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Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (Decapoda: Astacidea) through monitoring of invasive species barriers and relocated populations

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Abstract :

Native crayfish species often face competition and displacement by non-indigenous invasive crayfishes. Management responses implemented to preserve imperiled crayfishes may include the construction of physical barriers to prevent the spread of invasive crayfishes, and movement of native populations to "ark" sites that have not yet been invaded. These strategies require ongoing monitoring to determine their effectiveness. We propose that environmental DNA (eDNA), genetic material identified from environmental samples, can be useful for assessing advancing invasions and imperiled freshwater species associated with management interventions. We monitored a series of management interventions intending to isolate the endangered Shasta crayfish *Pacifastacus fortis* (Faxon, I941) from the invasive signal crayfish *Pacifastacus leniusculus* (Dana, 1852) in California, USA. We successfully detected P. fortis eDNA from two sites where it was known to occur, one site where its presence was uncertain, and one site (near an "ark" site) where it was believed absent. We also detected P. leniusculus eDNA from five sites it was known to occupy, but failed to detect its eDNA at two sites where it was believed to occur. We conclude with recommendations for improved eDNA monitoring of crayfish conservation and management interventions in the future.

Keywords : ark site, California, endangered crayfishes, *Pacifastacus fortis, Pacifastacus leniusculus*, translocation

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INTRODUCTION

Many native freshwater crayfish species are imperiled by impacts of non-indigenous invasive crayfishes, which often displace natives through mechanisms including competition, hybridization, and disease transmission (Lodge *et al.*, 2000). Tools available to prevent effects of

spreading invasive species on rare or imperiled native species in freshwater include managing habitat connectivity to isolate native species from invaders, often through barrier construction or maintenance (Fausch *et al.*, 2009), as well as relocation of native species to refuge or "ark" sites that have not yet been invaded (Fischer & Lindenmayer, 2000). Both of these management responses have been considered or implemented in protecting native crayfishes from impacts of invasive crayfishes, with examples ranging from studies evaluating barrier design for impassability to invasive crayfish (Frings *et al.*, 2013), to the active use of ark sites in conserving the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) in Great Britain (Kozák *et al.*, 2011; Haddaway *et al.*, 2012). Yet both of these management responses also require ongoing monitoring to confirm that invasive crayfishes have not spread above barriers or into ark sites where they may impact the native species, and that native species continue to persist where isolated. Detection of such populations is often difficult owing to their low densities or abundances, resulting in high research interest in developing more sensitive monitoring and surveillance tools (Lodge *et al.*, 2016).

Environmental DNA (eDNA) is one such methodology that shows considerable promise for both the early detection of new or spreading biological invasions (Ficetola *et al.*, 2008; Jerde *et al.*, 2011), as well as the monitoring of rare or endangered species that also frequently occur at low abundances (Thomsen *et al.*, 2012; Spear *et al.*, 2015). eDNA refers to genetic material extracted and identified from environmental samples, like water or sediment, to characterize the presence or abundance of focal organisms (Rees *et al.*, 2014; Barnes & Turner, 2016). Some studies have suggested that eDNA is more sensitive to the presence of organisms at low population densities or abundances than conventional sampling approaches (Jerde *et al.*, 2011; Thomsen *et al.*, 2012; Pilliod *et al.*, 2013), although this sensitivity also causes concerns about false positive detections arising from factors like eDNA transport or contamination of samples in

the field and laboratory (Deiner & Altermatt, 2014; Lahoz-Monfort *et al.*, 2015). Yet over the past decade, investigations into eDNA methodology have coalesced into recommended best practices (Goldberg *et al.*, 2016), and this approach is being increasingly applied to the real-world monitoring of important biological invasions (e.g., Jerde *et al.*, 2013) and surveillance for endangered species (e.g., de Souza *et al.*, 2016). Initial studies have so far shown promise for eDNA detection of both invasive and imperiled crayfishes (Dougherty *et al.*, 2016; Ikeda *et al.*, 2016), but also potential limitations for some crayfish taxa or habitats (Tréguier *et al.*, 2014). Given the growth of eDNA as a surveillance tool for both native and invasive biodiversity, we propose that this method may have high utility in simultaneously monitoring imperiled and invasive crayfishes around management activities like stream or river barrier construction and the use of ark sites.

The Shasta crayfish *Pacifastacus fortis* (Faxon, 1914) is narrowly endemic to middle reaches of the Pit River drainage of northeastern California, USA, where it generally occurs in headwater streams and springs (including spring-fed lakes) in lava boulder and cobble habitat (Daniels, 1980; Eng & Daniels, 1982; Light *et al.*, 1995; Ellis, 1999). *Pacifastacus fortis* has been severely impacted by competitive interactions and displacement by the invasive signal crayfish *P. leniusculus* (Dana, 1852; Ellis, 1999; Pintor *et al.*, 2008), in a parallel to effects of this same crayfish invader on imperiled, native crayfish species in both Europe and Japan (Vorburger & Ribi, 1999; Usio *et al.*, 2001). Owing to its small number of isolated populations and frequency of displacement by *P. leniusculus* where sympatric, *P. fortis* was listed as endangered under the U.S. Endangered Species Act in 1988 (U.S. Fish and Wildlife Service, 1988). To preserve remaining *P. fortis* populations from displacement by spreading *P. leniusculus*, crayfish impassable-barriers were proposed and constructed on several native-occupied stream or spring sites (Fig. 1, Table 1). *Pacifastacus leniusculus* unfortunately spread

upstream of barriers preceding or during their construction, although ongoing removal efforts using hand collection by snorkelers and divers seek to eradicate *P. leniusculus* from above barriers where *P. fortis* still persists. Additional conservation efforts have included relocating some *P. fortis* individuals to an isolated spring and pond site un-invaded by *P. leniusculus* with the intention of establishing a new allopatric (isolated) population (Fig. 1, Table 1). Evaluating the success of this conservation translocation has nevertheless been difficult due to the potential sensitivity of the habitat and organisms to intensive sampling for *P. fortis* survival.

These conservation interventions for the endangered *P. fortis* might benefit from eDNA surveillance, both for this imperiled crayfish as well as for invasive *P. leniusculus* that threaten its persistence. If eDNA is highly sensitive to detection of organisms at low population abundances, then this tool might be useful in investigating the distribution and persistence of hard-to-find *P. fortis* individuals in habitats throughout its narrowly endemic range. Further, eDNA may reveal whether *P. fortis* successfully established at its relocated site or persists above existing barriers, as well as if *P. leniusculus* has spread to or established in new locations. We sought to develop and field-validate an eDNA assay for *P. fortis,* while simultaneously applying an existing eDNA assay for *P. leniusculus* (Larson *et al.*, 2017), with a focus on efficacy of the above management measures for native crayfish conservation. Beyond implications for the conservation of this endangered crayfish species, our study also offers insights on utility, challenges, and limitations of using eDNA to monitor the effectiveness of management actions for imperiled crayfishes elsewhere (Kozák *et al.*, 2011).

