, where traditional sampling had been used for over 5 years to monitor their relative abundance and distributions. At the downstream end of each traditional survey reach, we collected eDNA samples in areas of flowing water, typically within 2–3 m of the stream bank. Because traditional sampling could stress fish and cause them to move, the eDNA samples were collected immediately before sampling with traditional techniques (see details of eDNA sample collection and analysis below).

Traditional sampling methods varied by state, reflecting the standard sampling methods for Spikedace and Loach

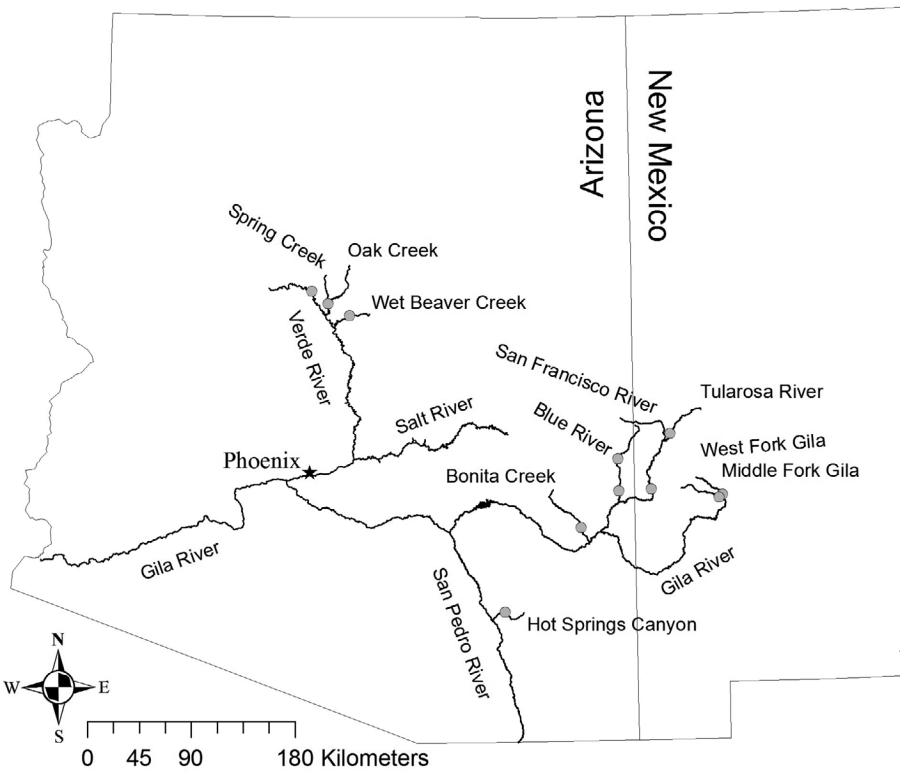

FIGURE 1. Map showing locations (gray circles) where eDNA was collected in streams of the Gila River basin in Arizona and New Mexico.

Minnows practiced by each state. In Arizona, a three-person crew consisting of a backpack electrofisher operator and two netters sampled all sites with a single, upstream pass. Sample sites (reaches) were 100 m long, except for in the Blue River, where they were 200 m long to encompass at least one pool–riffle–run sequence. We did not use block nets. Electrofisher settings varied by stream because of different stream sizes and conductivity, but operators set output to maximize catch while minimizing harm to fish (NCTC 2018). We collected stunned fish with dip nets and identified them to species. We counted all Spikedace and Loach Minnows and recorded total electroshocking time (in seconds) for each surveyed site. In New Mexico, we sampled 138–258-m-long reaches in a single pass, but sampling method depended upon mesohabitat (Paroz et al. 2006). We seined smooth-bottomed, slow-velocity mesohabitats, backpack electrofished slow-velocity, cobble- or debris-bottomed mesohabitats, and used a seine and backpack electrofisher in tandem to collect fish from riffles, cascades, and chutes. We calculated catch per unit effort (CPUE) for both states as the number of fish captured per 100 m.

Detection-at-distance experiments.— We examined longitudinal variation in the amount of eDNA collected downstream from caged Spikedace and Loach Minnows in two Arizona streams, the Verde River and Wet Beaver Creek, to assess if eDNA production and persistence rates differed among two different-sized streams. We chose these streams because they were historically occupied by both species but with no recent observations of either (Loach Minnows were last reported in both systems in 1938, whereas Spikedace were last reported in Wet Beaver Creek in 1938 and in the Verde River in 1999). The absence of these species from the systems meant that eDNA results were unlikely to be influenced by fish present outside the experimental cages. While there were no approved plans to stock the species into these streams, if cages did malfunction the fish would escape into previously occupied systems. The Verde River and Wet Beaver Creek are in a different drainage and more than 480 km from any of the previously mentioned traditional sampling sites (Figure 1). During the experiment, flows in the Verde River were 1.64–1.67 m^3/s at the U.S. Geological Survey (USGS) gauge (09504000) approximately 2.3 km downstream of the study reach; wetted width of the reach was 15–25 m. Because of the width and frequency of flooding, the riparian canopy covered 15–25% of the stream width. The study reach was a low-gradient C channel (Rosgen and Silvey 1996). In Wet Beaver Creek, flows during the experiment were 0.17–0.20 m^3/s at the USGS gauge (09505200) located 4.3 km upstream of the experiment location. Stream width in the study reach was 3–10 m,

TABLE 1. Reach lengths and measured discharge or mean daily discharge at USGS flow gauges nearest to the locations of paired eDNA and traditional surveys, on the day of sampling. Multiple sites were sampled in most streams, and reach lengths varied in the Middle Fork Gila River.

