Using eDNA to estimate the distribution of California Floater (*Anodonta californiensis/nuttalliana* clade) and Western Pearlshell (*Margaritifera falcata*) mussels in the Bear River basin of Wyoming

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Abstract

Mussels are cryptic animals that are often difficult to study due to their biology and characteristics of the aquatic ecosystem. Environmental DNA (eDNA) can be a useful tool to direct field surveys by identifying areas where eDNA from the target species is present. Mussels are ideal animals for eDNA surveys because they slough cells while filtering large volumes of water during feeding. We collected water samples for eDNA throughout the Bear River basin in Wyoming and analyzed samples for DNA from California Floater (*Anodonta californiensis/nuttalliana* clade) and Western Pearlshell (*Margaritifera falcata*) mussels. California Floater was detected in Wyoming from north of Evanston to where the Bear River flows out of the state north of Cokeville, with the highest concentrations of eDNA in the northern portion of the basin. Western Pearlshell were present throughout the basin in Wyoming from the southern border of Wyoming to where the Bear River flows out of the state north of Cokeville, and concentrations peaked in the southern and northern portions of the basin. Locations identified by eDNA provided new locations of likely presence for both mussel species to direct future field efforts.

Introduction

Mussels are critical animals in aquatic ecosystems but assessing their distribution and status can be difficult. As filter feeders, mussels clean the water by filtering large volumes of water (e.g., Howard and Cuffey 2006b). They also provide nutrients to bottom dwelling organisms (e.g., Vaughn and Hakenkamp 2001) and can have very high biomass (Dame 2011). Despite their roles in aquatic ecosystems, they are difficult to study. Mussels can burrow completely beneath the sediments and are often difficult to see, especially juveniles (Degerman et al. 2006). The shells of adults look like rocks requiring a trained eye to detect them in ideal conditions. Conditions are often far from optimal, such as high turbidity, deep water or high flow making visual survey techniques difficult. Visual surveys are also very labor intensive and surveying an entire basin can be resource intensive. Therefore, other methods to direct visual surveys are useful to focus field work.

Environmental DNA (eDNA) technology can help target survey locations. Environmental DNA is DNA shed naturally into the water column by target organisms (Sansom and Sassoubre 2017). Genetic assays can be designed to target either entire communities of organisms through eDNA metabarcoding or species-specific assays can be designed to target one or a few target species with quantitative PCR (qPCR). Environmental DNA has been used for a variety of aquatic species, including invasive (Shogren et al. 2019) and Endangered mussels (Lor et al. 2020), including the species targeted in this study (Rodgers et al. 2020). Sources of eDNA from mussels include sloughing cells during filter feeding, releasing excreta, and expelling gametes and glochidia during reproductive periods (Sansom and Sassoubre 2017). The amount of water mussels filter can exceed the discharge of the stream when abundant explaining why these filter feeders are ideal for eDNA techniques. Using eDNA in flowing water can be more complicated than in ponds or lakes because eDNA can be transported downstream between 5 m (Pilliod et al. 2014) and 12.3 km (Deiner and Altermatt 2014). This can make it difficult to determine how far from the original source population a detection is. Many factors determine how far an eDNA fragment is transported including water temperature, light and pH (Strickler et al. 2015). On the other hand, better mixing in rivers and streams can lead to higher detection probabilities than in lakes and ponds (T. Rodgers, personal observation). Quantitative PCR allows us to measure the concentration of DNA fragments in each sample (i.e., copies of DNA/L) which may correspond to the density of mussels in the

upstream reach (Shogren et al. 2019). Together, identifying where eDNA is located and the concentration can help direct traditional field surveys.

We used eDNA to estimate the distribution of California Floater (*Anodonta californiensis/nuttalliana* clade) and Western Pearlshell (*Margaritifera falcata*) in the Bear River basin of Wyoming. California Floater is a freshwater mussel native to western North America; however, in Wyoming it only occurs in the Bear River basin, and previous surveys collected adults suggesting a lack of recruitment (Mathias 2014). Mock et al. (2004) performed a phylogenetic analysis of California floater across the species range, including 18 individuals from the Bear River in Utah, and found limited gene flow between populations due to isolation in district hydrologic basins. In addition, the California Floater is imperiled in its native range (Blevins et al. 2017), likely due to a combination of anthropogenic factors (e.g., damming, sedimentation, loss of host species) as seen generally with freshwater mussels (Williams et al. 1993). Western Pearlshell also live in the Bear River basin and they are more secure throughout their range (Blevins et al. 2017), but only locally abundant in the Bear River basin. Live juvenile and adult Western Pearlshell were found in a few locations (Mathias 2014). Environmental DNA is especially usefully because many reaches of the Bear River had high turbidity and deep water making visual surveys difficult. Our specific questions were 1.) Where do California Floater and Western Pearlshell occur in the Bear River basin?, 2.) Did the concentration of eDNA differ within the basin? and 3.) What habitat conditions were related to the concentration of DNA for each species? Results will guide future mussel surveys in the Bear River basin of Wyoming.

Study Area and Species

The Bear River basin is a closed basin (river that does not drain into the ocean; 560 km river length; 18,200 km^2) that begins in the Uinta Mountains of Utah (~3000 m elevation) and flows north into southwestern Wyoming. The river turns and flows west into Idaho north of Cokeville, Wyoming and eventually flows south into Utah terminating in the Great Salt Lake (1284 m elevation). Interestingly, the Bear River flowed into the Snake River in the past; however, lava flows diverted its path long ago. The Bear River is smaller near the Utah boarder in southern Wyoming (1147 km^2 basin area; 2051 m elevation; average discharge between 2003 and 2019 was 5.3 m^3/s at Evanston) and is 2 times larger near Cokeville (6338 km^2 basin area; 1871 m elevation; average discharge between 2003 and 2019 was 10.4 m^3/s at Cokeville below confluence with Smith's Fork; https://waterdata.usgs.gov/nwis/rt).

Recent analysis of western US *Anodonta* species revealed inconsistent genetic differentiation between *A. californiensis* and *A. nuttalliana* using nuclear and mitochondrial DNA, and these mussels formed groups based on hydrologic basins (river drainages) with *Anodonta* in the Bear River included in the Bonneville Basin group (Mock et al. 2010). We refer to California Floater as the *Anodonta californiensis/nuttalliana* clade. California Floaters have a thin shell, can grow ≤130 mm in length and live 10-15 years (Tronstad et al. 2015). These mussels tend to live in rivers and low gradient streams, but they can also occur in the fine sediments of lakes and reservoirs. California Floaters live in the western United States and historically were found from California to Washington and east to Arizona, Utah, Wyoming and Idaho (Hovingh 2004). In Wyoming, they are limited to the Bear River basin (Tronstad et al. 2015). The host fish of California Floaters in Wyoming include Speckled Dace (*Rhinichthys osculus*), Longnose Dace (*Rhinichthys cataractae*), Cutthroat Trout (*Oncorhynchus clarkii*), Chubs (*Squalius cephalus*) and Green Sunfish (*Lepomis cyanellus*). All states rank the mussel as

imperiled (S2) except for Idaho (vulnerable, S3), Nevada and Arizona (critically imperiled, S1; NatureServe 2021). The California Floater is currently identified as NSS1 Species of Greatest Conservation Need in the 2017 Wyoming State Wildlife Action Plan.