METHODS

Study area

The historic distribution of *P. fortis* was highly restricted to the middle reaches of the Pit River in northeastern California and its tributaries, such as the Fall River, Hat Creek, Lava Creek, Rising River, and the Tule River, where *P. fortis* occurred primarily in headwater streams and springs or spring-fed lakes characterized by coarse lava substrates (Daniels, 1980; Eng & Daniels, 1982; Ellis, 1999). Recognizing its vulnerability as a highly-localized species, which had likely been impacted by disturbances over the 20th century, including hydropower development and the 1915 eruption of nearby Lassen Peak, the U.S. Fish and Wildlife Service listed *P. fortis* as endangered in 1988 (U.S. Fish and Wildlife Service, 1988). Range and population declines of *P. fortis* continued, however, largely through the expansion of non-native crayfishes into its habitats. These invasions included the virile crayfish *Faxonius virilis* (Hagen, 1870)*,* which rarely occurs in sympatry (together) with *P. fortis,* and the signal crayfish *P. leniusculus,* which frequently occurs in sympatry with *P. fortis* (Light *et al.*, 1995; Ellis, 1999; Pintor *et al.*, 2008). *Pacifastacus leniusculus* invasions, in particular, lead to extirpation of native *P. fortis* where sympatric over time. For example, *P. fortis* is currently presumed extirpated from approximately a third of sites where it was known to occur in 1978, and at each of these locations *P. leniusculus* replaced *P. fortis* as the numerically dominant crayfish species over a matter of decades (M.J.E. and K.G.H.B., unpublished data).

In response to the threat of invasive *P. leniusculus* to remaining populations of P. *fortis,* several management interventions were attempted over the past decade (Table 1). At two locations (Sucker Springs Creek, Upper Fall River), crayfish barriers were designed and installed, with the intent of preventing *P. leniusculus* from spreading upstream to impact allopatric *P. fortis* in isolated headwater streams originating from springs (Fig. 1; Ellis, 2005). In each case, *P. leniusculus* unfortunately arrived at these sites prior to completion of crayfish barriers. At the time of our study, *P. fortis* was believed to have been possibly extirpated by *P.*

leniusculus at Sucker Springs Creek, where no native crayfish have been observed since 2014 following a steady population decline. *Pacifastacus fortis* nevertheless persists above the barrier in the Upper Fall River, where active hand removal of invasive crayfish by divers or snorkelers seeks to reduce or eradicate the population of *P. leniusculus.* At an additional site (Spring Creek), road culverts may act as an effective if unplanned crayfish barrier, and hand removal by managers is similarly attempting to reduce population densities of *P. leniusculus* where sympatric with a remaining *P. fortis* population (Table 1).

Facing the loss of all remaining allopatric populations of *P. fortis* to invasion by *P. leniusculus*, 42 native crayfish were translocated to an isolated, spring-fed pond in 2013 and 2014, in an attempt to establish a new allopatric population (Fig. 1, Table 1). *Pacifastacus fortis* has not been observed from this spring-fed pond since shortly after its introduction, but this habitat is difficult to sample without potentially harming individuals of *P. fortis* while moving or searching lava rock substrate. Accordingly, eDNA could be an especially valuable tool for monitoring the status of this population in a non-destructive manner to both habitat and target animals. While our project involves an endangered species, the work performed did not involve the handling of either endangered or protected species, and was carried out under a U.S. Fish and Wildlife Service permit # TE806679-7 (01/30/2015 - 01/29/2019).

Field Sampling

We chose nine sites to sample for *P. fortis* and *P. leniusculus* using eDNA between 19 May and 2 June 2016 (Fig. 2, Table 1). These sites included the Upper Fall River and Sucker Springs Creek, where barriers were constructed to (unsuccessfully) exclude *P. leniusculus* from *P. fortis* populations (Ellis, 2005), as well as the relocation or ark site where *P. fortis* was introduced in 2013 and 2014 (Private Spring K). Additional sites selected included several where *P. fortis* has

been observed recently (Crystal Lake, Spring Creek), and a few sites where the status of *P. fortis* is unknown, because of private property access restrictions or the inability to survey large complex boulder substrate (Lava Creek, Pit River, Rising River). *Pacifastacus leniusculus* has been observed at all sample sites, where it is the numerically dominant crayfish, except for the relocation or ark site, as well as an adjacent spring-fed pond. This additional pond (Private Spring M) was sampled to evaluate potential movement of either *P. fortis* or its DNA from Spring K (potentially through a porous lava aquafer; Shogren *et al.*, 2016), as well as to confirm the absence of *P. leniusculus* in this isolated refuge system.

At most sample sites, we collected five total-eDNA water samples in proximity to each other (e.g., on a perpendicular transect across the width of a stream) from a single location, although at four sites we visited two to four total locations per site and took five replicated eDNA water samples at each location for 10–20 total eDNA samples (Table 1). Locations within sites were generally within a few hundred meters of each other, and included longitudinal stream gradients above and below invasive crayfish barriers at the Upper Fall River and Sucker Springs Creek sites, above and below the falls on the Pit River, and along the southern shoreline of Crystal Lake (Fig. 1). These efforts resulted in a total of 95 samples. Generally, eDNA water samples were deliberately collected on days that the authors had handled no crayfish; the exception was 19 May 2016 sampling at the Upper Fall River site, where eDNA water samples were taken and filtered immediately after the authors had been hand-removing *P. leniusculus* by snorkeling for several hours.

For each individual eDNA sample, we took 250 ml volumes of surface water in plastic bottles that had never been used. We stored water samples on ice in coolers for 1 to 2 hrs before drawing water through 1.0 µm cellulose nitrate filters using a hand vacuum pump (Actron CP7830, Bosch Automative Service Solution, Warren, MI, USA) connected to a side-arm flask,

replacing 300 ml plastic filter funnels between each individual eDNA water sample. We also filtered one field or cooler blank to test for contamination per every five field eDNA samples; these controls consisted of 250 ml sample volumes of store-bought, unopened bottled water. We changed nitrile gloves between collecting each individual eDNA water sample, as well as between filtering each individual eDNA water sample. We placed filters in 2 ml micro-centrifuge tubes where they were submerged in approximately 700 µl of cetyl trimethylammonium bromide (CTAB) buffer for transport back to the laboratory. Filtered field samples and controls were shipped to the University of Illinois, Urbana, IL, USA, where they were stored in a refrigerator prior to eDNA extraction in autumn of 2016. Storage of eDNA sample filters in CTAB and similar buffers has been found to reliably preserve eDNA concentrations at room temperature for long periods (Renshaw *et al.*, 2015; Wegleitner *et al.*, 2015).

DNA extraction protocol

Extractions of eDNA samples were performed in a clean laboratory that had never been exposed to crayfish DNA, and samples from only one site were handled per day. Extractions were completed following a modified chloroform-isoamyl alcohol (CI) and isopropanol precipitation protocol (Renshaw *et al.*, 2015). In brief, tubes were incubated in a 65 °C water bath for 1 h, after which 900 µl of 24:1 CI (VWR/Amresco, Radnor, PA, USA) was added to each tube. Tubes were next vortexed for 5 sec until the solution became milky and upon which they were then centrifuged at 15,000 g for 5 min to separate the top aqueous layer. Up to 700 µl of the aqueous layer was transferred to a clean 2 ml microcentrifuge tube, then 700 µl of ice-cold isopropyl alcohol and 350 µl of 5M NaCl were added to each tube for precipitation at –20 °C for 1 h. Tubes were centrifuged at 15,000 g for 10 min to pellet the precipitate, after which the remaining supernatant was decanted. Pellets were then washed twice by adding 150 µl of 70%

ethanol to each tube prior to centrifugation at 15,000 g for 5 min. After the ethanol was decanted, pellets were air dried by leaving tubes upside down on paper towels until no visible liquid remained. For resuspension, pellets were re-hydrated with 200 µl of double-distilled water, or ddH$_2$O. To test for potential contamination during the extraction process, we included an extraction blank for each sampling site, which were extracted on separate days. The blanks contained ddH$_2$O in the place of eDNA.