Location	Date	Discharge (m^3/s)	Gauge number or measured	Reach length (m)
New Mexico				
Gila River	Oct 4, 2016	6.23	09430500	200
	Oct 4, 2016	6.23	09430500	200
	Oct 2, 2017	1.42	09430500	190
Middle Fork Gila River	Jun 27, 2017			165–227
	Jun 28, 2017			138–145
	Jun 29, 2017			185–258
	Jun 30, 2017			177
West Fork Gila River	Oct 3, 2017	1.39	Measured	150
San Francisco River	Oct 4, 2016	1.22	09444000	200
	Oct 2, 2017	0.54	09444000	200
Tularosa River	Oct 3, 2016	0.06	Measured	165
	Oct 1, 2107	0.05	Measured	165
Arizona				
Blue River	Oct 5, 2016	0.40	09444200	200
	Oct 24, 2017	0.11	09444200	200
Bonita Creek	Sep 26, 2017	0.09	09447800	100
Hot Springs Canyon	Sep 19, 2016			100

and the riparian canopy extended across much of the channel, which was also a low-gradient C channel.

We acquired Spikedace and Loach Minnows used in the experiments from the Arizona Game and Fish Department's Aquatic Research and Conservation Center, and their use was permitted under a Native Endangered and Threatened Species Recovery permit from the U.S. Fish and Wildlife Service. We transported 20 Spikedace (total length = 67–93 mm; total fish weight = 72 g) and 15 Loach Minnows (total length = 44–68 mm; total fish weight = 23 g) in aerated coolers to the Verde River and Wet Beaver Creek. We set two cages, one for each species, in locations with moderate current velocities a few meters from shore. Before placing fish in cages, we collected three eDNA control samples 25–50 m upstream of the cages to ensure that eDNA of the target species was not already present. About 1 h before collecting the control samples, we used fluorescein dye to estimate water travel time from the cages to the sampling locations. We dispensed 50–100 mL of dye from a plastic bottle into the stream at the cage location and started a timer. We followed the dye downstream, adding more as necessary to ensure detection. We recorded the dye arrival time at each of six sampling locations (50, 100, 200, 300, 400, and 500 m downstream of the cages).

We placed fish in cages in the Verde River on June 17, 2016, and in Wet Beaver Creek on June 18, 2016 (Table 2). We collected three eDNA samples from each of the six sampling locations moving sequentially downstream from the cages. To ensure that the stream had been exposed to the target species long enough for DNA to accumulate and drift downstream, eDNA samples were collected after the sampling sites had been exposed to caged fish at least four times as long as the estimated travel time of fluorescein dye from the cages to the given sampling location.

Fish density experiment.—To understand the relationship between Spikedace density and eDNA quantity, we conducted experiments with caged fish held at varying densities in the Blue River and Spring Creek, Arizona (Figure 1). We chose these streams because there were already approved plans to introduce Spikedace to these streams. We conducted the experiments before, but on the same day that, Spikedace were stocked. We did not experiment with Loach Minnows because they were present in the Blue River and were not planned to be introduced into Spring Creek.

The Blue River is in east-central Arizona, near the New Mexico border. It flows for 82 km from its headwaters to its mouth at the San Francisco River. Discharge during the day of the experiment (September 13, 2017) was 0.09–0.12 m^3/s at a USGS gauge (09444200) 38 km downstream from the study area. Water temperatures at the cage locations on the day of the experiment were 19.3–22.5°C. A fish barrier was constructed near the mouth in 2012 to prevent the upstream movement of nonnative fish. There are no historical records of

TABLE 2. Site characteristics for the field experiment to detect eDNA at fixed distances downstream of caged fish. The negative values of collection distance indicate where the control samples were collected upstream of the cage location. Dye was added at the cage location to determine water travel duration, which was then used to estimate when eDNA arrived at each site after caged fish were put in the water. The estimated eDNA arrival time at the cage location (0 m) is when the cages of fish were set in the water.