Western Pearlshells live in the Snake, Green and Bear River basins of Wyoming. Despite their similar size (≤130 mm in length), they are distinct from California Floaters by the heart-shaped shell that is often purple on the inside (nacre) and thicker than the California Floater (Tronstad et al. 2015). Western Pearlshells also live much longer (>100 years) and can reproduce through parthenogenesis. These mussels live in clear rivers and streams with sand, gravel or cobble substrate. The range of Western Pearlshell extends from Alaska to California and east to British Columbia, Montana and Wyoming. They are host specialists on salmonids, and their host fish in Wyoming include Cutthroat Trout (*Oncorhynchus clarkii*), Speckled Dace (*Rhinichthys osculus*), Kokanee Salmon (*Oncorhynchus nerka*), Brown Trout (*Salmo trutta*), Rainbow Trout (*Oncorhynchus mykiss*) and Brook Trout (*Salvelinus fontinalis*). State ranks range from critically imperiled (S1; California, Nevada and Utah), imperiled (S2; Montana and Idaho), vulnerable (S3; Wyoming, Oregon and Washington) to secure (S5: British Columbia; NatureServe 2021).

Methods

We sampled in the Bear River basin to estimate the distribution of California Floater and Western Pearlshell mussels in Wyoming. At each site, we recorded the location using a GPS (Datum NAD83), noted land use and stream substrate, measured basic water quality, measured stream width and stream depth, and recorded the instream location where we collected the water sample. Basic water quality was recorded with a Yellow Springs Instrument (YSI) Professional Plus that measured water temperature (°C), dissolved oxygen (DO; percent saturation and mg/L), specific conductivity (µS/cm) and pH. Sensors were calibrated every four days except DO which was calibrated daily. We measured stream width using a range finder when the stream was ≥5 m wide or a meter stick for narrower streams. Stream depth was measured by marking depth on a pair of waders and recording the deepest depth while collecting water samples. Location of water samples was recorded as center of stream, left center, right center, right margin or left margin when facing upstream. Land use was recorded as hayland, rangeland or recreation. Stream substrate was noted as fines (sand, silt and clay; <2 mm diameter), gravel (2-64 mm diameter) or cobble (64-256 mm diameter). Additionally, we watched for mussel shells along the streams when we could walk between sampling locations, and noted the number and species.

We collected water samples at each site to estimate the presence of California Floater and Western Pearlshell using eDNA. To minimize cross-contamination of samples, we wore clean nitrile gloves and used new sterile 1 L Whirl Pak bags. Ideally, we waded into the center of the stream, rinsed the bag three times with stream water upstream from our position and filled the bag with stream water. We were careful to only touch the outer portion of the sample bag. We labeled the outside of the bag, placed the sample in a dark, cool location and transported it to a cooler where it was stored on ice until filtering (usually <12 hours after collection). If the river was too deep to sample in the center, we sampled on the right or left center, or at the margin.

Samples were filtered using 0.45 µm cellulose nitrate filters using GeoPump peristaltic pumps. We filtered up to 1 L of water or until the filter clogged. We used clean and sterile procedures to filter

samples to minimize contamination including clean nitrile gloves for each sample and a disposable filter cup. Forceps were soaked in 10% bleach solution and rinsed 3x in deionized water between uses. We ran a blank sample each day at the start of filtering and about every 5 samples thereafter. Blanks filtered deionized water using the same procedures and supplies as other samples. We noted the volume filtered for each sample. Filters were frozen in a 50 mL Falcon tube filled half to two-thirds full of silicone gel beads separated from the filter by a kimwipe. Samples were analyzed at the Molecular Ecology Laboratory, Utah State University.

Samples were extracted in a room dedicated for extracting eDNA using the DNeasy Blood & Tissue Kit (Qiagen, Inc.) with a modified eDNA protocol described in Carim et al. (2016). Each round of eDNA extraction included one 'extraction blank' negative control consisting of a clean filter to monitor for contamination. All samples were analyzed in triplicate TaqManTM qPCR with 4 µl of eDNA extract, TaqManTM Environmental Mastermix 2.0 (Thermo-Fisher Scientific), and a multiplex TaqManTM qPCR assay for detecting *Anodonta nuttalliana/californiensis*, and *Margaritifera falcata.* Further details on this assay can be found in Rodgers et al. (2020). Each qPCR run included six 'no template' negative control reactions to monitor for contamination. All reactions included an internal positive control to monitor for PCR inhibition. Any samples flagged by the internal positive control as inhibited were treated with a OneStep PCR inhibitor removal kit (Zymo Research) and re-run. eDNA extractions were conducted in a dedicated clean lab space physically separated from post-PCR spaces and tissue DNA extraction spaces, and all qPCR reactions were set up under a dedicated PCR hood sterilized with UV radiation prior to each qPCR run.

We analyzed the eDNA results using Program R (R Core Development Team 2013) and the plyr (Wickham 2011) package. We used Spearman's Rank (cor) to estimate the correlation between the number of replicate samples that amplified and the mean copies of DNA per reaction measured for each sample. The concentration of eDNA/L is calculated using the number of replicates that amplified and the volume of water filtered, but the mean copies of DNA per reaction is independent of these variables. Therefore, we used the mean copies of DNA per reaction to investigate how this varied with the number of replicates that amplified and the volume of water filtered. Linear regression (lm) calculated the relationship between the number of replicates that amplified and the mean copies of DNA per reaction compared to the number of shells we discovered along each stream reach from sites with shells and the volume of water we filtered for each sample. We used linear regression to estimate any relationships between the copies of DNA/L and continuous water quality variables (i.e., dissolved oxygen, specific conductivity, pH, water temperature, stream width and stream depth). Analysis of variance (aov) calculated the relationship between the copies of DNA/L and presence of fines, gravel or cobble.

Results

We filtered water and analyzed samples for the eDNA of California Floater and Western Pearlshell at 136 sites in the Bear River basin of Wyoming between 27 August and 26 September 2019. None of the blank samples were contaminated indicating that our techniques did not introduce mussel DNA across samples. We sampled 10 streams and Woodruff Narrows Reservoir, and 73% of samples were from the mainstem of the Bear River from the southern Wyoming border to where the Bear River flows out of Wyoming north of Cokeville (Figure 1). The elevation of sampled sites ranged between ~1850 to 2300 m. The dominant land use adjacent to the streams was rangeland (50%) and hayland (42%). Water

temperature varied between 7.1 and 20.8°C (Table 1). The percent saturation of dissolved oxygen was between 67 and 116% and the concentration of oxygen varied between 7.4 and 11.8 mg/L which is partially attributed to the time of day the measurement was collected. Specific conductivity ranged between 188 and 972 µS/cm and pH was basic (8.01 to 8.91). We sampled small streams (minimum width of 0.5 m and depth of 10 cm) to large rivers (maximum width of 53 m and depth of 130 cm, although pools were deeper). Fine substrate (clay, sand and silt) occurred at 59% of sites followed by cobble (54%) and gravel (16%). Most water samples (94%) were collected in the center of the stream. We filtered between 305 and 1090 mL of water for eDNA samples.