Primer selection and development for Pacifastacus

An eDNA assay for *P. leniusculus* was previously developed and field validated by Larson *et al.* (2017), successfully detecting this invasive crayfish from six of seven lakes in the Sierra Nevada mountains approximately 240 km southeast of the current study sites. For the present study, we used qPCR primers designed and tested by Larson *et al*. (2017): PacifastacusE_COI_F2 (5’-GGR GGA TTT GGT AAT TGG TTA ATT C -3’) and PacifastacusE_COI_R2b (5’- CAA TAG CCG CTG CTA GAG GA -3’), which produce an 184bp amplicon of the cytochrome c oxidase subunit 1 (COI) gene.

For *P. fortis*, we designed a series of primers in comparison to outgroup crayfishes of the genus *Pacifastacus* (Larson *et al.*, 2017) with the aid of Primer Hunter 1.0.2 (Duitama *et al.*, 2009) and Primer 3 v.0.4.0 (Untergasser *et al.*, 2012). Because *P. fortis* is listed as endangered, tissue samples are largely unavailable for use in the laboratory. To therefore evaluate and confirm resulting amplicons, candidate primers were instead tested against a gBlock Gene Fragment (Integrated DNA Technologies, Coralville, IA, USA), synthesized for a 637 bp fragment of the *P. fortis* COI gene (KU603502.1), as well as against our field samples. Evaluation against the field samples resulted in an occasional non-target amplification of distantly related (i.e., non-crayfish) organisms, including two COI sequences of freshwater

midges (Chironomidae) (KM937882.1) at 75% and 98% identity. Although most non-target amplifications could be differentiated from *P. fortis* by distinct melt-curve temperatures, the Chironomidae sequence at 98% shared a melt curve temperature with the target organism at 74.75 °C. Given limited access to tissue samples and the known low genetic diversity of *P. fortis* at the COI and 16S loci (Larson *et al.*, 2016), we were unable to find alternative primers that were specific only to *P. fortis* without amplification of non-crayfish taxa at these study sites. Accordingly, Sanger sequencing confirmation of positive amplifications was required, and we report the resulting sequences in Supplementary material Appendix S1.

We considered only as positive eDNA detections those samples that amplified with correct melt-curve temperatures and for which we had a Sanger sequencing confirmation matching to 99–100% identity for *P. fortis*, and 98–100% for *P. leniusculus*, which was consistent with high intraspecific variability within this species (Larson *et al.*, 2017), when compared against the NCBI public database. We identified the best performing primer pair of those tested to be the following: Pfortis_COI_F1 (5'- TCA CTT CAC TTA GCT GGA GTA TC -3') and Pfortis_COI_R3 (5'- CCG CTA ATA CCG GTA AAG ATA ATA A -3'), amplifying a 118 bp fragment. We performed non-target testing of this primer pair against genomic DNA extracted from the tissues of two *P. leniusculus* individuals (GenBank accessions KU603495, KU603493) which were selected to represent the major, invasive lineages of this species (Larson *et al.*, 2016; Usio *et al.*, 2016), and confirmed that the *P. fortis* primers did not amplify *P. leniusculus* mtDNA.

Quantitative PCR (qPCR) assays

To avoid cross-contamination, all quantitative PCR (qPCR) assays were prepared in a different laboratory from where eDNA extractions were completed. An additional, separate laboratory

housed the qPCR machines. Assays were prepared under an AirClean® 600 PCR Workstation (AirClean Systems, Creedmoor, NC, USA) with UV sterilization and filtered airflow. Multiplexing was not performed; each assay plate contained only one species primer pair, and assays for each primer pair were tested on different time schedules (i.e., all samples were assayed for one primer pair, before moving to the next primer pair). Further, work station sterilization was performed before and after each assay. Apart from field controls and extraction blanks that were run in duplicates, qPCR reactions were run as six replicates for eDNA extracts. Each 20 µl reaction contained 10 µl iTaq™ Universal SYBR® Green Supermix (BIO-RAD®, Hercules, CA, USA), 1 µl of primer mix (250 nM of each primer), 5 µl of ddH_2O and 4 µl of eDNA extract (none for negative controls). Reactions were run on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems®, Foster City, CA, USA) 96-well 0.2 ml thermal block under the following conditions: an initial denaturation at 95 °C for 3 min; 45 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 1 min, followed by a melting curve analysis that transitioned from 60 °C to 95 °C for 0.15 °C/sec.

To generate a standard curve for quantification of the eDNA extracts (unknowns), we synthesized gBlock Gene Fragments for COI fragments of each *Pacifastacus* species: a 487-bp fragment for *P. leniusculus* as described in Larson *et al.* (2017) and a 637-bp *P. fortis* fragment based on GenBank accession KU603502.1. The copy number for each gBlock is estimated by multiplying Avogadro's number by the number of moles, and eight 10-fold serial dilutions of each gBlock were added in duplicates to each assay to provide a range of copy numbers for quantification of the unknowns, as well as positive control for each assay plate (Renshaw *et al.,* 2015). All assays contained duplicated negative controls (ddH_2O in place of DNA extract) and these controls showed no evidence of contamination among samples. Apart from two assays, all

qPCR reaction efficiencies were between 90–100%; all r^2 values for the standard curves were 0.98–1.00. The two assays that had efficiencies of < 90% were 86.2% and 86.6%.

Field controls and extraction blanks were finally run as assays on plates separate from field samples to avoid contamination with any samples that may have contained *Pacifastacus* eDNA. There were no positive detections for controls/blanks corresponding to *P. leniusculus* primers; however, there were two field controls with positive detections for the *P. fortis* primer. Both of these field controls were from the Upper Fall River site where crayfishes had been handled prior to taking eDNA water samples and collectors had been snorkeling in *P. fortis* occupied water for several hours (see below). Amplified qPCR products were purified using the ExoSAP-IT protocol (Thermofisher, Waltham, MA, USA); products were submitted for Sanger sequencing in both directions at the University of Illinois at Urbana-Champaign Keck Center for Comparative and Functional Genomics to be read on an ABI 3730xl sequencer. Sequences were assembled and edited using Geneious v.10.0.5 (Kearse *et al.*, 2012).

RESULTS

While the amount of target eDNA in our water samples was too low to quantify DNA copy number for either species using the qPCR calibration curves (i.e., outside the limit of quantification but within the limit of detection), the sensitive, species-specific eDNA assay allowed for the positive detection of *P. fortis* eDNA in five of the 95 field samples from four sites: Upper Fall River, Sucker Springs Creek, Private Spring M, and Crystal Lake (Table 2). We detected *P. fortis* eDNA at two of the four sites where it was believed to still occur, one site where its status was uncertain, and one site where it was believed to be absent (Table 2). We failed to detect *P. fortis* eDNA from two sites where it was believed to still occur, and three sites where its status is currently unknown. *Pacifastacus fortis* eDNA was detected from two of 20

samples taken at Upper Fall River, one at 193 m, and the other at 280 m downstream of the constructed barrier. We detected *P. fortis* eDNA from a single sample at Crystal Lake where the species is still routinely observed, including in 2017 after our eDNA sampling. Although its status was unknown at Sucker Springs Creek, a single detection of *P. fortis* was obtained from one sample located above the lowest of several constructed crayfish barriers at this site. After our eDNA sampling, two male *P. fortis* were observed from Sucker Springs Creek in late 2017 (Table 1), demonstrating that this crayfish had not been extirpated from the site and our eDNA detection was likely a true positive. While no *P. fortis* eDNA was detected at the ark site Private Spring K, we identified a single detection from one sample taken at Private Spring M, located approximately 60 m from Spring K (Fig. 2, Table 2). We did not detect *P. fortis* eDNA from Spring Creek (last observed after eDNA sampling in 2017), Pit River (last observed in 2008), Lava Creek (last observed in 2007), or Rising River (last observed in 1995; Table 1).