Stream	Collection distance from cage (m)	Dye travel duration (min)	Estimated eDNA arrival time (hours)	Sample collection time (hours)	Exposure duration (min)
Verde River	−38			1230	
	0		1034		
	50	1.8	1036	1300	144
	100	7.8	1042	1335	173
	200	11.0	1045	1401	196
	300	15.9	1050	1435	225
	400	19.0	1053	1515	262
	500	40.0	1114	1545	271
Wet Beaver Creek	−22			0920	
	0		0745		
	50	4.4	0750	0949	119
	100	10.2	0755	1016	141
	200	18.2	0803	1045	162
	300	36.4	0821	1118	177
	400	46.3	0836	1152	196
	500	54.3	0839	1225	226

Spikedace in the Blue River, but they were stocked into the lower portion of the river in 2012 and became established. However, because portions of the Blue River are intermittent, Spikedace are restricted to the lower portion of the river and have not been observed at the location of the experiment, 33 km upstream of the introduced population.

Spring Creek is a perennial stream that flows 5.5 km from its spring source to its confluence with the Verde River near Cornville in central Arizona. There is no USGS gauge on Spring Creek, but median monthly discharge ranges from 0.10 to 0.13 m^3/s (ADWR 2008). There are no historical records of Spikedace in Spring Creek, but they were stocked into the middle portion of the creek in 2015 and 2016. During poststocking monitoring, 3 Spikedace were captured in 2015, 1 in 2016, and 11 in 2017, so it was unclear if the species had established. The location chosen for the density experiment was the same as the stocking location, and it was assumed that Spikedace were either absent or at very low abundance in the stream. We performed the experiment on February 21, 2018. Water temperatures at the cage locations on the day of the experiment were 14.8–17.1°C.

To test the relationship between fish density and eDNA production, we conducted a trial in two locations in each stream. Each trial had seven density treatments, including a no-fish control, but densities varied among trials (Table 3). We collected Spikedace used in the Blue River experiments from 33 km downstream and transported them to the study reach in aerated coolers. We acquired Spikedace used in the Spring Creek experiments from the Aquatic Research and Conservation Center.

For each trial in each stream, we placed new cages that had never been exposed to fish DNA in the study reach 100 m upstream of the eDNA sampling site. Before any fish were put in the cages, we determined water travel time between the cage and the eDNA sampling site using dye tracers as described for the detection-at-distance experiments above. Water travel times in the Blue River were 8.53 min for the first trial and 10.23 min for the second trial and in Spring Creek were 4.03 min for the first trial and 8.22 min for the second trial. Prior to adding fish to the cages, we collected a control sample at the eDNA sampling site to verify that Spikedace were absent from the study areas. We recorded the duration of filtering and sample processing for this control sample. Thereafter, we added fish to the cages at intervals of approximately twice the water travel time plus the filtering and processing time. For each density, we began eDNA sample collection at twice the water travel time from when the fish were added to the cages in an effort to allow eDNA concentrations associated with a specific density of fish to equilibrate. To ensure independence between trials, we conducted the second trial in each stream in new cages, 150 m upstream of the first cage location. We used a unique batch of Spikedace individuals in each trial.

TABLE 3. The number and mass (g) of fish held in cages for each treatment and each trial of the experiment comparing Spikedace density with eDNA quantity.

Fish per treatment	Blue River fish mass (g)		Spring Creek fish mass (g)	
	Trial 1	Trial 2	Trial 1	Trial 2
0 (control)	0		0	0
1			0.3	0.3
2	1	1		
4	2	3	1	3
8	4	7	2	6
16	7	12	5	10
32		28		
64	30		32	39
128	82	87	63	75
256	149	175	133	143

Environmental DNA sample collection and laboratory analysis.— We collected all eDNA samples following methods outlined by Carim et al. (2016b). Briefly, for each sample we pumped 5 L of water through a 1.5-µm-pore-size, glass-fiber filter. We placed the filter in a plastic bag containing silica desiccant, and then sealed it in an envelope labeled with sampling information. Samples were kept at ambient temperature in the field and stored in a freezer before being shipped to the National Genomics Center for Wildlife and Fish Conservation in Missoula, Montana, for analysis.

Upon receipt of samples, we catalogued and stored them at −20°C until DNA was extracted. For each sample, we extracted eDNA from half of the sample filter using the Qiagen DNEasy Blood and Tissue Kit following a modified protocol described in Carim et al. (2016a). The other half of the sample filter was retained and stored at −20°C. If more than one filter was used to collect the sample, as a consequence of a filter clogging from suspended sediment or biological material, DNA from all filters for a given sample was combined during DNA extraction. Samples were analyzed for the presence of DNA of the target species in triplicate on a qPCR instrument (StepOnePlus or QuantStudio 3 Real-Time PCR System; Life Technologies) following methods described by Dysthe et al. (2016). We conducted all laboratory experiments with negative controls to ensure there was no contamination during DNA extraction or qPCR setup.

A sample was considered positive for the presence of the target species if at least one of the three qPCR reactions amplified DNA of that species. All reactions included an internal positive control (TaqMan Exogenous Internal Positive Control Reagents; Life Technologies) to screen for PCR inhibition. A sample was considered inhibited if amplification of the internal positive control was delayed by ≥ 1 cycle relative to the negative control. If any sample appeared inhibited, we extracted DNA from the second half of the filter, combined DNA from both filter halves, and processed them using the OneStep PCR Inhibitor Removal Kit (Zymo Research; www.zymoresearch.com). We then reanalyzed each sample in triplicate qPCR reactions as described above.

To estimate the number of target DNA copies in each sample from the caged-fish experiments, we analyzed samples alongside a seven-level standard curve (31,250, 6,250, 1,250, 250, 50, 10, and 2 copies per reaction) following the same conditions mentioned above. Standard curves were created for each target species by purifying qPCR product with the GeneJET PCR Purification Kit (ThermoFisher Scientific), quantifying the cleaned product on a Qubit 2.0 Fluorometer, and serially diluting to the specified concentrations in sterile tris–EDTA buffer. Using the linear regression from each standard curve, we estimated the number of target DNA copies in each reaction. All laboratory experiments were set up inside of a hood that was irradiated with UV for at least 30 min prior to PCR setup.

Statistical analysis.— We recorded the number of locations with detections using eDNA sampling and traditional methods and the congruence between the two methods. We used contingency table analysis and the Phi coefficient (IBM SPSS Statistics, version 23) to evaluate the consistency in species detection between eDNA and traditional sampling in areas where Spikedace and Loach Minnows were documented at least once between 2007 and 2016.

We estimated the relationship between DNA concentration (copies/L) and the distance from caged fish using an exponential regression model: $N_x = N_0 e^{-kx}$, where N_x is the eDNA concentration at distance x, N_0 is the eDNA concentration at distance 0, and k is the longitudinal loss rate of eDNA (Paul and Hall 2002). The values N_x and x are data measured in our field experiments, while N_0 and k are parameters estimated by the model. We obtained N_x by averaging the measured eDNA concentration across the three samples at each distance. We selected an exponential regression model both because the model parameters have a sensible biological interpretation and because similar models have been used for similar research questions (Paul and Hall 2002; Wilcox et al. 2016; Nukazawa et al. 2018). The loss rate, k, is closely related to the transport length, $1/k$, which is the average distance traveled by eDNA. In addition, e^{-k} equals the persistence rate, or the percent of eDNA retained in the stream flow over each unit of stream distance. Finally, the depositional velocity of eDNA, which is the rate at which eDNA moves towards the stream bed, can be calculated as $v = kQ/w$, where v is depositional velocity, Q is stream discharge in

cubic meters per second, and w is the wetted stream width in meters.

We extended the basic model described above to estimate the effect of each stream (Wet Beaver Creek and Verde River) and each fish species (Spikedace and Loach Minnow) and their interactions on eDNA production, N_0, and loss rate, k. The most general model we fit included interactions on both N_0 and k:

$$N_x = \begin{bmatrix} S \times V \times N_{(SV)} + S \times W \times N_{(SW)} + L \times V \\ \times N_{(LV)} + L \times W \times N_{(SW)} \end{bmatrix} \times e^{-[S \times V \times k(SV) + S \times W \times k(SW) + L \times V \times k(LV) + L \times W \times k(LW)]x},$$

where S, L, V, and W are dummy variables indicating if the trial was conducted with Spikedace, with Loach Minnows, in the Verde River, or in Wet Beaver Creek, respectively, and production and loss rates are estimated for each interaction (e.g., $N_{(SV)}$ is the production rate for Spikedace in the Verde River).

We fit six additional models, including a null model with no covariates, by removing variables from this general model (Table 4). Because production rate may vary between species and because differences in abiotic factors, such as temperature, flow, and channel roughness, may cause persistence of DNA to differ between streams (Pilliod et al. 2013; Pont et al. 2018), we developed a model set that emphasized the effect of fish species on production rates and the effect of stream on persistence rates. Because we fit nonnested models to small data sets, we used Akaike information criterion corrected for small sample size (AIC_c) to identify the most parsimonious model from this set (Hurvich and Tsai 1989). We performed these analyses in Program R version 3.5.1 (R Core Team 2018) using the "nls" function in the "stats" package. We estimated standard errors for all transformed parameters (i.e., transport length, persistence, and depositional velocity) using the delta method (Williams et al. 2002:736–737).

We estimated the relationship between the density of Spikedace (number of fish or grams of fish) and DNA concentration (copies/L) using a linear regression model with no intercept. We excluded the intercept because this ensured that the model would never predict a negative quantity of eDNA and that the model would never predict a positive quantity of eDNA in the absence of fish. We used a linear model because we expected each fish to add a similar quantity of eDNA to the stream. For each independent variable—number of fish or biomass (g) of fish—we fit three models. In the simplest model, the quantity of eDNA recovered was only affected by the number of fish. In the second model, the quantity of eDNA was also affected by the stream in which data were collected. In the third model, eDNA was also affected by the specific trial (reach) in which data were collected. We compared the fit of these nested models using likelihood ratio tests. Because we initially observed high heteroscedasticity in the residuals (the variance increased with the density of fish), we used the sandwich variance estimator, also known as the robust variance estimator or White's variance estimator, which does not rely on an assumption of homoscedasticity (White 1980). We present the resulting estimates and compare the fit of models using number of fish and grams of fish, using the R^2 value. We performed these analyses in Program R (version 3.5.1) using packages "sandwich" and "lmtest" for the sandwich variance estimator.

RESULTS

Environmental DNA Sampling versus Traditional Sampling

The detection of Spikedace and Loach Minnows by eDNA sampling was significantly correlated to detection using traditional sampling (Phi = 0.734, $P < 0.001$ for Loach Minnows; Phi = 0.603, $P = 0.001$ for Spikedace). Both methods detected Loach Minnows at 22 sites and neither technique detected this species at 7 sites (Table 5). At four sites, however, Loach Minnow DNA was detected where the species was not captured with traditional methods. At one of those three sites, Loach Minnow was detected further upstream (about 3.8 km upstream) by both methods. Spikedace were detected with both

TABLE 4. Model set, ranked by AIC_c, for estimating the relationship between DNA quantity (copies/L) and the distance (in 100-m increments) separating caged fish and the eDNA sampling location.

Production rate covariates	Persistence rate covariates	Parameters	ΔAIC_c	w_i
Fish species × stream	Stream	7	0.00	0.93
Fish species × stream	Fish species × stream	9	5.23	0.07
Species	None	4	17.67	0.00
Species	Stream	5	20.49	0.00
Species	Fish species × stream	7	25.96	0.00
None	None	3	36.08	0.00
None	Stream	4	38.90	0.00

TABLE 5. Detection of Loach Minnows using traditional sampling techniques (captures/100 m) and with eDNA collected at the downstream end of fish survey locations in several streams in Arizona and New Mexico. In the historical occupancy column, Y denotes locations where this species has been collected within the last 10 years and question marks denote locations where its presence at a site was unknown before the paired traditional and eDNA sampling. Survey locations are specified as river kilometers (RKM), measuring up from the mouth of the given stream.

Location	Date	RKM	Historical occupancy	Captures/100 m	Detected eDNA
New Mexico					
Gila River	Oct 4, 2016[a]	942.6	Y	6.0	Yes
	Oct 4, 2016	953.9	Y	4.0	Yes
	Oct 2, 2017	954.1	Y	33.0	Yes
Middle Fork Gila River	Jun 27, 2017	4.1	Y	12.3	Yes
	Jun 28, 2017	6.7	Y	20.0	Yes
	Jun 28, 2017	8.4	Y	12.3	Yes
	Jun 29, 2017	11.3	Y	0.0	Yes
	Jun 29, 2017	15.1	Y	6.3	Yes
	Jun 29, 2017	17.5	?	0.0	No
	Jun 27, 2017	20.3	Y	0.0	Yes
	Jun 27, 2017	23.8	?	0.0	No
	Jun 27, 2017	25.0	?	0.0	No
	Jun 29, 2017	26.3	?	0.0	No
West Fork Gila River	Oct 3, 2017	9.9	Y	27.0	Yes
San Francisco River	Oct 4, 2016	107.6	Y	40.8	Yes
	Oct 2, 2017	107.6	Y	128.0	Yes
Tularosa River	Oct 3, 2016	16.0	Y	0.0	Yes
	Oct 1, 2107	16.0	Y	10.3	Yes
Arizona					
Blue River	Oct 24, 2017	11.6	Y	14.5	Yes
	Oct 5, 2016[a]	12.6	Y	2.5	Yes
	Oct 24, 2017	12.6	Y	34.5	Yes
	Oct 24, 2017	13.7	Y	6.5	Yes
	Oct 24, 2017	14.6	Y	9.0	Yes
	Oct 24, 2017	17.5	Y	11.5	Yes
Bonita Creek	Sep 26, 2017	17.2	?	0.0	No
	Sep 26, 2017	17.6	?	0.0	No
	Sep 26, 2017	17.7	?	0.0	Yes
Hot Springs Canyon	Sep 19, 2017	16.1	Y	2.0	Yes
	Sep 19, 2017	16.4	Y	2.0	Yes
	Sep 19, 2017	17.1	Y	10.0	Yes
	Sep 19, 2016	17.8	Y	32.0	Yes
	Sep 19, 2016	17.9	Y	15.0	Yes
	Sep 19, 2016	18.1	?	0.0	No

[a]Sample treated to remove PCR inhibitors.

techniques at 15 sites, were not detected by either technique at 8 sites, and were detected with eDNA sampling alone at 7 sites (Table 6). At two of those seven sites, Spikedace were detected further upstream (1.7 and 11.0 km) by both methods. Within the last 10 years, there were observations of the target species at all of the 10 sites in which only eDNA sampling detected these species, suggesting that they may have been present in low numbers but not detected by traditional means. At one site in Hot Springs Canyon, neither method detected either species despite observations of Loach Minnows in 2014 and Spikedace in 2010, which may indicate recent loss of these species from this site.

Detection-at-Distance Experiments

In the Verde River and Wet Beaver Creek, Spikedace and Loach Minnow eDNA were detected in at least one of three samples at all sampling locations within the study

TABLE 6. Detection of Spikedace using traditional sampling techniques (captures/100 m) and with eDNA collected at the downstream end of fish survey locations in several streams in Arizona and New Mexico. In the historical occupancy column, Y denotes locations where this species has been collected within the last 10 years, N denotes locations where this species has not been collected, and question marks denote locations where its presence at a site was unknown before the paired traditional and eDNA sampling. Survey locations are specified as river kilometers (RKM), measuring up from the mouth of the given stream.

Location	Date	RKM	Historical occupancy	Captures/100 m	Detected eDNA
New Mexico					
Gila River	Oct 4, 2016[a]	942.6	Y	0	Yes
	Oct 4, 2016	953.9	Y	16.0	Yes
	Oct 2, 2017	954.1	Y	84.2	Yes
Middle Fork Gila River	Jun 27, 2017	4.1	Y	5.7	Yes
	Jun 28, 2017	6.7	Y	0	Yes
	Jun 28, 2017	8.4	Y	10.9	Yes
	Jun 29, 2017	11.3	Y	8.5	Yes
	Jun 29, 2017	15.1	Y	0	Yes
	Jun 29, 2017	17.5	?	0	No
	Jun 27, 2017	20.3	?	0	No
	Jun 27, 2017	23.8	?	0	No
	Jun 27, 2017	25.0	?	0	No
	Jun 29, 2017	26.3	?	0	No
West Fork Gila River	Oct 3, 2017	9.9	Y	50.2	Yes
San Francisco River	Oct 4, 2016	107.6	Y	0	Yes
	Oct 2, 2017	107.6	Y	3.2	Yes
Tularosa River	Oct 3, 2016	16.0	N	0	No
	Oct 1, 2107	16.0	N	0	No
Arizona					
Blue River	Oct 24, 2017	11.6	Y	56.0	Yes
	Oct 5, 2016[a]	12.6	Y	20.0	Yes
	Oct 24, 2017	12.6	Y	20.5	Yes
	Oct 24, 2017	13.7	Y	26.0	Yes
	Oct 24, 2017	14.6	Y	13.0	Yes
	Oct 24, 2017	17.5	Y	40.0	Yes
Hot Springs Canyon	Sep 19, 2016	16.1	Y	1.0	Yes
	Sep 19, 2016	16.4	Y	3.0	Yes
	Sep 19, 2016	17.1	Y	0	Yes
	Sep 19, 2016	17.8	Y	0	Yes
	Sep 19, 2016	17.9	Y	0	Yes
	Sep 19, 2016	18.1	?	0	No

[a]Sample treated to remove PCR inhibitors.

reaches (Table 7). However, species eDNA was detected in more of the samples from Wet Beaver Creek than from the Verde River.

The best-supported model relating eDNA quantity and distance from experimental fish, with 93% of the AIC_c weight, indicated that production rates were different for each stream × species combination and persistence rates differed among streams (Table 4). This model indicated that production rates were greater for Spikedace than for Loach Minnows and they were greater in the Verde River than in Wet Beaver Creek (Figure 2; Table 8). Under this model, persistence rates over the 100-m reach were very high (94%; with 95% CI = 84–105%) in Wet Beaver Creek and much lower in the Verde River (47%; with 95% CI = 36–58%). Persistence rates did not differ among fish species. Equivalently, transport length was much greater in Wet Beaver Creek (1,697 m; 95% CI = 0–49,307 m) than in the Verde River (132 m; 95% CI = 90–175 m). Consequently, depositional velocity was much lower in Wet Beaver Creek (0.0017 m/s; 95% CI = 0.0–0.0050 m/s) than in the Verde River (0.0627 m/s; 95% CI = 0.0426–0.0828 m/s).

TABLE 7. Positive detections and DNA quantities in samples collected at increasing distances downstream from caged Spikedace and Loach Minnows in two Arizona streams. For each species and each stream, we list the number of eDNA samples with positive detections ($N = 1$–3) and the mean DNA copies/L.

		Distance from cages (m)					
Stream and species	Metric	50	100	200	300	400	500
Verde River							
Spikedace	*N*	3	3	2	3	3	1
	Mean	38.9	25.8	6.4	9.6	11.0	2.5
	Range	16.9–66.3	19.3–30.2	0.0–13.2	7.2–12.8	2.6–23.3	0.0–7.6
Loach Minnow	*N*	3	3	1	2	3	1
	Mean	14.5	4.0	0.5	1.4	1.7	0.2
	Range	7.7–18.5	2.0–5.1	0.0–1.4	0.0–2.8	0.9–3.3	0.0–0.5
Wet Beaver Creek							
Spikedace	*N*	3	3	3	3	3	3
	Mean	18.3	13.0	16.8	12.7	11.4	14.7
	Range	10.3–27.8	2.6–30.0	5.4–24.7	3.6–27.9	5.6–18.7	9.4–17.9
Loach Minnow	*N*	3	3	3	2	2	3
	Mean	5.3	2.7	2.9	4.3	1.1	3.3
	Range	2.1–7.1	1.6–3.2	0.8–4.0	0–9.5	0.0–2.0	2.3–4.4

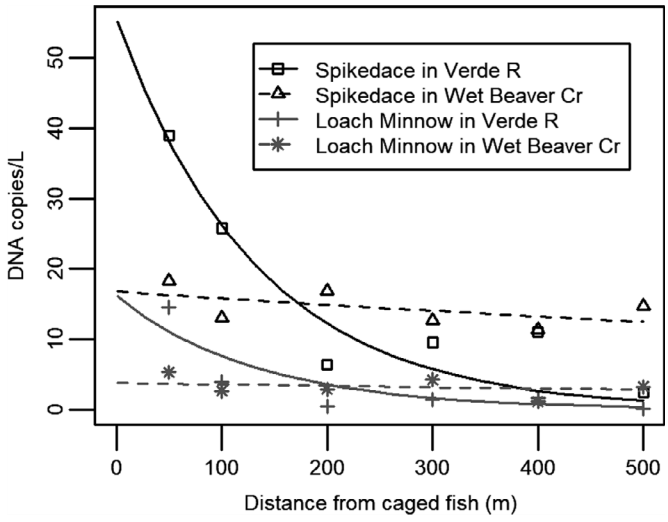

FIGURE 2. Variation in eDNA concentrations with respect to distance from caged specimens of Spikedace and Loach Minnow in the Verde River and Wet Beaver Creek, Arizona. The points represent the means of measured values, and the lines are estimated from the models.

Fish Density Experiments

The amount of Spikedace eDNA collected 100 m downstream of experimental cages was positively related to the number of Spikedace held in those cages (Figure 3). Considering the number of fish as a predictor, the model including the effect of each stream fit significantly better than the model that excluded any stream effect [$\chi^2(1) = 72.25$, $P < 0.0001$]. The model including the effect of each trial did not significantly improve model fit [$\chi^2(2) = 3.19$, $P = 0.20$] and was disregarded. Under the preferred model, the effect of fish numbers was significantly positive in both streams, although each fish in Blue River ($\beta = 12.39$, $P < 0.0001$) was associated with nearly nine times as many eDNA copies/L as each fish in Spring Creek ($\beta = 1.42$, $P < 0.0001$; Figure 3). Results using the biomass of Spikedace were similar, as each gram of fish in Blue River ($\beta = 16.65$, $P < 0.0001$) was associated with about six times as many eDNA copies/L as each gram of fish in Spring Creek ($\beta = 2.60$, $P = 0.018$), but the coefficient of determination was lower for the model using biomass ($R^2 = 0.94$) than for model using number of fish ($R^2 = 0.96$).

DISCUSSION

Our results indicate that eDNA sampling methods may be more sensitive for detecting target species than traditional sampling methods and may increase the probability of detection for rare, small-bodied fish in stream settings. We followed extensively tested protocols to avoid sample contamination in the field (Carim et al. 2016b) and laboratory (Goldberg et al. 2016) and so think it unlikely that the eDNA detections where the species was not detected with traditional sampling were in error. Both Spikedace and Loach Minnows were detected at more sites using eDNA sampling than using traditional sampling, but at 3 of 11 sites where eDNA alone detected the species, they were detected 1.7 to 11 km further upstream by both methods. However, all 11 sites were known to be occupied in the last 10 years based on monitoring by Arizona and New Mexico

TABLE 8. Estimated parameters in the top-ranked models relating DNA quantity and the distance to the water sampling location.

Rate	Estimate	SE	*t*	*P*
Production, Spikedace, Verde River (DNA copies/L)	55.49	6.60	8.41	<0.001
Production, Spikedace, Wet Beaver Creek (DNA copies/L)	16.79	2.68	6.26	<0.001
Production, Loach Minnow, Verde River (DNA copies/L)	16.18	4.06	3.98	0.001
Production, Loach Minnow, Wet Beaver Creek (DNA copies/L)	3.82	1.61	2.37	0.029
Persistence, Verde River (proportion retained per 100 m)	0.47	0.06	8.10	<0.001
Persistence, Wet Beaver Creek (proportion retained per 100 m)	0.94	0.05	17.45	<0.001

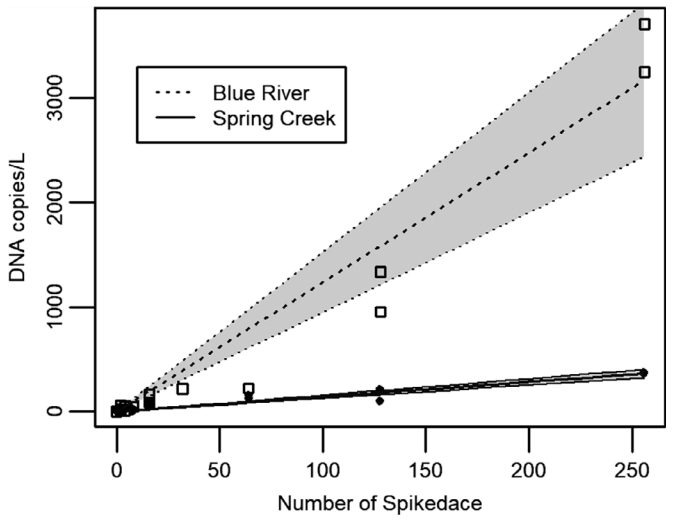

FIGURE 3. Relationship between fish density and eDNA quantity in two Arizona streams. Open squares and dashed lines represent Spikedace in the Blue River. Dots and solid lines represent Spikedace in Spring Creek. The shaded areas represent the 95% confidence intervals.

Game and Fish agencies. As a result, it is unknown if eDNA detections at these three sites is the result of downstream drift of DNA from fish upstream or if traditional sampling methods were simply not sensitive enough to detect the fish present within the study reach. Other experiments have demonstrated that the detection rates of stream fishes can be several-fold higher for eDNA sampling than for more traditional methods (Wilcox et al. 2016), although this effectiveness is contingent upon eDNA sampling with sufficient intensity (Perez et al. 2017; Ulibarri et al. 2017; Wilcox et al. 2018). In our density experiments, Spikedace DNA was detected in 100% of eDNA samples collected within 100 m of as few as two fish. In our detection-at-distance experiments, we detected the DNA of each species in at least one of three samples at distances up to 500 m downstream from caged individuals. We did not sample for eDNA further downstream, so the downstream detection limit is uncertain. However, our model provided estimated transport lengths of 1,697 m in Wet Beaver Creek and 132 m in the Verde River, but the confidence interval for the Wet Beaver Creek estimate was very wide, indicating the estimate may not be very accurate.

Models of fine particulate organic matter dynamics in streams (Thomas et al. 2001; Paul and Hall 2002) also appear to capture the behavior of eDNA in streams (Wilcox et al. 2016; Carraro et al. 2018; Pont et al. 2018). Those models suggest that downstream detection limits are primarily a function of DNA transport distance (the mean distance traveled by each molecule) and dilution. Our observations of apparent production and persistence in the distance experiment are consistent with these processes. There is an exponential decline in particulate concentrations downstream from point sources (Minshall et al. 2000), with the decline well approximated by a linear relationship at greater distances from a source (also see Jane et al. 2015). It may seem puzzling that the apparent production of eDNA by both species was greater in the Verde River than in Wet Beaver Creek, but this is consistent with the order-of-magnitude differences in discharge between these streams. The greater discharge in the Verde River would lead to greater DNA transport rates in the vicinity of the cages and greater apparent production, whereas transport rates over the initial measurement interval (50 m) in the much smaller Wet Beaver Creek would have been substantially lower (and associated with greater local deposition), approaching a near-linear decline at the distance where the first collections of DNA were made. A collection of samples in Wet Beaver Creek between the cages and 50 m downstream would have likely revealed the initial exponential decline. The higher estimate of eDNA persistence in Wet Beaver Creek compared with the Verde River can be explained by the greater dilution in the larger system. The DNA persistence patterns for a given creek were similar between species, indicating that variation in persistence was driven more strongly by differences between streams than between species. The greater apparent production for Spikedace than for Loach Minnows was expected given the greater number and biomass of the former.

Similar to other studies (Takahara et al. 2012; Klymus et al. 2015; Wilcox et al. 2016; Doi et al. 2017), we found a positive relationship between fish abundance and the amount of eDNA collected, although this relationship was stream-specific. Temperature is reported to affect DNA

production (Jo et al. 2019), as is body size (Maruyama et al. 2014), which may have contributed to less eDNA detected in Spring Creek, which was 5°C colder and had less total fish mass than in the Blue River. We attempted to maximize any relationships between abundance or biomass and the amount of eDNA in the caged-fish experiments by controlling for fish size and environmental conditions within each stream. For free-swimming wild populations, these relationships will be complicated by other factors (e.g., fish-size-related or seasonal variation in eDNA production; Maruyama et al. 2014; Bylemans et al. 2017). Furthermore, others have shown that persistence rates differ among streams of various sizes and characteristics (Wilcox et al. 2016; Pont et al. 2018). Therefore, it may be some time before eDNA concentrations can be used as a reliable index of abundance to make valid comparisons among reaches and streams.

To our knowledge, this is among the first studies to effectively use eDNA sampling in southwestern USA streams. Our results validate eDNA sampling as an effective tool for detection of small-bodied stream fish that may be present in low abundance and adds to a larger body of research aimed at understanding detection probabilities for aquatic species across a range of stream ecosystems. With the sensitivity to detect even a single small-bodied fish in a 100-m reach, managers will be able to more effectively identify reaches occupied by threatened or endangered fish, even if a population is in decline. Coupled with the ability to detect species without the risk of injury or mortality of the species of concern, eDNA sampling is an increasingly valuable addition to the toolbox of fisheries managers.

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