According to eDNA samples and shells, California Floater occurred between north of Evanston to the northern section of the Bear River basin in Wyoming (Figure 1). Thirty-eight percent of all sites were positive for eDNA where 17 samples amplified one replicate, 13 samples amplified two replicates and 21 samples amplified three replicates. More amplified replicates indicated more mean copies of DNA per reaction present in water ($r = 0.51$). The highest concentration of eDNA were in the northern portion of the Bear River in Wyoming, and measured ≤7593 copies of DNA/L (Figure 2, 3a). Additionally, we found 68 shells of California Floaters at 20 sites (Figure 1) that varied between 65 and 99 mm in length (Figure 4a). Generally, more replicates amplified (lm, $t = 2.9$, $df = 14$, $R^2 = 0.34$, $p = 0.01$; Figure 3b) and more mean copies of DNA per reaction (lm, $t = 3.6$, $df = 18$, $R^2 = 0.39$, $p = 0.002$; Figure 3c) were measured at sites where we discovered more California Floater shells. A larger volume of water filtered did not increase the number of replicates that amplified (lm, $t = -0.08$, $df = 48$, $R^2 = 0.02$, $p = 0.94$) or the mean copies of DNA per reaction (lm, $t = 0.43$, $df = 48$, $R^2 = 0.02$, $p = 0.67$; Figure 3d).

California Floaters were found primarily in the Bear River itself. All positive eDNA results and shells of California Floaters were in the mainstem of the Bear River; however, one positive eDNA result was found each in the Smith's Fork River and Woodruff Narrows Reservoir. The number of DNA copies/L did not differ at sites with fines (aov, $F = 0.6$, $df = 1$, $p = 0.46$) or cobble present (aov, $F = 2.7$, $df = 1$, $p = 0.1$), but more copies of California Floater DNA were measured at sites with gravel (aov, $F = 35$, $df = 1$, $p < 0.001$). Characteristics of the water (i.e., temperature, dissolved oxygen, specific conductivity, pH, stream width and stream depth) were not related to the concentration of California Floater DNA measured (lm, $p > 0.05$).

Western Pearlshell were found throughout the Bear River basin in Wyoming using eDNA and shells. Samples indicated that their presence extended from the southern to northern portion of the Bear River basin in Wyoming (Figure 5). Of the sampled sites, 32% were positive for their eDNA where 9 samples amplified one replicate, 11 samples amplified two replicates and 23 samples amplified three replicates. More amplified replicates indicated more mean copies of DNA per reaction present in water ($r = 0.57$). The highest concentrations peaked in the southern and northern portions of the basin where we measured ≤8409 copies of DNA/L (Figure 3a, 6). We found 70 shells of Western Pearlshells along 13 stream reaches (Figure 3) and shell length varied between 34 and 94 mm (Figure 4b). More shells found along the stream reach did not indicate that more replicates amplified (lm, $t = -0.3$, $df = 10$, $R^2 = 9\%$, $p = 0.74$; Figure 3b) or more copies of DNA/L (lm, $t = 0.7$, $df = 11$, $R^2 = 4\%$, $p = 0.51$; Figure 3c). The volume of water filtered did not predict the number of replicates that amplified (lm, $t = 0.65$, $df = 41$, $R^2 = 0.01$, $p = 0.52$) or the mean copies of DNA per reaction (lm, $t = 0.25$, $df = 41$, $R^2 = 0.02$, $p = 0.81$; Figure 3d).

Western Pearlshells were primarily found in the Bear River, but we collected two positive samples in the Smith's Fork River and another positive sample in a tributary of the Bear River (Figure 5). We found

shells of Western Pearlshell in the mainstem of the Bear River and the Smith's Fork River (Figure 5). The number of DNA copies/L were higher at sites lacking fines (aov, $F = 7.1$, $df = 1$, $p = 0.009$), where gravel was present (aov, $F = 7.1$, $df = 1$, $p = 0.009$), and with cobble but the presence of cobble was not significant (aov, $F = 1.9$, $df = 1$, $p = 0.17$). Characteristics of the water (i.e., temperature, dissolved oxygen, specific conductivity, pH, stream width and stream depth) were not related to the concentration of Western Pearlshell DNA measured (lm, $p > 0.05$).

Discussion

The combination of being a cryptic species and high turbidity makes searching for mussels in the Bear River extremely difficult using visual methods. The turbidity limited the volume of water we filtered but it did not appear to limit detection. Environmental DNA was useful to identify locations for future visual surveys and provide information about the distribution of each species in the basin. Our eDNA surveys expanded the known range of California Floater and Western Pearlshell in Wyoming, and we hope to verify the eDNA results with visual surveys. Environmental DNA has been used to detect if eradication methods removed invasive carp (Furlan et al. 2019), the presence of cave dwelling crayfish (Boyd et al. 2020), discover new populations of protected mussels (Lor et al. 2020), modeling occurrences of hellbender salamanders (Neto et al. 2020) and we used eDNA to better establish the distribution of mussels in a river basin. Additionally, one sample allowed us to investigate the distribution of two species simultaneously using a multiplex assay (Rodgers et al. 2020) and amplified DNA from these samples may be used to assess the eDNA of other species (Dysthe et al. 2018). Environmental DNA can be a useful tool for many aquatic species, especially those that are difficult to detect using traditional methods.

New locations for California Floater were discovered using eDNA. The first known observation of California Floater in Wyoming was a shell in the Bear River near Woodruff Narrows Reservoir (built in 1961) in 1979 (Mathias 2014). California Floater was noted in the Bear River by Hovingh (2004) and the Wyoming Game and Fish Department discovered 12 live California Floaters in 2008 and 2010 near Cokeville. The most recent surveys in the Bear River basin searched for mussels using visual and tactile methods (Mathias 2014, Wilmot et al. 2017). California Floaters were discovered along four reaches (3 reaches with 13 live individuals; Mathias 2014) and live California Floaters were found in the Bear River at Cokeville Meadows National Wildlife Refuge (2 individuals), below Woodruff Narrows Reservoir (10 individuals) and in lower Yellow Creek (1 individual). Shells but no live individuals were found at an additional site at Cokeville Meadows National Wildlife Refuge. No live California Floaters were found in the Bear River basin after searching nearly 4 river km in 2016 (Wilmot et al. 2017). Our surveys using eDNA indicated that California Floater live in Woodruff Narrows Reservoir and upstream of the reservoir in the Bear River. This observation is the farthest upstream location in the Bear River reported, but one individual was found in Yellow Creek previously (Mathias 2014), which is upstream and flows into the Bear River near Evanston. Our surveys also found that California Floater live in the northern reaches of the Bear River in Wyoming and the Smith's Fork River.