Nineteen of the 95 field samples were positive for the non-native *P. leniusculus* at five sites: Upper Fall River, Crystal Lake, Lava Creek, Pit River, and Rising River. We detected *P. leniusculus* eDNA at five of seven sites where it was known to occur, failed to detect eDNA of this species at two sites where it was believed to occur, and did not detect its eDNA from the two sites where it was not anticipated to occur (Table 2). *Pacifastacus leniusculus* was previously known from Upper Fall River, where it was observed during sampling for eDNA in 2016, from Crystal Lake and Pit River, where it is routinely observed, including after eDNA sampling in 2017, and at Lava Creek and Rising River, where it was documented as the dominant species when these sites were last surveyed for *P. fortis* (Table 1). Detections at Upper Fall River originated from three samples taken along the barrier (Table 2). Two samples from the southwest cove of Crystal Lake were positive for *P. leniusculus* DNA. We detected *P. leniusculus* eDNA from two and four samples at Lava Creek and Pit River, respectively. Rising River had the most

abundant signatures of *P. leniusculus* eDNA, producing detections across eight of ten samples collected from this site. While recently observed at Sucker Springs Creek and Spring Creek (Table 1), our assay did not detect *P. leniusculus* eDNA; however, we also did not detect this species at Private Springs K or M, as expected.

DISCUSSION

The detection of eDNA of the endangered *P. fortis* at a number of locations where it is known to occur, or was rediscovered after our sampling, shows promise for applying this tool to monitoring of imperiled freshwater species around management interventions like invasive species barriers or relocated populations. Our assays were sensitive enough to identify the presence of both *Pacifastacus* species in several of the nine streams, ponds, or lakes sampled. Notably, the eDNA assay developed for invasive *P. leniusculus* (Larson *et al.*, 2017) detected this species at the majority of sites where it is known to occur (Table 2). In contrast, we detected *P. fortis* eDNA at relatively few sites, and failed to detect eDNA of this species from some locations where it is believed to still occur. Cumulatively, our study suggests a potential role for eDNA in monitoring both rare native and spreading invasive crayfishes, but also a need for ongoing methods improvements to this tool in application to rare or low abundance freshwater organisms.

Our study provides some evidence that recent management interventions for *P. fortis* could have been effective, but further study and monitoring is needed. We did not detect *P. fortis* eDNA at the ark site, Private Spring K, where it was introduced in 2013 and 2014, which may indicate that this effort to establish a new population of an endangered crayfish failed. The lack of *P. fortis* eDNA detections at Private Spring K could also be the result of low power due to the limited number of field samples (five) taken during our study. Additional eDNA sampling should

be pursued to investigate the status of *P. fortis* at the ark site, potentially using more field replicates, larger water sample volumes, or samples taken at different times of the year that may capture more eDNA due to seasonal patterns of organism behavior or reproduction (de Souza *et al.*, 2016; Dunn *et al.*, 2017). Our detection of *P. fortis* eDNA at Private Spring M immediately adjacent to the ark site Private Spring K may alternatively support persistence of the introduced population, either through movement of individuals into this neighboring site or DNA transport through a possible porous aquafer connection (Shogren *et al.*, 2016). A particularly encouraging finding of our study was the detection of *P. fortis* eDNA at Sucker Springs Creek where the status of this species was uncertain and extirpation by *P. leniusculus* in recent years was suspected. Monitoring by the authors after the eDNA study discovered two *P. fortis* males in Sucker Springs Creek in late 2017, demonstrating that the species has persisted at this site, and the eDNA detection was likely a true positive. Given the rediscovery of *P. fortis* at Sucker Springs Creek, an opportunity still exists to manage this endangered crayfish at a site where invasive species barriers have already been installed, such as by removing invasive crayfishes.

Future applications of eDNA to monitoring the performance of management interventions in this particular study system would benefit from improvements to our *P. fortis* eDNA assay. Non-target amplification of distantly related organisms such as Chironomidae midges is undesirable, even if Sanger sequence confirmation can be used to distinguish *P. fortis* DNA from non-target DNA. Larson *et al.* (2016) found low genetic diversity at COI and 16S mtDNA loci for *P. fortis*, which excluded our ability to find alternative primers that were more specific to this species. As mtDNA has higher cellular copy numbers and is more resistant to degradation when compared to nuclear DNA, it is frequently targeted in environmental studies where DNA is present at low concentrations or may be degraded (see Mills *et al.*, 2000; Turner *et al.*, 2014). Future development of improved primers for *P. fortis* might therefore benefit from

a full mitogenome for this species, to provide a broad range of mtDNA regions with enough variability to differentiate non-target sequences. A carefully designed primer/probe eDNA assay that allows for maximizing base-pair mismatches against non-targets might also prove more specific than the primer-only protocol we used here (Goldberg *et al.*, 2016).

The low frequency of eDNA detections for *P. fortis*, especially relative to *P. leniusculus* at the same sites, is likely due to the exceptional rarity of this species, perhaps occurring as only a few hundred total individuals in some of the large habitats we sampled. If detection of the absolute rarest organisms and populations must be the goal, then eDNA is to be useful for management in this and similar contexts. In our case, eDNA detection probability for *P. fortis* might be improved by collecting more samples and larger water volumes to improve the likelihood of finding eDNA of the species when present (Wilcox *et al.*, 2013; Goldberg *et al.*, 2016), especially given that some of the exceptionally clear springs inhabited by *P. fortis* (Fig. 1) should allow passage of multiple liters of water through single filters. The five field water samples taken per location or study site could have been under-powered to detect eDNA of this rare animal, as has been observed in other studies for imperiled or endangered freshwater organisms that conducted formal power analyses (e.g. de Souza *et al.*, 2016). Furthermore, the use of digital droplet PCR (ddPCR) may be more sensitive than qPCR as another method for detecting and quantifying rare molecules. One comparison between these two platforms produced similar estimates of eDNA concentrations (Nathan *et al.,* 2014), whereas other comparisons favored ddPCR as more sensitive for estimating low concentrations and in the presence of PCR inhibitors (Doi *et al.*, 2015a,b). Nevertheless, the lack of utility of qPCR calibration curves in the present study suggests that the use of ddPCR for direct quantification of *Pacifastacus* and similar organisms may be worth exploring.

We detected eDNA of the invasive *P. leniusculus* more frequently than eDNA of the endangered *P. fortis*, but we still failed to detect *P. leniusculus* eDNA at two sites where this invader is known to occur. Such false negatives may reflect either a failure to collect target DNA, or a failure to amplify it in the laboratory (Darling & Mahon, 2011). One reason for failed or reduced DNA amplification could be PCR inhibition. Among the most common PCR inhibitors in freshwater environmental samples are humic and tannic acids, and the addition of substances like bovine serum albumin (BSA) to the PCR mixture can be effective at reducing inhibition from these substances (Schrader *et al.*, 2012). We did not add BSA to our BIO-RAD qPCR master mix because we did not anticipate issues from these particular inhibitors associated with high dissolved organic matter (DOM) in freshwaters (Wetzel, 1992), largely because of the exceptionally high clarity (and associated low DOM) of our study sites (Fig. 1). Other PCR inhibitors can nevertheless occur in freshwater environmental samples, such as dissolved calcium or sodium chloride (Opel *et al.*, 2010; Schrader *et al.*, 2012), and future studies in our system could investigate the role of water chemistry associated with lava springs and groundwater on PCR inhibition.