Results from eDNA provided new locations of Western Pearlshell living in the Bear River basin. The first known observation of Western Pearlshell in the Bear River was a shell in 1895 near Evanston (Henderson 1924). Hovingh (2004) reported finding 100 Western Pearlshell in the Bear River of Wyoming in 1956 and only five live individuals in 1998. Wyoming Game and Fish Department discovered one live Western Pearlshell in 2003 near Cokeville. The most recent surveys in the Bear River

basin searched for mussels using visual and tactile methods along 11 reaches in 7 streams (Mathias 2014) and 10 reaches in the Bear River (Wilmot et al. 2017). Western Pearlshell mussels were found in four reaches (2 reaches with 526 live individuals; Mathias 2014). Western Pearlshell were abundant at a site along the lower Smith's Fork River (524 individuals) and two individuals were found in Mill Creek in the upper Bear River basin. Only shells of Western Pearlshells were found at Cokeville Meadows National Wildlife Refuge and below Woodruff Narrows Reservoir (Mathias 2014). Only ten live Western Pearlshells were found in the Bear River basin after searching nearly 4 river km in 2016 (Wilmot et al. 2017). In our study, eDNA of Western Pearlshell was not detected in the Bear River at the Cokeville Meadows National Wildlife Refuge nor below Woodruff Narrows Reservoir despite previous surveys finding shells there (Mathias 2014). The shells of Western Pearlshell are thick and likely persist in the environment for decades. We sampled farther upstream on the Smith's Fork River than previous surveys, and eDNA evidence and shells indicated that Western Pearlshell inhabited some reaches. Environmental DNA suggested Western Pearlshell live in the upper Bear River of Wyoming and these are the most upstream locations on record for Wyoming. No current populations are known upstream in Utah (Hovingh 2004, Jepsen et al. 2010). Additional, positive eDNA results south of Evanston are the first to indicate Western Pearlshells inhabit this section of the Bear River.

Current taxonomy considers *A. californiensis* and *A. nuttalliana* to be separate species (Turgeon et al. 1998); however, recent genetic evidence suggested they are in the same clade (Chong et al. 2008). Morphology used to separate *A. californiensis* and *A. nuttalliana* did not vary consistently with nuclear and mitochondrial DNA evidence thus these species cannot be distinguished by shell characteristics or genetics. The clade is composed of six genetically distinct groups that cluster by hydrologic basin (Mock et al. 2010). The eighteen individuals collected in the Bear River near Woodruff, Utah used in the analysis clustered in the Bonneville Basin clade along with populations from the Snake and Sevier Rivers. The Snake River was distinct from the Columbia River Basin and more closely related to individuals in the Bear River likely because of past connections between the Snake and Bear Rivers (Malde 1968). In fact, a unique haplotype (group of genes inherited together) was only found in the Snake and Bear Rivers. The other hydrologically distinct units were the Columbia River Basin, Lahontan Basin, Pit and Owens Rivers, Colorado River Basin, and Coastal and Central California. Interestingly, genetic diversity was low in the Bear River (Mock et al. 2004), but increased downstream, potentially indicating better habitat or more host fish (Mock et al. 2010). Overall, Mock et al. (2010) recommended that *Anodonta* from the six hydrologic units be considered as evolutionarily distinct units and decisions should be based upon information from populations within that hydrologic unit. If management actions are used, they recommend using individuals from within that hydrologic unit (e.g., Bonneville Basin) and individuals from farther downstream should be used to increase genetic diversity.

Environmental DNA has been used to study several mussel species and the distance mussels can be detected downstream varies. The modelled distance that a bed of Fatmuckets (*Lampsilis siliquoidea*) could be detected was predicted to be 36.7 km downstream, but eDNA samples only detected the mussels 1 km downstream when experiments were done in a natural stream (Sansom and Sassoubre 2017). A variety of factors control how quickly eDNA degrades and were likely not incorporated into their model, such as water temperature, ultraviolet light (Strickler et al. 2015), pH (Seymour et al. 2018), eDNA particle size (Turner et al. 2014), sediment (Jerde et al. 2016, Shogren et al. 2016), hydrology (Shogren et al. 2017) and biofilm (Shogren et al. 2018). Shogren et al. (2019) modeled the dynamics of eDNA using nutrient cycling theory and discovered that uptake length was 1052 m (distance eDNA

travels before being removed from the water) and they detected Zebra mussels (*Dreissena polymopha*) 4 km downstream of known occurrences in a Danish stream. Twenty caged Western Ridged mussels (*Gonidae angulate*) were detected 8 km downstream in Central Valley, California (Preece et al. 2020), but the variation in eDNA concentration increased as samples were collected farther from the mussel bed (Shogren et al. 2019). Environmental DNA likely degraded quickly in the Bear River because detections quickly went from high concentrations to zero within a short distance. In fact, 80-90% of eDNA particles may degrade within the first day (Shogren et al. 2018). In the Bear River, we assumed that mussels were living upstream when they were detected because eDNA of animals was not detected 7 to 31 days after the species was removed from mesocosms (Sansom and Sassoubre 2017) and 1 hour in a stream (Pilliod et al. 2014); however, eDNA may persist in the environment in shells (Hawk and Geller 2019) or in sediments (Turner et al. 2015). The shell of an endemic mussel in New Zealand had 0.002 to 21.48 ng DNA/mg shell (Ferreira et al. 2020) which probably contributes a small amount to the water via weathering and is a tiny source of eDNA compared to live mussels (54,000 to 2,400,000 copies DNA/hr/mussel; Sansom and Sassoubre 2017).

The concentration of eDNA depends on mussel density and other characteristics of the stream. The detection rate of eDNA was positively correlated to the number of hand collected mussels in Japanese ponds (Togaki et al. 2020). In streams, the concentration of eDNA can vary with mussel density, but ammonium and phosphate concentrations, algal biomass, water velocity (Shogren et al. 2019), where the water sample was collected and the time of year may also explained concentrations. Water collected in the middle of the stream (Shogren et al. 2019), near the benthos (Lor et al. 2020) and in riffles (Preece et al. 2020) collected higher concentrations of mussel eDNA compared to the edge of the stream, surface water samples, and pools or runs. Samples collected during larval release had higher detection rates (73-93%) compared to after the reproductive period (56-71%) for the Endangered Spectacle Case mussel (*Margaritifera monodonta*) in Wisconsin (Lor et al. 2020); however, the mussels in the Bear River reproduce in spring when discharge is high and the increase in eDNA from reproduction may be overshadowed by dilution during runoff (T. Rodgers, personal observation). One study modeled the density of Zebra mussels and the best predictors were the concentration of eDNA, nitrate concentration, substrate type and sampling location (Shogren et al. 2019). We cannot model mussel density in the Bear River because few surveys have been done, but many factors likely effect the concentration of eDNA we observed, including mussel density. Thick biofilms can contribute to the rapid degradation of eDNA (Shogren et al. 2018). We observed well-developed biofilm at most locations along the Bear River and may at least partially explain why eDNA appeared to degrade quickly in the Bear River basin. Based on these factors, we believe that detection of mussel eDNA in the Bear River is likely indicative of local presence, and not from eDNA originating far upstream.

Shells were a good indicator that mussels were present in a reach of the Bear River. The presence of thin, fragile shells, such as those from California Floater, are likely more indicative that a mussel species recently or currently inhabited a river reach compared to species with thicker shells, such as Western Pearlshell and Plain Pocketbook (*Lampsilis cardium*) whose shells likely persist for decades. We are not aware of any studies that specifically measured how long mussel shells persisted in the environment, but shells are often used in archeological studies and can be thousands of years old (e.g., Burkli and Wilson 2017). A study measured decomposition rates of snail shells and found that shells persisted longer in dry, alkaline environments such as found in Wyoming and larger shells lasted longer (Rihova et al. 2018). We imagine that thicker shells also decompose more slowly. Previous sites where only

Western Pearlshell shells were found (no live individuals) were reaches where eDNA did not detect Western Pearlshell in this study. This indicates that Western Pearlshell may be extirpated from these reaches currently or if they occur, densities are very low. Conversely, the number of shells we found along a reach was positively related to the concentration of California Floater eDNA. How long shells persist may explain why the relationship between the concentration of eDNA and the number of shells found was significant for California Floater and not for Western Pearlshell. The presence of shells does not replace eDNA analysis as several reaches had positive eDNA samples but we did not find shells.

The size frequency of shell length for live individuals is an indicator of the age distribution of a population and can provide insight about recruitment. Twenty live *Anodonta* collected from the Bear River in Utah in 2001 had an average shell length of 50 mm (±11.2, standard error), and an equal number of males and females (Mock et al. 2004). In 2011, 13 live California Floater were found in the Bear River in Wyoming and lengths ranged between 58 and 103 mm (mean 69 mm; Mathias 2014). The Wyoming Game and Fish Department sampled a 1 km reach on Cokeville Meadows National Wildlife Refuge since 2018 to estimate mussel density and establish proper monitoring protocols for California Floater. They tagged four live California Floaters in 2018, three live individuals in 2019 and two live mussels in 2020, one of which was a recapture from 2018. California Floater length ranged from 83 - 114 mm, and no juveniles were detected live or dead. We are not aware of any studies that documented at what age or shell length California Floater can begin reproducing; however, *Anodonta woodiana* became sexually mature at 2 years old and 58 mm while in culture (Chen et al. 2015). We recognize that the size at first reproduction will differ among mussels and locations as growth depends on the quality and quantity of food, but estimates can be useful to interpret shell sizes. *Anodonta woodiana* maximum length is longer (167 mm length; Kamburska et al. 2013) than California Floater (130 mm; Tronstad et al. 2015), and if we assume a proportional relationship and similar growth, California Floater would start reproducing at ~45 mm length. Based on shells from the Bear River, 2 to 3 years old individuals were ~45 mm in length and we would expect growth to be slower in streams than in culture. Individuals <45 mm in length were detected in 2001, but not in 2011; however, smaller individuals are more difficult to detect in visual and tactile searches. The shells we discovered in 2019 (not live individuals) were larger indicating low mortality of juveniles, smaller shells were more fragile or juveniles were absent. Regardless, recruitment may be a limiting factor for this species and should be investigated, given the lack of evidence of recruitment during the past 20 years.

Shell lengths of Western Pearlshell indicate that recruitment is occurring for this species in the Bear River basin. Western Pearlshells that Mathias (2014) found in the Bear River basin of Wyoming in 2011 ranged between 28 and 75 mm length (mean 52 mm) which varied in age between <10 year old and ~30 years old according to a regression relating shell length to age from the Chehalis River, Washington (Waterstrate 2013) if we assume growth is similar between streams. Shells we discovered (not live individuals) ranged in age between <10 and 40 years old, indicating mortality in a variety of size classes. The age at which Western Pearlshell can begin reproducing is unknown; however, *Margaritifera margaritifera* became sexually mature at 15 to 20 years old (Bauer 1987, Degerman et al. 2006) which is 52 to 57 mm long (Waterstrate 2013). Therefore, recruitment appeared to be occurring in Western Pearlshell populations of the Bear River basin.

Mussels are declining due to a variety of anthropogenic stressors. Non-marine mollusks have the highest recorded number of extinctions, and this group includes terrestrial snails, aquatic snails and

mussels (Lydeard et al. 2004). North America has the highest diversity of mussels with 300 species recorded (NatureServe 2021). Of these species, 40% are ranked as critically imperiled or imperiled (G1 or G2 rank) in their global ranges. Mussels are declining due to a variety of anthropogenic causes, such as sedimentation (Hansen et al. 2016), impoundments (Watters 1999), land use (Howard and Cuffey 2006a), water quality (Haag et al. 2019), loss of host fish (Williams et al. 1993) and drought (DuBose et al. 2019). California Floater are ranked as critically imperiled or imperiled in all states they occur in (except Idaho where they are vulnerable) and appear to occur at low densities with no evidence of recruitment in the Bear River basin of Wyoming. Western Pearlshell are ranked between critically imperiled and vulnerable in the United States where they occur. They can be more abundant in localized areas compared to the California Floater and we found evidence of recruitment in the Bear River basin. To learn more about mussels in the Bear River basin, we recommend surveys based on locations identified using eDNA samples, prioritizing sites by the concentration of eDNA. Studying the reproduction of California Floaters (e.g., release of glochidia, fish parasitized by glochidia, development of juveniles) in the Bear River basin may identify what is limiting the populations. Glochidia (parasitic stage of mussels) of California Floater and Western Pearlshell can be distinguished (O'Brien et al. 2013) and such a study would provide information about what fish are used as hosts. A similar study is currently being conducted on the Western Pearlshell in Montana and evidence suggest that juvenile recruitment (after glochidia release from fish hosts) is limiting populations there (Stagliano et al. 2020). Collecting information about the reproduction and size structure of mussels in the Bear River basin will provide more information about their status and eDNA samples targeted locations to conduct such studies.

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Table 1. Minimum and maximum values of water quality and habitat conditions at sites with California Floaters, Western Pearlshells and all sites sampled in the Bear River basin of Wyoming.

	California Floater		Western Pearlshell		All stream reaches	
Parameter	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
Temperature (°C)	9.4	20.8	9.0	16.3	7.1	20.8
Dissolved oxygen (% sat)	67.2	116.1	67.2	102.3	67.2	116.1
Dissolved oxygen (mg/L)	7.5	11.8	7.4	11.1	7.4	11.8
Specific conductivity (µS/cm)	271.7	738.0	187.6	550.8	145.6	791.0
pH	8.36	8.82	8.36	8.69	8.01	8.91
Stream depth (cm)	20	125	15	130	10	130
Stream width (m)	13.3	53	3.2	53	0.5	53

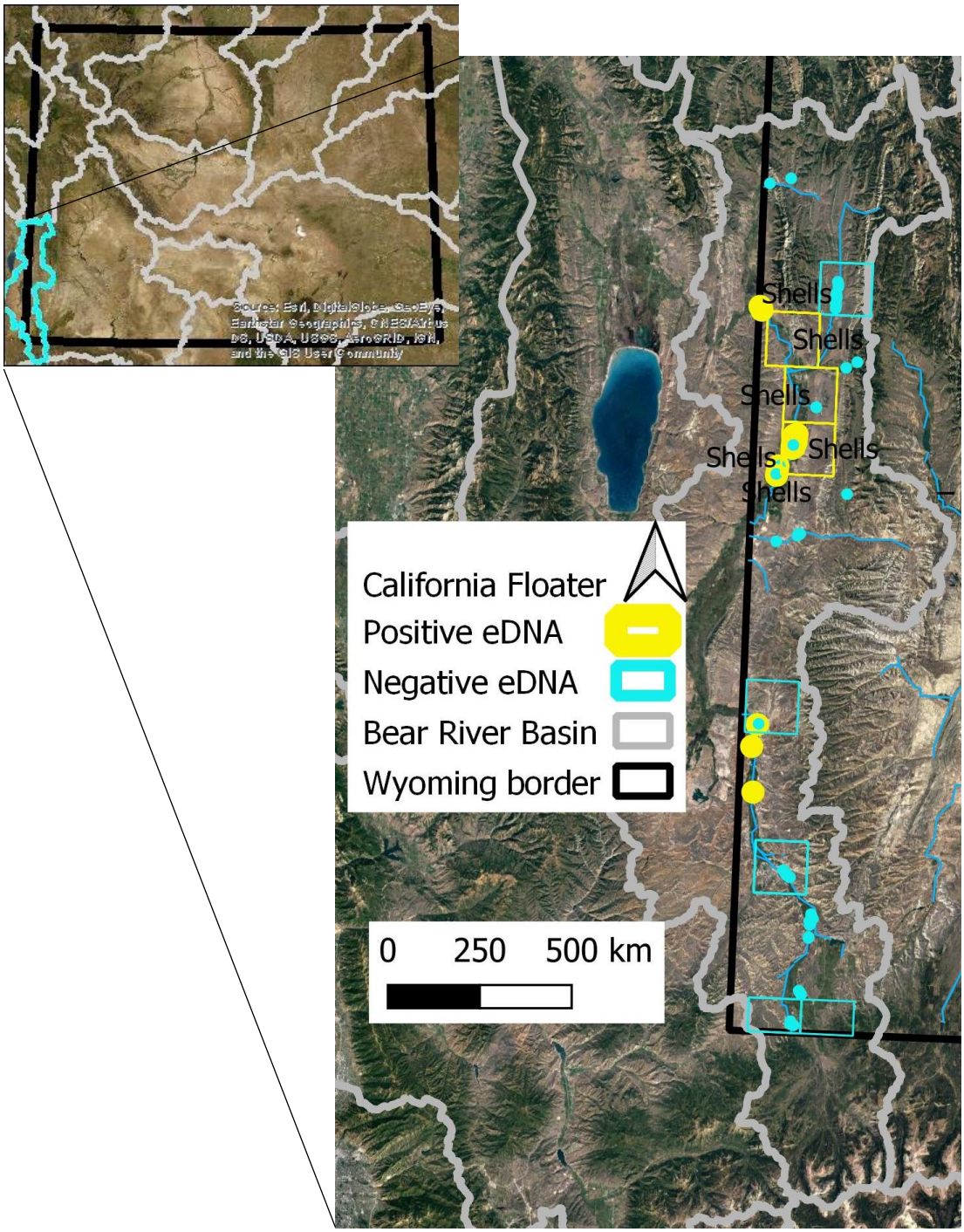

Figure 1. Presence and absence of California Floater environmental DNA (eDNA) in water samples collected at 136 sites in the Bear River basin of Wyoming (hydrologic unit code 6; grey outline). Shells were found at labeled locations. Squares indicated that a positive or negative eDNA result was within that area. The inset map shows the location of the Bear River basin relative to Wyoming.

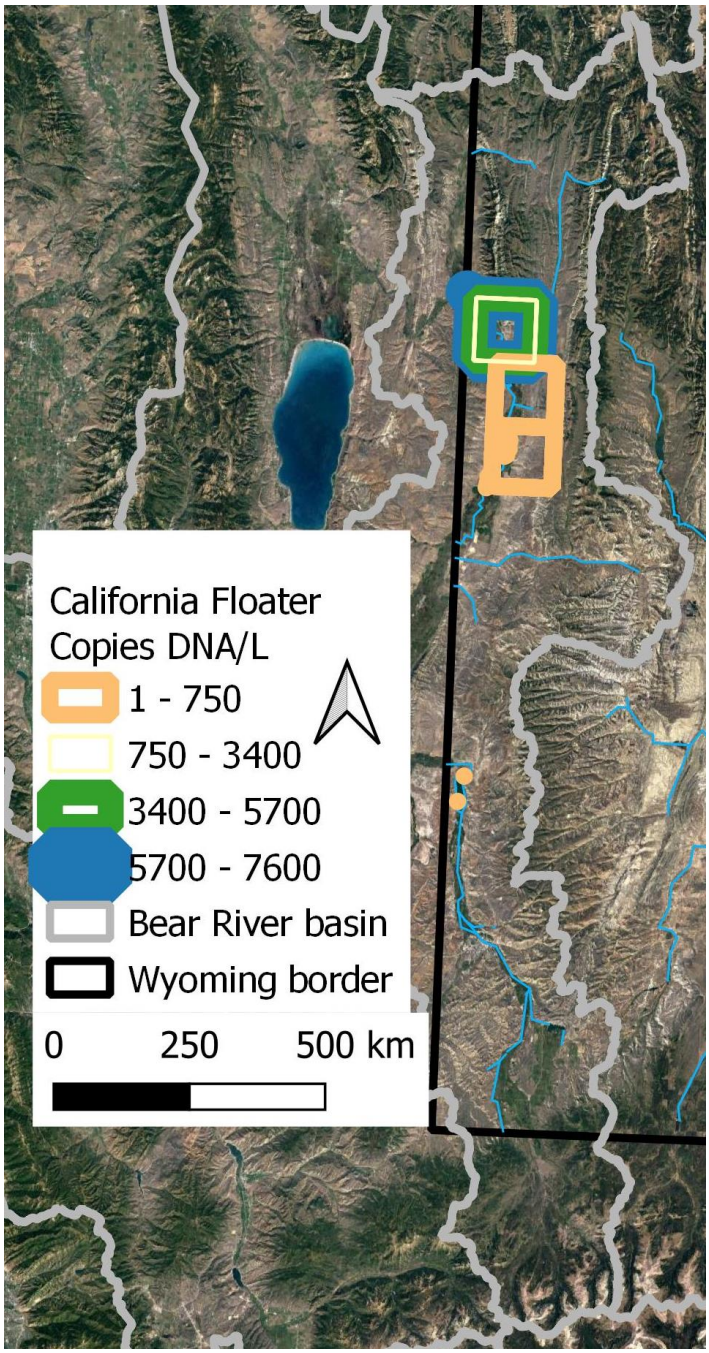

Figure 2. The concentration of California Floater DNA fragments in water samples collected from the Bear River basin of Wyoming. Higher concentrations of DNA fragments may indicate higher densities of mussels upstream. Squares indicated that the concentration of environmental DNA (eDNA) was measured within that area.