Our study sites also vary from small streams to medium-sized rivers, small ponds, and medium-sized lakes, and performance of eDNA might differ across these lotic and lentic freshwater environments for a variety of reasons. The eDNA assay for *P. leniusculus* has previously been effective at detecting this species in large lakes (Larson *et al.*, 2017), but performance of eDNA for crayfishes in swiftly flowing lotic environments where downstream DNA transport (e.g. Deiner & Altermatt, 2014) or dilution could be a problem has been minimally investigated. There is a need for studies on eDNA degradation, residence times, and transport distances across disparate freshwater environments (Dejean *et al.*, 2011, Thomsen *et al.,* 2012, Deiner & Altermatt, 2014). Some studies have found higher eDNA concentrations in

benthic sediments relative to surface water samples, including a laboratory mesocosm study on crayfish (Figiel & Bohn, 2015). We might reduce the incidence of eDNA false negatives for both *P. fortis* and *P. leniusculus* by taking benthic sediment samples rather than surface water samples. We caution, however, that because benthic sediments retain DNA longer than surface waters, this can potentially inflate the rate of false positives, particularly through detecting eDNA at sites where the target organism was previously present but no longer occurs (Turner *et al.*, 2015). We are similarly concerned that crayfish carcasses or molted carapaces might release detectable eDNA for unknown periods of time after all individuals in a population have died, an issue that could suggest a *P. fortis* population still persists at a location where it was actually extirpated months or even potentially years earlier (Barnes & Turner, 2016).

Most concerning in our study was contamination of two field controls at one site with *P. fortis* eDNA. We are relatively confident that this contamination occurred because eDNA water sample collection and filtering was conducted immediately after several hours snorkeling in a site occupied by both *P. fortis* and *P. leniusculus*, including handling of some organisms; this decision was made as a logistical constraint to avoid needing to visit a privately owned (rather than public) property twice on adjoining days. As per previous suggestions for best practices in eDNA sample collection (Dougherty *et al.*, 2016; Goldberg *et al.*, 2016), we recommend against handling or being in contact with any target organisms before or during sample collection for eDNA. We chose not to exclude our positive eDNA detections from this site with contamination because both species of interest were observed on the day of sampling, reducing concerns that our eDNA results represent false positives. Our field-control contamination nevertheless stands as a warning of this contamination risk, even for an organism that otherwise was remarkably rare.

False positive detections have consequences and costs; they can misdirect limited resources to managing populations, whether of native or invasive species, that do not exist. Most eDNA field studies implicitly prioritize the risk of false negatives (not detecting an organism where present) over the risk of false positives from contamination in the choice to replicate field samples at a higher rate than both field and laboratory contamination controls. In cases where false positive detections may be particularly costly, such as conservation of an endangered species, more effort can be expended to quantify incidence or rates of false positives by increasing replication of these contamination controls. For example, we used qPCR duplicates for field and extraction blanks, which was lower than our level of qPCR replication for field samples. Increased replication of field and laboratory controls might be used to increase sensitivity to potential contamination and false positives in future applications of eDNA for *P. fortis* or *P. leniusculus*.

CONCLUSIONS

Cumulatively, our study demonstrates the promising capacity for eDNA to be used in monitoring conservation and management interventions for crayfishes, while also highlighting opportunities for improvement in implementation of this tool. Invasive species barriers and conservation translocation to ark sites are already important management approaches for rare and imperiled crayfishes (Kozák *et al*., 2011; Haddaway *et al.*, 2012; Frings *et al*., 2013), and may see increased implementation in the future in response to stressors like climate change and ongoing introductions and spread of invasive species. We propose that eDNA may be useful in monitoring whether invasive crayfishes have passed barriers or expanded into ark sites, while simultaneously monitoring for the presence and abundance of imperiled crayfishes in sometimes difficult to sample habitats. We were able to successfully detect eDNA of the endangered *P.*

fortis and the invasive *P. leniusculus* from a number of sites where they were known to occur at present around such management interventions, but also had some false negatives for these species that demonstrate methodological improvement is still needed (Goldberg *et al.*, 2016). Yet, despite some of these present limitations, we believe our project offers a promising case study in using eDNA to monitor outcomes of conservation interventions for crayfishes that may inform subsequent efforts to use this tool to better manage both imperiled and invasive populations of this taxonomic group.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Appendix. Environmental DNA (eDNA) Sanger sequence confirmation for *Pacifastacus fortis* and *Pacifastacus leniusculus*. Sequencing was performed for every positive qPCR replicate.

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REFERENCES

Barnes, M.A. & Turner, C.R. 2016. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, **17**: 1–17.

Dana, J.D. 1852. *United States Exploring Expedition during the years 1838 to 1842 under the command of Charles Wilkes, U.S.N.* Crustacea. C. Sherman, Philadelphia.

Daniels, R.A. 1980. Distribution and status of crayfishes in the Pit River drainage, California. *Crustaceana*, **38**: 131–138.

Darling, J.A. & Mahon, A.R. 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic envrionments. *Environmental Research*, **111**: 978–988.

Deiner, K. & Altermatt F. 2014. Transport distance of invertebrate environmental DNA in a natural river. *PLoS One*, **9**: e88786.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P. & Miaud, C. 2011. Persistence of environmental DNA in freshwater ecosystems. *PloS one*, **6**: e23398.

de Souza, L.S., Godwin, J.C., Renshaw, M.A. & Larson, E.R. 2016. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One*, **11**: e0165273.

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K. & Yamanaka, H. 2015a. Droplet digital polymerase chain reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. *Environmental Science & Technology*, **49**: 5601–5608.

Doi, H, Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H. & Minamoto, T. 2015b. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS One*, **10**: e0122763.

Dougherty, M.M., Larson, E.R., Renshaw, M.A., Gantz, C.A., Egan, S.P., Erickson, D.M. & Lodge, D.M. 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *Journal of Applied Ecology*, **53**: 722–732.

Duitama, J., Kumar, D.M., Hemphill, E., Khan, M., Măndoiu, I.I. & Nelson, C.E. 2009. PrimerHunter: a primer design tool for PCR-based virus subtype identification. *Nucleic Acids Research*, **37**: 2483–2492.

Dunn, N., Priestley, V., Herraiz, A., Arnold, R. & Savolainen, V. 2017. Behavior and season affect crayfish detection and density inference using environmental DNA. *Ecology and Evolution,* **7**: 7777–7785.

Ellis, M.J. 1999. *Species invasions and replacements in a native crayfish community*. University of Michigan, Ann Arbor, MI, USA.

Ellis, M.J. 2005. Crayfish Barrier Flume Study Final Report. Contract Number 101812M634. Spring Rivers Ecological Sciences, United States Fish and Wildlife Services, Cassel, CA, USA. [give city, state and USA] [not clear what type of pubication this is…]

Eng, L.L. & Daniels, R.A. 1982. Life history, distribution, and status of *Pacifastacus fortis* (Decapoda: Astacidae). *California Fish and Game*, **68**: 197–212.

Fausch, K.D., Rieman, B.E., Dunham, J.B., Young, M.K. & Peterson, D.P. 2009. Invasion versus isolation: trade-offs in managing native salmonids with barriers to upstream movement. *Conservation Biology*, **23**: 859–870.

Faxon, W. 1914. Notes on crayfishes in the U.S. National Museum and the Museum of Comparative Zoology with descriptions of new species and subspecies to which is appended a catalogue of the known species and subspecies. *Memoirs of the Museum of Comparative Zoology at Harvard College*, **10**: 1–186.

Ficetola, G.F., Miaud, C., Pompanon, F. & Taberlet, P. 2008. Species detection using environmental DNA from water samples. *Biology Letters*, **4**: 423–425.