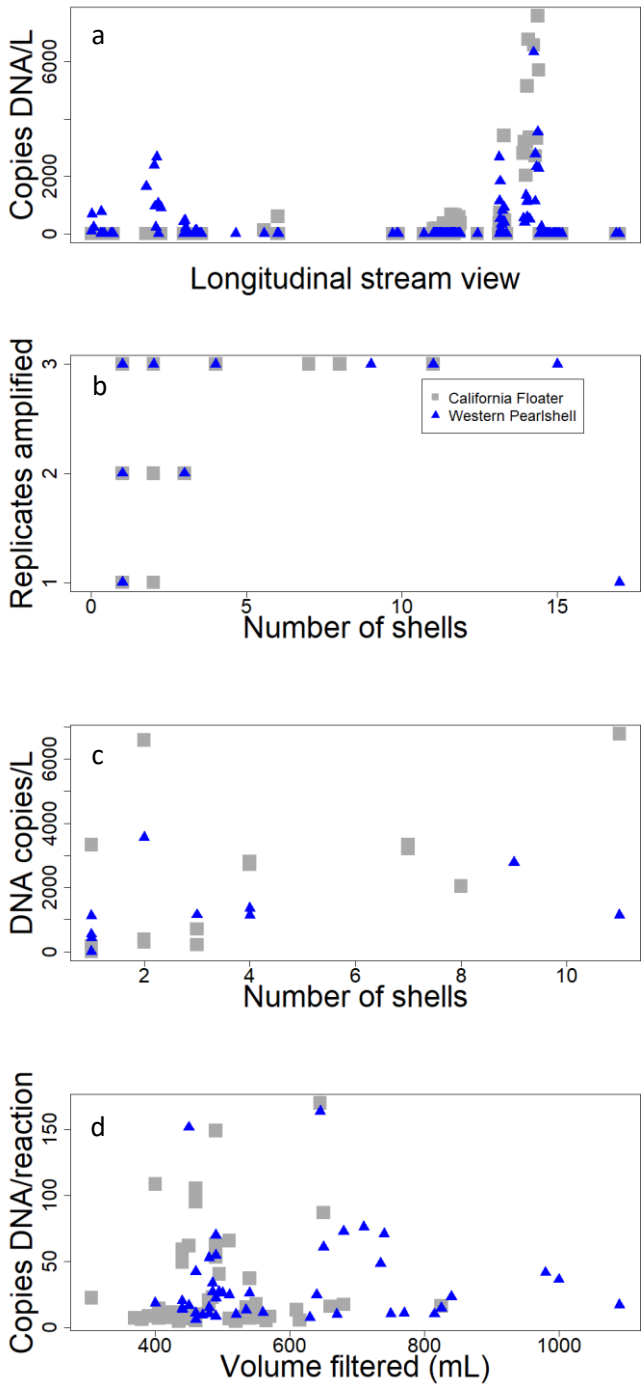

Figure 3. a.) The concentration of DNA varied along the length of the stream. The more shells we discovered along each reach for California Floater (grey squares) generally meant more b.) replicates amplified and c.) more copies of DNA/L measured in environmental DNA samples, but the relationships was not significant for and Western Pearlshell (blue triangles). d.) A larger volume of water filtered did not produce more mean copies of DNA per reaction for either species of mussel.

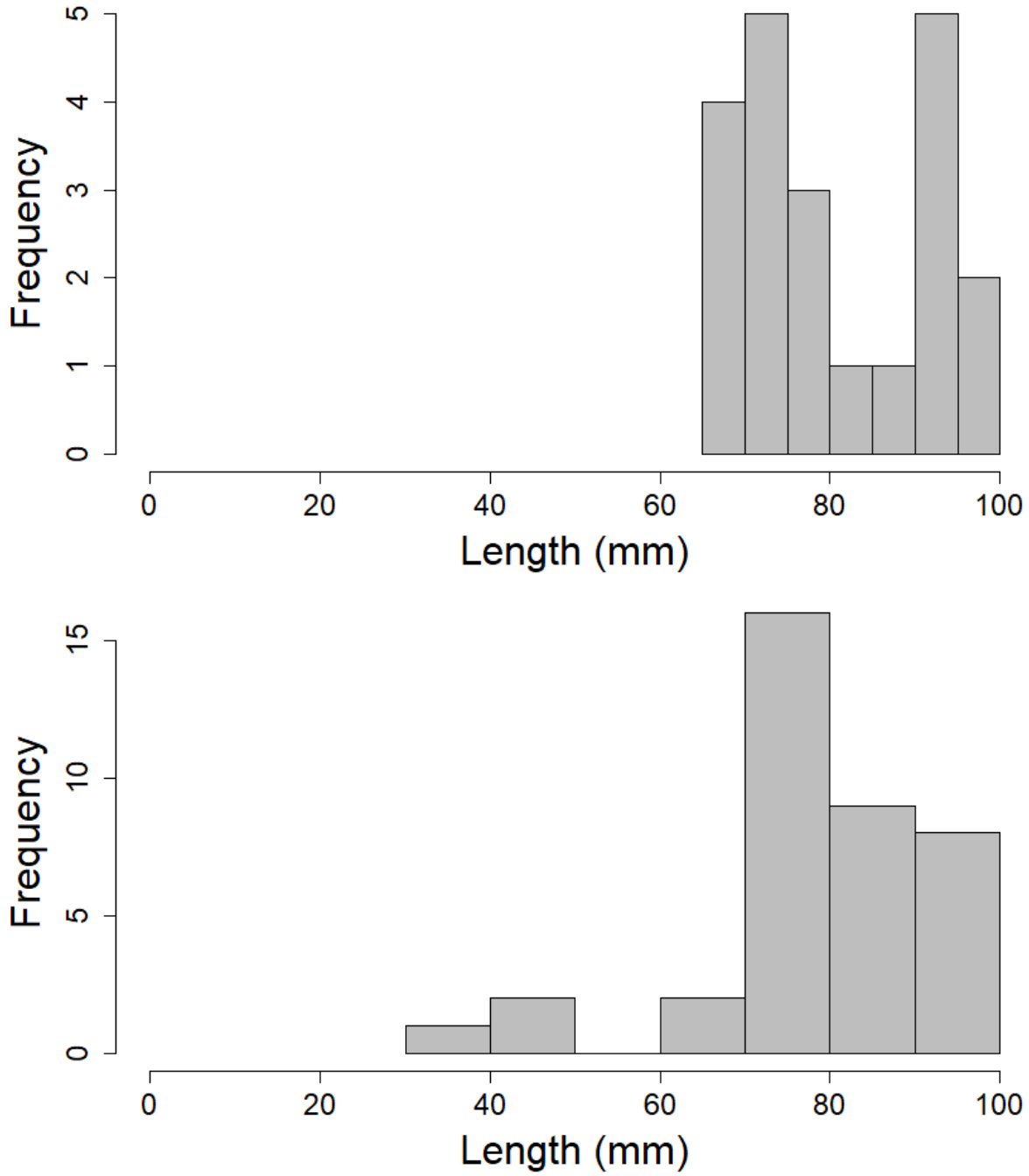

Figure 4. The number and shell length for a.) California Floaters and b.) Western Pearlshells found in the Bear River basin, Wyoming.

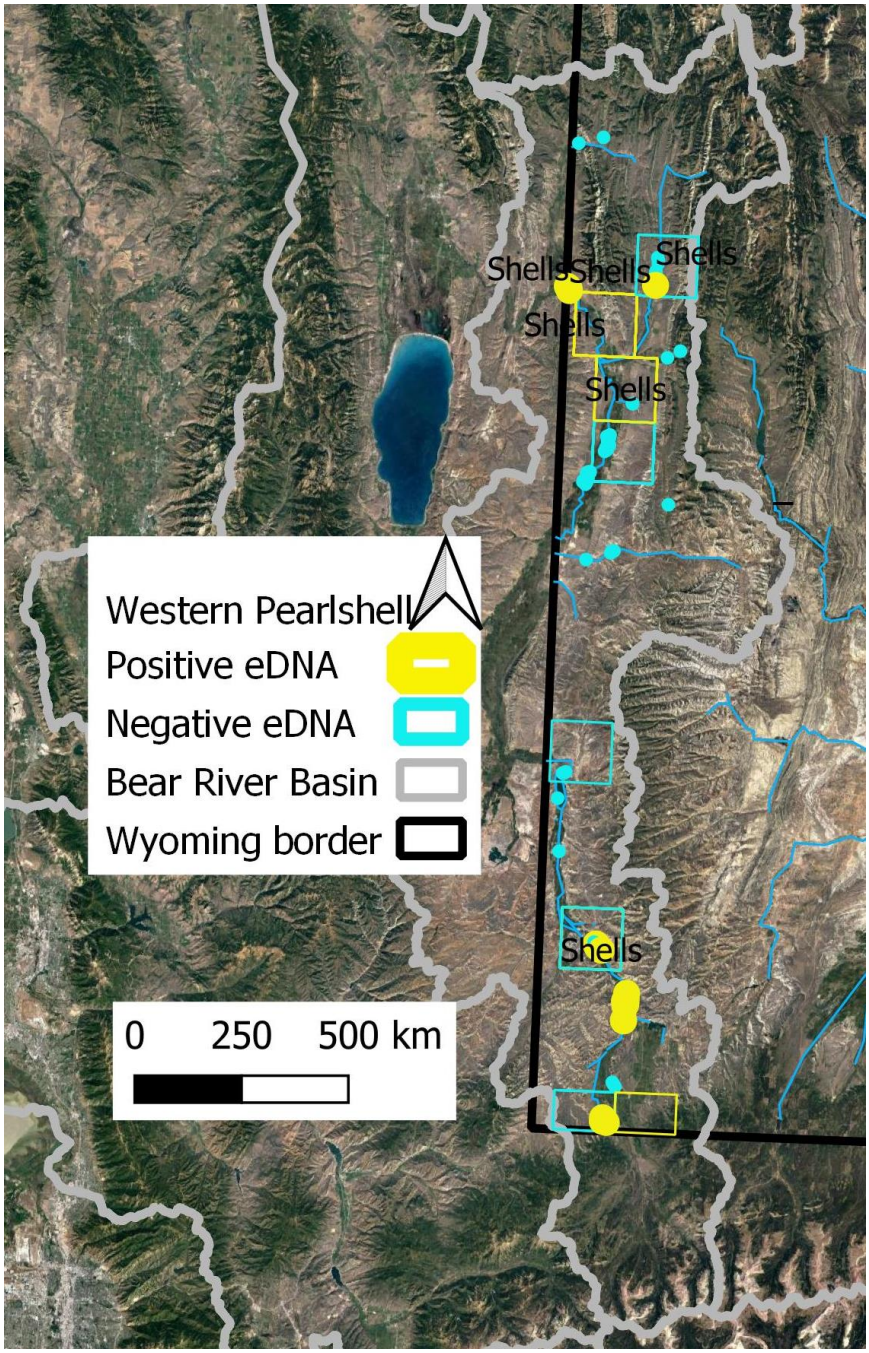

Figure 5. Presence and absence of Western Pearlshell environmental DNA (eDNA) in water samples collected at 136 sites in the Bear River basin of Wyoming (hydrologic unit code 6; grey outline). Squares indicated that a positive or negative eDNA result was within that area. Shells were found at labeled locations.

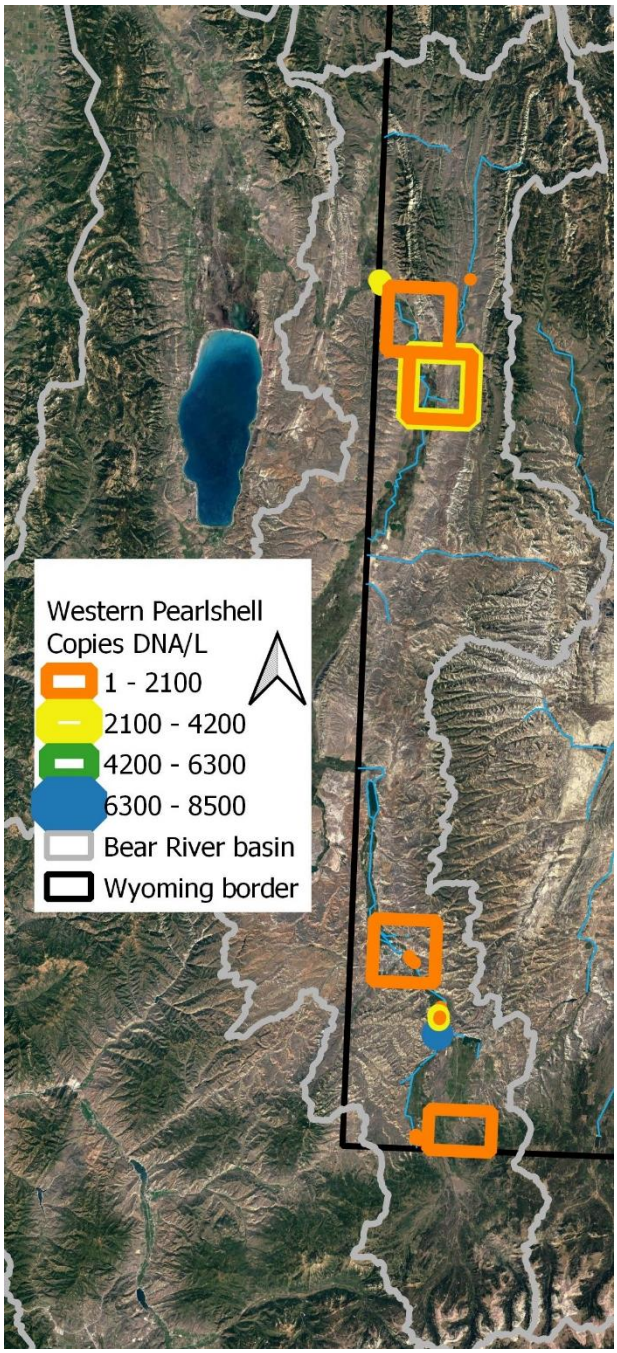

Figure 6. The concentration of Western Pearlshell DNA fragments in water samples collected from the Bear River basin of Wyoming. Higher concentrations of DNA fragments may indicate higher densities of mussels upstream. Squares indicated that the concentration of environmental DNA (eDNA) was measured within that area.