Figiel, C.R. & Bohn, S. 2015. Laboratory experiments for the detection of environmental DNA of crayfish: examining the potential. *Freshwater Crayfish*, **21**: 159–164.

Fischer, J. & Lindenmayer, D.B. 2000. An assessment of the published results of animal relocations. *Biological Conservation*, **96**: 1–11.

Frings, R.M., Vaeßen, S.C., Groß, H., Roger, S., Schüttrumpf, H. & Hollert, H. 2013. A fish-passable barrier to stop the invasion of non-indigenous crayfish. *Biological Conservation*, **159**: 521–529.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Olyer-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E. & Taberlet, P. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, **7**: 1299–1307.

Haddaway, N.R., Mortimer, R.J., Christmas, M., Grahame, J.W. & Dunn, A.M. 2012. Morphological diversity and phenotypic plasticity in the threatened British white-clawed crayfish (*Austropotamobius pallipes*). *Aquatic Conservation: Marine and Freshwater Ecosystems*, **22**: 220–231.

Hagen, H.A. 1870. Monograph of the North American Astacidae. *Memoirs of the Museum of Comparative Zoology at Harvard College,* **2**: 1–109.

Ikeda, K., Doi, H., Tanaka, K., Kawai, T. & Negishi, J. 2016. Using environmental DNA to detect an endangered crayfish *Cambaroides japonicus* in streams. *Conservation Genetics Resources*, **8**: 231–234.

Jerde, C.L., Chadderton, W.L., Mahon, A.R., Renshaw, M.A., Corush, J., Budny, M.L., Myosrekar, S. & Lodge, D.M. 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences*, **70**: 522–526.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. & Lodge, D.M. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation Letters*, **4**: 150–157.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**: 1647–1649.

Kozák, P., Füreder, L., Kouba, A., Reynolds, J. & Souty-Grosset, C. 2011. Current conservation strategies for European crayfish. *Knowledge and Management of Aquatic Ecosystems*, **401**: 1–8. [only one page??]

Lahoz-Monfort, J.J., Guillera-Arroita, G. & Tingley, R. 2015. Statistical approaches to account for false-positive errors in environmental DNA samples. *Molecular Ecology Resources*, **16**: 673–685.

Larson, E.R., Castelin, M., Williams, B.W., Olden, J.D. & Abbott, C.L. 2016. Phylogenetic species delimitation for crayfishes of the genus *Pacifastacus*. *PeerJ*, **4:** e1915.

Larson, E.R., Renshaw, M.A., Gantz, C.A. Umek, J., Chandra, S., Lodge, D.M. & Egan S. 2017. Environmental DNA (eDNA) detects the invasive crayfish *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes of North America. *Hydrobiologia*, **800**: 173–185.

Lereboullet, A. 1858. Description de deux nouvelles espèces d’écrevisses de nos rivières. *Mémoires de la Société des Sciences naturelles*, **5**: 1–11.

Light, T., Erman, D.C., Myrick, C. & Clarke, J. 1995. Decline of the Shasta crayfish (*Pacifastacus fortis* Faxon) of northeastern California. *Conservation Biology*, **9**: 1567–1577.

Lodge, D.M., Taylor, C.A., Holdich, D.M. & Skurdal, J. 2000. Nonindigenous crayfishes threaten North American freshwater biodiversity: lessons from Europe. *Fisheries,* **25**: 7–20.

Lodge, D.M., Simonin P.W., Burgiel, S.W., Keller, R.P., Bossenbroek, J.M., Jerde, C.L., Kramer, A.M., Rutherford, E.S., Barnes, M.A., Wittmann, M.E., Chadderton, W.L., Apriesnig, J.L., Beletsky, D., Cooke, R.M., Drake, J.M., Egan, S.P., Finnoff, D.C., Gantz, C.A., Grey, E.K., Hoff, M.H., Howeth, J.G., Jensen, R.A., Larson, E.R., Mandrak, N.E., Mason, D.M., Martinez, F.A., Newcomb, T.J., Rothlisberger, J.D., Tucker, A.J., Warziniack, T.W. & Zhang, H. 2016. Risk analysis and bioeconomics of invasive species to inform policy and management. *Annual Review of Environment and Resources*, **41**: 453–488.

Mills, L.S., Pilgrim, K.L., Schwartz, M.K. & McKelvey, K. 2000. Identifying lynx and other North American felids based on mtDNA analysis. *Conservation Genetics*, **1**: 285–288.

Nathan, L.M., Simmons, M., Wegleitner, B.J., Jerde, C.L. & Mahon, A.R. 2014. Quantifying environmental DNA signals for aquatic invasive species across multiple detection platforms. *Environmental Science & Technology*, **48**: 12800–12806.

Opel, K.L., Chung, D. & McCord, B.R. 2010. A study of PCR inhibition mechanisms using real time PCR. *Journal of Forensic Sciences*, **55**: 25–33.

Pilliod, D.S., Goldberg, C.S., Arkle, R.S. & Waits, L.P. 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*, **70**: 1123–1130.

Pintor, L.M., Sih, A. & Bauer, M.L. 2008. Differences in aggression, activity and boldness between native and introduced populations of an invasive crayfish. *Okios*, **117**: 1629–1636.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R. & Gough, K.C. 2014. The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, **51**: 1450–1459.

Renshaw, M.A., Olds. B.P., Jerde, C.L., McVeigh, M.M. & Lodge, D.M. 2015. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol–chloroform–isoamyl alcohol DNA extraction. *Molecular Ecology Resources*, **15**: 168–176.

Schrader, C., Schielke, A., Ellerbroek, L. & Johne, R. 2012. PCR inhibitors–occurrence, properties and removal. *Journal of Applied Microbiology*, **113**: 1014–1026.

Shogren, A.J., Tank, J.L., Andruszkiewicz, E.A., Olds, B., Jerde, C. & Bolster, D. 2016. Modelling the transport of environmental DNA through a porous substrate using continuous flow-through column experiments. *Journal of the Royal Society Interface*,**13**: DOI: 10.1098/rsif.2016.0290. [PLEASE INSERT DOI NUMBER]

Spear, S.F., Groves, J.D., Williams, L.A. & Waits, L.P. 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation*, **183**: 38–45.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L. & Willerslev, E. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, **21**: 2565–2573.

Tréguier, A., Paillisson, J., Dejean, T., Valentini, A., Schlaepfer, M.A. & Roussel, J.M. 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, **51**: 871–879.

Turner, C.R., Barnes, M.A., Xu, C.C., Jones, S.E., Jerde, C.L. & Lodge, D.M. 2014. Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, **5**: 676–684.

Turner, C.R., Uy, K.L. & Everhart, R.C. 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, **183**: 93–102.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. & Rozen, S.G. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Research*, **40**: DOI: 10.1093/nar/gks596.[PLEASE PROVIDE DOI NUMBER]

United States Fish and Wildlife Service [NO AUTHORS?]. 1988. Endangered and threatened wildlife and plants; determination of endangered status for the Shasta crayfish. *Federal Register*, **53**: 38460–38465.

Usio, N., Konishi, M. & Nakano, S. 2001. Species displacement between an introduced and a 'vulnerable' crayfish: the role of aggressive interactions and shelter competition. *Biological Invasions*, **3**: 179–185.

Usio, N., Azuma, N., Larson, E.R., Abbott, C.L., Olden, J.D., Akanuma, H., Takamura, K. & Takamura, N., 2016. Phylogeographic insights into the invasion history and secondary spread of the signal crayfish in Japan. *Ecology and Evolution*, **6**: 5366–5382.

Vorburger, C., & Ribi, G. 1999. Aggression and competition for shelter between a native and an introduced crayfish in Europe. *Freshwater Biology*, **42**: 111–119.

Wegleitner, B.J., Jerde, C.L., Tucker, A., Chadderton, W.L. & Mahon, A.R. 2015. Long duration, room temperature preservation of filtered eDNA samples. *Conservation Genetics Resources*, **7**: 789–791.

Wetzel, R.G. 1992. Gradient-dominated ecosystems: sources and regulatory functions of dissolved organic matter in freshwater ecosystems. *Hydrobiologia*, **229**: 181–198.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R. & Schwartz, M.K. 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS*, One **8**: e59520.

FIGURES

Figure 1. The Shasta (*Pacifastacus fortis*) (**A**), and the signal (*Pacifastacus leniusculus*) (**B**) crayfishes. A crayfish barrier at Upper Fall River, California (**C**); crayfish barrier at Sucker Springs Creek, California (**D**). *Pacifastacus fortis* habitat above the Upper Fall River barrier (**E**). Relocation (or "ark") site of *P. fortis* in isolated spring-fed pond with appropriate substrate (**F**). Photos by K.G.H. Breedveld and E.R. Larson.

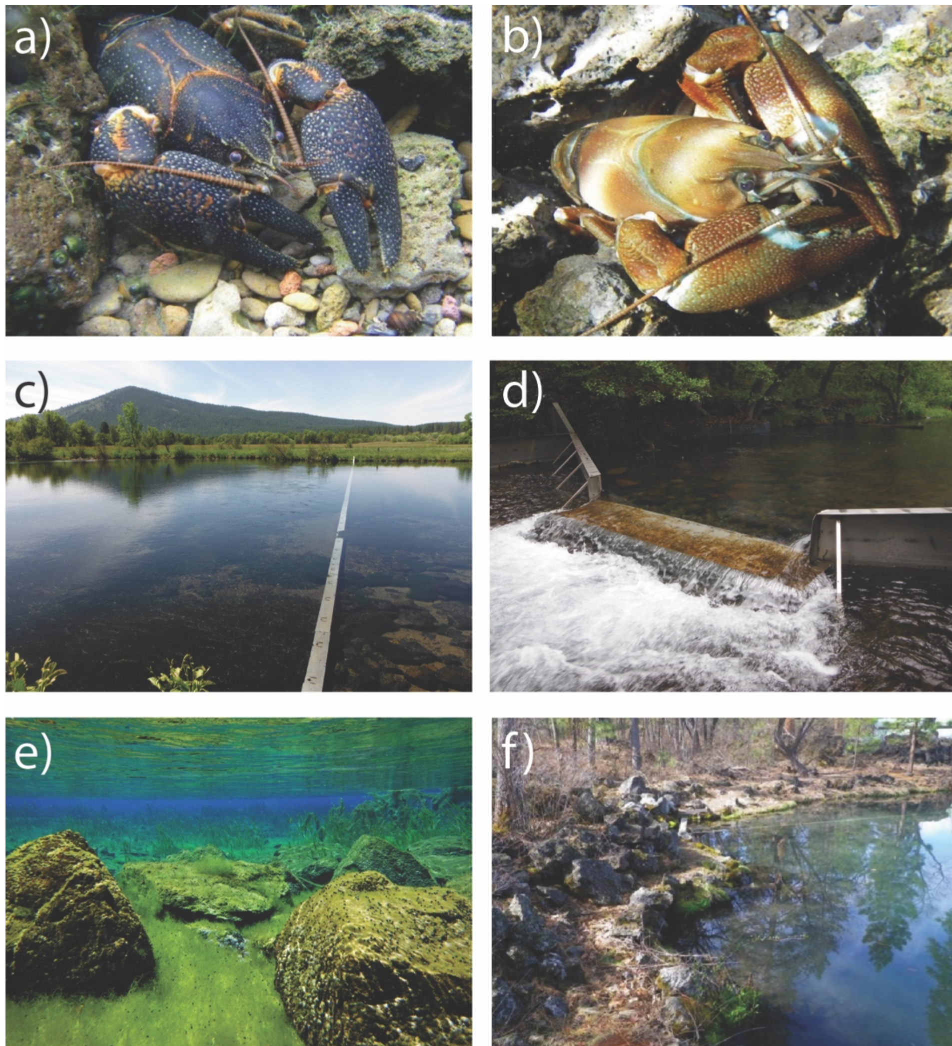

Figure 2. Study sites for environmental DNA (eDNA) sampling for the endangered crayfish *Pacifastacus fortis* and the invasive *Pacifastacus leniusculus* in the middle reaches of the Pit River, California (inset star). Four sample locations were used at Upper Fall River, four at Sucker Springs Creek, three at Crystal Lake, and two at Pit River. All other study sites used a single sample location.

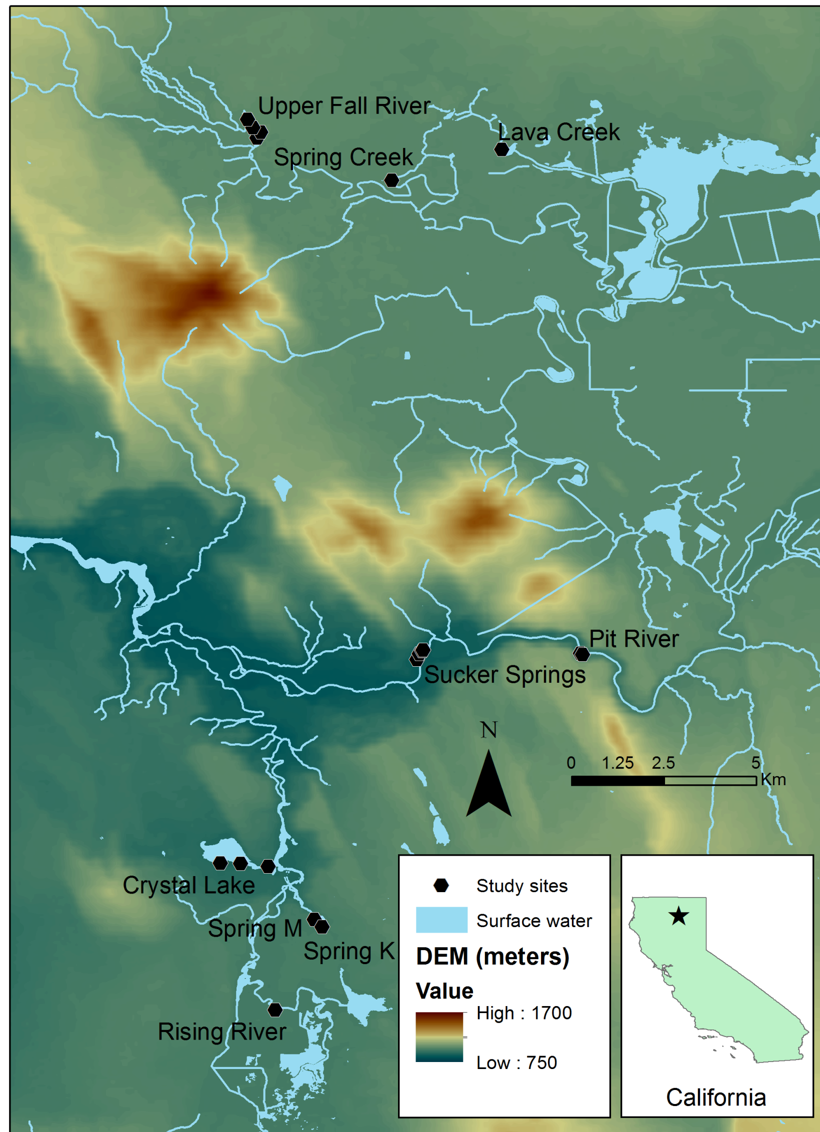

Table 1. Study sites with the number of eDNA sample locations and total samples, most recent status for the endangered crayfish *Pacifastacus fortis* and the invasive crayfish *Pacifastacus leniusculus*, and management details for each study site.

Site (locations, samples)	*Pacifastacus fortis*	*Pacifastacus leniusculus*	Management details
Upper Fall River (4, 20)	Present; observed date of eDNA sampling	Present; observed date of eDNA sampling	Barrier constructed to exclude *P. leniusculus* from spring; active hand removal of *P. leniusculus* above barrier.
Sucker Springs Creek (4, 20)	Last observed in 2014 (female with eggs); suspected absent, but rediscovered in 2017 (two males)	Last observed in 2017	Multiple barriers constructed to exclude *P. leniusculus* but unsuccessful. *P. fortis* believed extirpated after 2014, but two males rediscovered in 2017.
Private Spring K (1, 5)	Last observed in 2013 (2 fresh molts), not in 2015	Believed absent	*P. fortis* introduced to pond in 2013 and 2014; 42 individuals (incl. 5 females and 2 males of reproductive sizes). Pond area is approximately 0.12 hectares.
Private Spring M (1, 5)	Believed absent	Believed absent	Spring K pond has a possible connection to Spring M through porous lava aquafer; pond area is approximately 1.56 hectares.
Crystal Lake (3, 15)	Last observed in 2017	Last observed in 2017	No active management
Lava Creek (1, 5)	Last observed in 2007	Last observed in 2007	No active management
Pit River (2, 10)	Last observed in 2008	Last observed in 2017	No active management
Rising River (1, 10)	Last observed in 1995	Last observed in 1995	No active management

Spring Creek (1, 5)	Last observed in 2017	Last observed in 2017	Culverts act as flow barriers, active hand removal of *P. leniusculus* above barrier.

Table 2. Summary of eDNA results for *Pacifastacus fortis* and *Pacifastacus leniusculus* by site, whether each species was observed at site, and whether we detected eDNA and the number of eDNA detections per set of samples collected.

Pacifastacus fortis			
Site	**Expected Present?**	**eDNA detection**	**Detections per sample set**
Upper Fall River	Yes	Yes	2/20
Sucker Springs Creek	No	Yes	1/20
Private Spring K	Yes	No	0/5
Private Spring M	No	Yes	1/5
Crystal Lake	Yes	Yes	1/15
Lava Creek	Unknown	No	0/5
Pit River	Unknown	No	0/10
Rising River	Unknown	No	0/10
Spring Creek	Yes	No	0/5

Pacifastacus leniusculus			
Site	**Expected?**	**eDNA detection**	**Detections per sample set**
Upper Fall River	Yes	Yes	3/20
Sucker Springs Creek	Yes	No	0/20
Private Spring K	No	No	0/5
Private Spring M	No	No	0/5
Crystal Lake	Yes	Yes	2/15
Lava Creek	Yes	Yes	2/5
Pit River	Yes	Yes	4/10
Rising River	Yes	Yes	8/10
Spring Creek	Yes	No	0/5

Table S1. Environmental DNA (eDNA) Sanger sequence confirmation for *Pacifastacus fortis* and *P. leniusculus*. Sequencing was performed for every positive qPCR replicate.

Primer target	Location	Length (bp)	Result	Result Name
P. fortis	Crystal Lake	119	100% query cover, 99% identity	*Pacifastacus fortis*
P. fortis	Private Spring M	118	100% query cover, 99% identity	*Pacifastacus fortis*
P. fortis	Sucker Springs Creek	118	100% query cover, 100% identity	*Pacifastacus fortis*
P. fortis	Upper Fall River - Bridge	93	100% query cover, 100% identity	*Pacifastacus fortis*
P. fortis	Upper Fall River – Sand Spring	90	100% query cover, 100% identity	*Pacifastacus fortis*
P. leniusculus	Crystal Lake	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Crystal Lake	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Crystal Lake	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Crystal Lake	136	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Crystal Lake	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Crystal Lake	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Lava Creek	139	100% query cover, 99% identity	*Pacifastacus leniusculus*
P. leniusculus	Lava Creek	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Lava Creek	139	100% query cover, 99% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	127	100% query cover, 98% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	138	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*

P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	133	100% query cover, 99% identity	*Pacifastacus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	126	100% query cover, 98% identity	*Pacifastacus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	140	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Rising River	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Downstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Downstream	139	100% query cover, 99% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Downstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Downstream	139	100% query cover, 99% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*

P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Pit River - Upstream	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Upper Fall River - Barrier	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Upper Fall River - Barrier	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Upper Fall River - Barrier	139	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*
P. leniusculus	Upper Fall River - Barrier	119	100% query cover, 100% identity	*Pacifastacus leniusculus leniusculus*

Primer target	**Sequence**
P. fortis	TAAAAGAACTGCTGTAATAAACACAGATCAAACAAATAAGGGTATACGATCTATAGTTATACCTACCCCTTCGTATATTAATAGCCGTAGTTATAAAATTTACAGCACCTAAAATAGAA
P. fortis	TAAAAGAACTGCTGTAATAAACACAGATCAAACAAATAAGGGTATACGATCTGTAGTTATACCTACCCTTCGTATATTAATAGCCGTAGTTATAAAATTTACAGCACCTAAAATAGAA
P. fortis	TAAAAGAACTGCTGTAATAAACACAGATCAAACAAATAAGGGTATACGATCTATAGTTATACCTACCCTTCGTATATTAATAGCCGTAGTTATAAAATTTACAGCACCTAAAATAGAA
P. fortis	GATCAAACAAATAAGGGTATACGATCTATAGTTATACCTACCCTTCGTATATTAATAGCCGTAGTTATAAAATTTACAGCACCTAAAATAGAA
P. fortis	TAAAAGAACTGCTGTAATAAACACAGATCAAACAAATAAGGGTATACGATCTATAGTTATACCTACCCTTCGTATATTAATAGCCGTAGT
P. leniusculus	CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTA
P. leniusculus	CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTAATATTAGGGGCTCCTGATATAGCATTCCCCCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACGTTGTTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC
P. leniusculus	CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAGCTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus TATTCCTCTAGGTTAATAATAAAGTTAAAGAAAATGGAAGTAATCAAAATCTTATATTATTTATACGAGGAAATGCTATATCAGGGGCCCCTAATATCAAAGGAATTAACCAATTACCAAATCCCCC

P. leniusculus TTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus GGATAAACAGTTCATCCAGTACCCACTCCTCTTTCAACTATTCCTCTAGTTAATAATAAAGTTAAAGAAAATGGAAGTAATCAAAATCTTATATTATTTATACGAGGAAATGCTATATCAGGGGCCCCTAATATTAAAG

P. leniusculus TCAATAGCCGCTGCTAGAGGAGGATAAACAGTTCATCCAGTACCCACTCCTCTTTCAACTATTCCTCTAGTTAATAATAAAGTTAAAGAAAATGGAAGTAATCAAAATCTTATATTATTTATACCGAGGAAAT

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus TATTCCTCTAGTTAATAATAAAGTTAAAGAAAATGGAAGTAATCCAAATCTTATATTATTTATACGAGGAAATGCTATATCAGGGGCCCCTAATATTAAAGGAATTAACCAATTACCAAATCCCCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CCTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

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P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

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P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGGATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

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P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTAATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

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P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

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P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

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P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGTACTGGATGAACTGTTTATCC

P. leniusculus CTTTGATATTAGGGGCCCCTGATATAGCATTTCCTCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGAGTGGGT