

Rapid Communication

The first occurrence of the southern plains crayfish *Procambarus simulans* (Faxon, 1884) documented in Montana, USA

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Abstract

During a statewide crayfish survey of Montana, we documented the southern plains crayfish *Procambarus simulans* for the first time in the state. *Procambarus simulans* is native to, and commonly found in, southern and central US states, including Arkansas, Colorado, Kansas, Louisiana, New Mexico, Oklahoma, and Texas. We found this species in a single location, the Miles City State Fish Hatchery, in eastern Montana. At 46.38 degrees north latitude, this is the northernmost population of the species reported. The presence of multiple size classes suggests that the crayfish can overwinter and reproduce in Montana and have been at the location for at least several years. The source of introduction, current extent of spread, and ecosystem impacts are unknown, and investigation is needed to understand the source and effects of this novel species on native crayfishes and other aquatic species.

Key words: aquatic invasive species, fish hatchery, introduction, detection

Introduction

Crayfishes are among the largest freshwater invertebrates in North America in size and species diversity (Thorp and Covich 2010; Taylor et al. 2015). Whereas 350 species have been documented in the United States (Helfrich and DiStefano 2020), only three have been documented in Montana. Due in part to their diverse trophic roles, crayfishes often have strong impacts on ecosystems (Dorn and Mittelbach 1999). They consume living and dead plant and animal material and are eaten by a wide variety of aquatic and terrestrial species (including humans) (Hobbs 1993).

Multiple pathways of crayfish introductions are known. Crayfish trade, in both the pet and food industries, has increased over recent decades, leading to more introductions (Faulkes 2015; Kawai et al. 2016). Crayfishes have been intentionally introduced, both legally and illegally, as forage for many game fish species, including nonnative fishes (Lowery and Holdich 1988; Lodge et al. 2000; Larson and Olden 2008). They have also been introduced

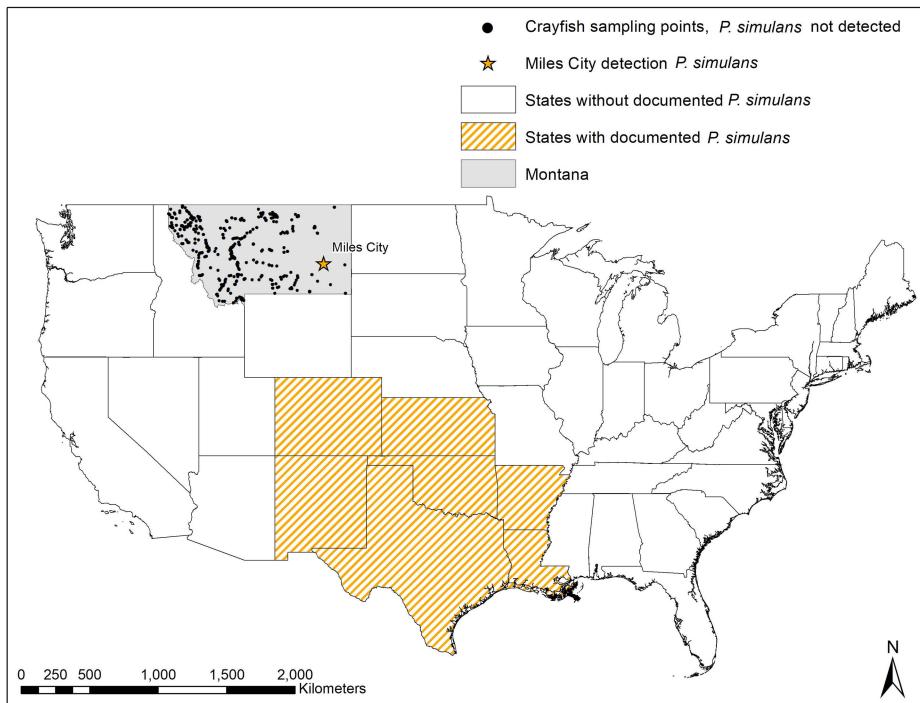


Figure 1. States with documented occurrences (putative native range) of *Procambarus simulans* in contiguous United States and crayfish sampling sites in Montana with no detection of *P. simulans* and the one location in Miles City, MT, with a detection of *P. simulans*.

while used as bait for fishing (DiStefano et al. 2016). Anecdotes indicate that crayfish also have been unintentionally introduced during fish stocking or inter-hatchery fish transfers (Sheldon 1989; Thomas 1991).

In Montana, crayfish distributions are only well-documented from parts of the Columbia River basin west of the Continental Divide (Sheldon 1989). The remainder of the state has only scattered crayfish records (Hobbs 1989; Montana Natural Heritage Program 2022; Smithsonian National Museum of Natural History 2022), and species identifications for some records were questionable. Historically, Montana Fish, Wildlife, and Parks (FWP) personnel opportunistically recorded crayfish encounters while sampling for fishes or aquatic invasive species in waterbodies across the state. However, crayfish presence or absence was not consistently recorded, and species were not always identified correctly, or at all.

In 2021, we conducted the first year of a two-year statewide crayfish survey. Two of our objectives were to document current crayfish species distributions in the state and to inform managers about nonnative crayfishes. In this paper, we report the first record of the nonnative southern plains crayfish *Procambarus simulans* (Faxon, 1884) in Montana. We discovered the species in the Miles City State Fish Hatchery, a warmwater fish hatchery located two miles southwest of Miles City, Montana (Figure 1), that supplies fishes to the entire state. *Procambarus simulans* is native to, and commonly found in, southern and central US states, including Arkansas, Colorado, Kansas, Louisiana, New Mexico, Oklahoma, and Texas.

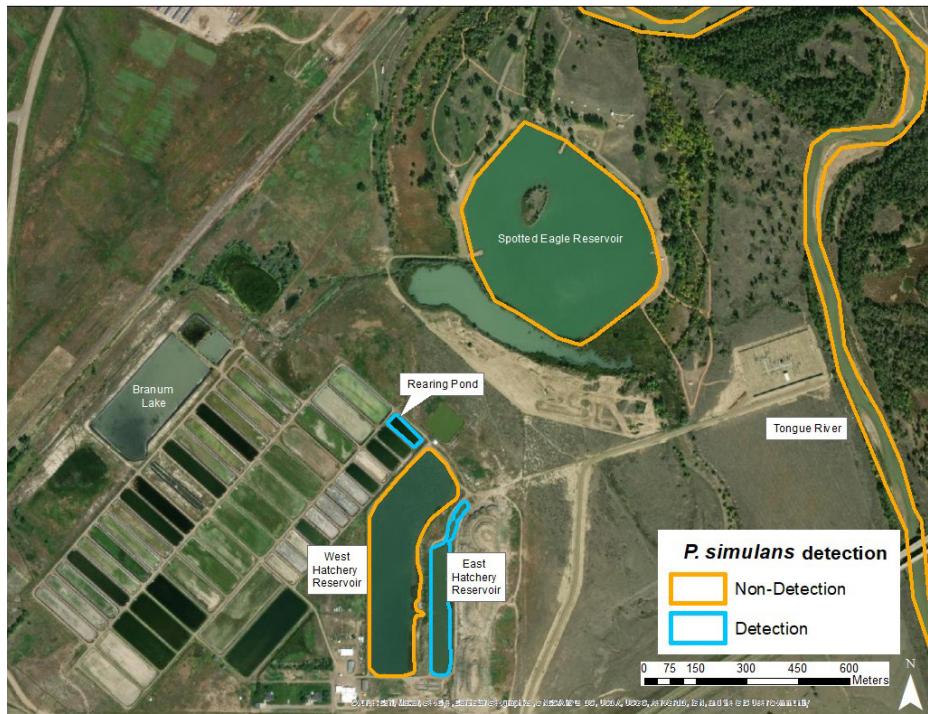


Figure 2. Satellite view of the Miles City Fish Hatchery located at 46.382°N; -105.860°W. Water bodies sampled in 2021 with or without detection of *P. simulans* outlined in blue and orange, respectively. Water intake for the hatchery located on Yellowstone River north of facility and not in photo frame. Intake water from the Yellowstone River is collected in the east and west hatchery reservoirs and used as supply for the other hatchery infrastructure. World Imagery – Source: Esri, Maxar, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community.

Materials and methods

Miles City State Fish Hatchery

The Miles City State Fish Hatchery began operating in 1928 as the Miles City Pond Culture Station (Alvord 1991) located off Fort Keogh Road about 1.5 km west of the current location. The U.S. Bureau of Fisheries assumed ownership in 1933, and the name changed to Miles City National Fish Hatchery (Alvord 1991). Then in 1959, the hatchery moved to its current location (US Department of the Interior 1960). The facility was transferred to Montana FWP in 1983, when the hatchery assumed its current name (MFWP 2001). Historically this hatchery raised warmwater fishes, many of which came from out-of-state hatcheries. The hatchery's source water is typically drawn from the Yellowstone River but can also be taken from the Tongue River, and water is stored in the East and West Hatchery reservoirs before flowing throughout the hatchery. Effluent from the hatchery flows into the Tongue River via Spotted Eagle Reservoir (Figure 2).

Field surveys

In 2021, targeted crayfish sampling for our statewide survey consisted of sampling over 100 sites across the state (Figure 1) using various combinations

Table 1. Crayfishes caught at the Miles City State Fish Hatchery, Montana. Sampling methods, dates (for traps, date retrieved), and locations: numbers of crayfishes caught. Abbreviations as follows: East = East Hatchery Reservoir; West = West Hatchery Reservoir; Spotted Eagle = Spotted Eagle Reservoir; Rearing Pond 45 = rearing pond number 45; *simulans* = *Procambarus simulans*; and *virilis* = *Faxonius virilis*. Oct. 22 results are from subsample of crayfishes collected.

Method	Date sampled, 2021					
	Sep. 14		Sep. 15		Oct. 22	
	Location	Crayfish	Location	Crayfish	Location	Crayfish
Crayster® trap	East	8 <i>P. simulans</i>	West	6 <i>F. virilis</i>	Spotted Eagle	0
Minnow trap	West	0	West	0	Spotted Eagle	0
Visual search	East, levee	1 <i>P. simulans</i>	Rearing pond (45)	1 <i>P. simulans</i>	East, drained (subsample)	1742 <i>F. virilis</i> 22 <i>P. simulans</i>

of dipnetting, visual searches while wading or snorkeling, kick-seining, and occasionally trapping with baited traps. In most sites, we sampled for a minimum of 45 person-minutes. We identified nearly all crayfishes to species, but some smaller juveniles were too difficult for us to identify due to size and recent molting.

In addition, FWP's Aquatic Invasive Species (AIS) Early Detection Program technicians annually sample > 800 sites distributed among 350 waterbodies to search for AIS. The technicians use standardized sampling protocols, and in 2021, we added targeted crayfish sampling, documentation, and voucher collection to the protocols. Technicians vouchered crayfish specimens from each of the > 200 sites where they captured crayfishes. Finally, 62 sites were sampled by FWP biologists, who caught crayfishes during their fish sampling or recorded the absence of crayfish from their samples.

One component of the AIS program's annual early detection sampling is routine inspections of fish hatcheries (federal, state, and commercial). We sampled for invasive species at the Miles City State Fish Hatchery from September 13–15, 2021 (Figure 2). Hatchery staff informed us that they had observed two crayfish species in the facility's outdoor infrastructure. The staff did not know what species they were but noted that both had likely been present at the hatchery for decades (C. Hagemeister, FWP, *personal communication*, September 14, 2021).

To determine which species were present, we set baited traps over one night in East and two nights in West Hatchery reservoirs (Figure 2; Table 1). We used two traps: a Crayster® stream cylinder trap (51 × 22 × 22 cm made of 2.5 × 1.0 cm, 16-gauge, powder-coated-steel mesh, with two 6.5-cm-diameter openings); and a cylindrical minnow trap (42 × 22 cm made of 5 mm galvanized steel with two 2.5-cm-diameter openings). We set both traps on September 13 and 14 (Table 2). We baited traps with canned sardines in water the first night and a combination of canned tuna and canned cat food the second night. In both reservoirs, intake structures that supplied water to other parts of the hatchery provided shelter for crayfishes and were near two other areas of suitable crayfish habitat: rocky cobble and cattail (*Typha* sp.) cover. We set traps at depths of about 1.8–2.4 m approximately 3–5 m from shore between the intake structures and the rock and vegetation cover. We captured the as-yet unidentified crayfish in the traps.

Table 2. Subsampled crayfishes from the Miles City State Fish Hatchery, East Hatchery Reservoir, October 22, 2021. Species, reproductive form, number (n), and the minimum (Min.) and maximum (Max.) post-orbital carapace lengths (POCL, mm) of each species x form group in a random subsample of the crayfishes collected from the reservoir. Other abbreviations as follows: F. = *Faxonius*, P. = *Procambarus*, M1 = reproductive form male, M2 = non-reproductive form male, F w/g or F w/o g = female with or without, respectively, glair visible in glair glands.

Totals	Form	n	Min. POCL	Max. POCL
<i>F. virilis</i>	M1	651	17.1	40.6
<i>F. virilis</i>	M2	127	12.3	26.8
<i>F. virilis</i>	F w/g	831	15.6	41.0
<i>F. virilis</i>	F w/o g	133	11.0	26.3
<i>P. simulans</i>	M1	7	32.9	37.6
<i>P. simulans</i>	M2	4	32.4	41.1
<i>P. simulans</i>	F w/o g	11	26.0	35.1

During our inspection in September, hatchery staff drained rearing pond number 45 (“rearing pond” in Figure 2) as part of routine hatchery operations for fish collection. On September 15th while the pond was mostly drained (with a few centimeters of water remaining in the lowest places and the pond bed and liner exposed), we visually surveyed the entire pond by walking the entirety of the pond bed looking for dead or live crayfish, burrows, and holes occupied by crayfish.

In October 2021, as a result of our findings and routine maintenance needs, hatchery staff drained East Hatchery Reservoir, collected crayfishes from the drained area, and held them live in one indoor raceway. On October 21, 2021, we returned to the hatchery to identify and count the captured crayfishes and to set baited traps (the same two described above) overnight in Spotted Eagle Reservoir. Because of the large number of crayfishes captured in East Hatchery Reservoir, we randomly subsampled the total catch, processing 1764 crayfish. The catch consisted of mostly virile or northern crayfish, *Faxonius virilis* (Hagen, 1870) with a few of the still-unidentified species mixed in (see Results). We sorted the subsampled crayfishes by species, sex, and reproductive status (reproductive form 1 versus non-reproductive form 2 males, and females with versus without visible glair glands). We measured post-orbital carapace lengths (mm) of the smallest and largest individuals from each of the above groups, preserved whole specimens of the unidentified species in 70% ethanol for species identification, and preserved gill tissue from four individuals in 95% ethanol for genetic sequencing.

We tentatively identified specimens as *P. simulans* based on morphology. We then sent preserved specimens to Dr. Chris Taylor (Illinois Natural History Survey, USA) and Mr. Dan Johnson (Texas, USA) for confirmation of identification, and live specimens to Dr. Guenter Schuster (emeritus, Eastern Kentucky University, USA) for identification and photography. Six specimens were deposited in the Illinois Natural History Survey Crustacean Collection (Catalog number: INHS 17337; Champaign, IL). Additional specimens are in the US Forest Service, Center for Bottomland Hardwoods

Research Crayfish Collection (Oxford, MS) and will ultimately be deposited in Montana State University's Montana Entomology Collection (Bozeman, MT).

Genetic identification

We extracted DNA from abdominal or gill tissue using the Qiagen DNEasy Blood and Tissue kit following the standard protocol (Qiagen, Valencia, CA). We amplified the *Cytochrome Oxidase 1 (CO1)* mitochondrial gene using Polymerase Chain Reaction (PCR) with ORCO1R and ORCO1F primers (Taylor and Hardman 2002). Each reaction consisted of 1.0 µL of template DNA, 10.2 µL of dH₂O, 2.0 µL of 25 mM MgCl₂ (New England Biolabs Inc., Ipswich, MA, Cat. #B0510A), 1.5 µL of 10mM dNTP Mix, 4.0 µL of 5X PCR Buffer (Promega, Madison, WI, Cat # M891A), 0.1 µL Bovine Serum Alumbin (New England Biolabs Inc., Ipswich, MA, Cat. #B9000S), 0.5 µL of each primer (10 µM) and 0.2 µL of Taq Polymerase (New England Biolabs Inc., Ipswich, MA, Cat. #M0267L). We ran PCR in a thermocycler, where samples were first held at 94 °C for 3 minutes for the initial denaturation. They then underwent the following cycle 30 times: denaturation at 94 °C for 30 seconds, annealing at 50 °C for 45 seconds, and extension at 72 °C for 90 seconds. Samples were held at 72 °C for 5 minutes for the final extension. We cleaned the PCR product using the Thermo Fisher ExoSAP-IT PCR cleanup reagent and protocol (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania, cat # 78201.1.ML) using a 1:10 ExoSAP to PCR product ratio. Samples were sent to the Keck DNA Sequencing Facility (Yale School of Medicine) for Sanger sequencing.

We created consensus sequences for each of the four specimens in Geneious R7.1.9 (<https://www.geneious.com/>), and then blasted them in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the identity of the specimens. These sequences were accessioned to GenBank under OQ282953–OQ282956. All 4 specimens were MUSCLE aligned in MegaX 11.0.11 (<https://www.megasoftware.net/>), along with the other *P. simulans* CO1 sequences on GenBank (accession numbers EU583575, KU527881, and KX238232), *Procambarus ceruleus* (Fitzpatrick and Wicksten, 1998), *Procambarus liberorum* (Fitzpatrick, 1978), *Procambarus steigmani* (Hobbs, 1991), and *Procambarus kensleyi* (Hobbs, 1990) (KX238183, KF827978, KX238233, and KX238198), and *Cambarus friaufi* (Hobbs, 1953) as an outgroup (DQ411784). All sequences were trimmed to include only nucleotides within the defined range of the CO1 gene. To generate a Maximum Likelihood phylogeny from this alignment, we ran IQ-TREE 1.6.12 (Nguyen et al. 2015) with default settings including the ModelFinderPlus option (Kalyaanamoorthy et al. 2017) and assessed topological support with 1000 ultrafast bootstrap replicates. IQTREE chose the HKY + F + G4 model of evolution because it had the most Bayesian support.



Figure 3. Southern plains crayfish (*Procambarus simulans*) collected from Miles City State Fish Hatchery, MT. Photo of recently preserved specimen. Photo courtesy of Guenter Schuster.

Results

The novel specimens exhibited all of the diagnostic characteristics of *P. simulans* (Faxon 1884; Penn 1956; Hobbs and Robison 1982; Figure 3). The species was easily distinguished from all other crayfishes (*Faxonius* and *Pacifastacus* spp.) known to occur in Montana by the five terminal elements on the form 1 male gonopods (as opposed to two terminal elements on the other species) and by the dark tubercles contrasted against a lighter background on the chelae (as opposed to no obvious tubercles or light tubercles against a darker background on other species). All three outside experts confirmed our identification of *P. simulans* based on morphological features.

Among the four specimens for which we collected molecular data, mtDNA CO1 sequences were 100% identical. Comparison of the specimens' CO1 sequences to those previously uploaded in GenBank was consistent with their identification as *P. simulans*, with sequence overlap (percent identity) as high as 96.7–99.5%. The Maximum Likelihood phylogeny suggested that the four specimens were nested within the *P. simulans* clade (Figure 4), which further supported this identification.

Of the nearly 400 sites sampled for crayfishes in Montana in 2021, we found *P. simulans* only in the Miles City State Fish Hatchery. In September, we trapped eight *P. simulans* in East Hatchery Reservoir and none in the West Hatchery Reservoir (Table 1). We found one crayfish, a male *P. simulans*, in the drained rearing pond number 45. In West Hatchery Reservoir, we trapped only *F. virilis* (Table 1). *Faxonius virilis* are native to eastern Montana presumably including the Miles City area.

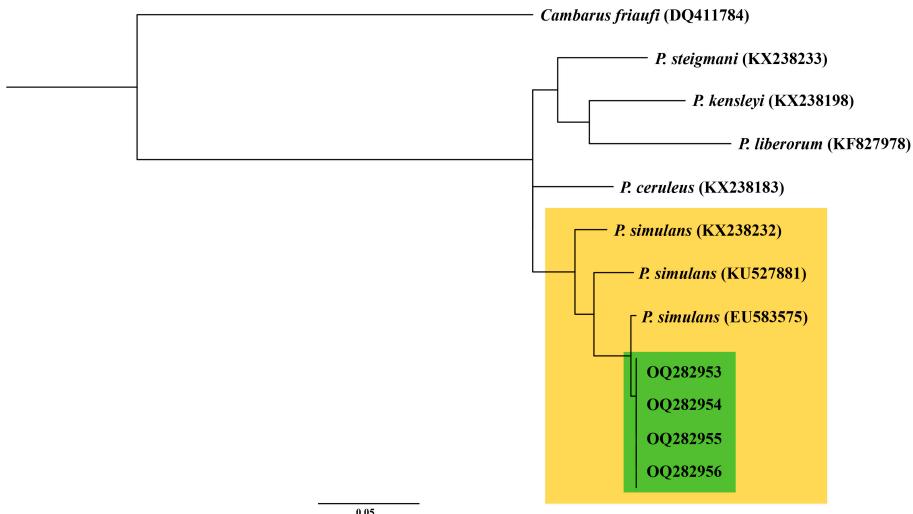


Figure 4. Maximum Likelihood phylogeny of the four specimens sequenced in this study and the sequences of five *Procambarus* species from GenBank, with *Cambarus friaufi* as an outgroup. Specimens sequenced in this study are shaded green while all *P. simulans* are shaded yellow.

Faxonius virilis were far more abundant than *P. simulans* at the hatchery. In October when East Hatchery Reservoir was drained, hatchery staff recovered both *F. virilis* and *P. simulans* from the reservoir, but the former outnumbered the latter by nearly 80:1 in the subset of crayfishes we counted (Table 1). In addition, we saw many shallow burrow openings in the drained reservoir but saw only 2–3 large burrow openings topped by tall chimneys. We extracted *F. virilis* from shallow burrows lacking chimneys but dug down approximately 0.75 m into a large burrow that had a chimney without capturing a crayfish or reaching the bottom of the burrow. Both reproductive form (M1) and non-reproductive form (M2) males of both species were present, but the only females captured with visible glair (an indication of physiological preparation for egg extrusion) were *F. virilis* (Table 2). No crayfishes were captured in Spotted Eagle Reservoir in October or during AIS crew routine sampling on May 17 and August 7, 2021.

Discussion

Our unexpected discovery of *P. simulans* in Montana represents the northernmost population of the species documented in the primary literature. We suspect that the species has been at the hatchery for at least several years and possibly for decades. This supposition is supported by the presence of multiple size classes and the fact that hatchery staff had known for years that two species were present, and yet the only two species collected in 2021 were *P. simulans* and *F. virilis*. However, the AIS staff had been sampling this facility since 2004 and had never previously observed crayfish on the shore, which could suggest a recent introduction. Routine AIS sampling included using a 500 µm kicknet, plankton sampling and

using a thatching rake on a rope to sample for aquatic macrophytes in addition to visual and hand-grab samples. To reiterate, routine AIS sampling had not yielded crayfish capture at this location. Crayfish were collected in 2021 due to our statewide crayfish sampling project (trapping).

Although we collected no juvenile *P. simulans*, the larger mesh size of the Crayster® trap would likely not have retained small individuals of either species in September (Chadwick et al. 2021; Green et al. 2018). Crayfishes collected from the drained East Hatchery Reservoir in October would have likely provided a more accurate representation of size classes of both species (assuming larger individuals did not consume smaller individuals), but juvenile *P. simulans* may have burrowed as the water receded. The species' possible ability to overwinter and persist for years in Montana is concerning because it suggests that they could invade areas with climates colder than those in their native range (Chucholl 2011; Haubrock et al. 2019; Veselý et al. 2015), similar to invasions by the red swamp crayfish, *Procambarus clarkii* (Girard, 1852) (Peruzza et al. 2015).

Both hatchery and AIS staff had assumed that the two crayfish species present at the hatchery were most likely the common *F. virilis* and the less-common calico crayfish *Faxonius immunis* (Hagen, 1870), which also occurs in the Yellowstone River basin. This case study provides an excellent example of how non-target introduced species can be easily overlooked by both experts and non-experts alike. To sample for rare or newly introduced species effectively, one must use the selection of tools that are most likely to result in species detection for each individual species or group of species of interest, and even then, species can be missed. Crayfish trapping is not part of the Montana AIS program's standardized sampling protocols for hatchery inspections because crayfish traps are most effective when deployed overnight, but inspections typically last less than one day. Our detection of *P. simulans* indicates that crayfish trapping should be part of routine hatchery AIS inspections, and that in Montana, this could be done by hatchery staff to accommodate overnight trapping. Both the East and West Hatchery reservoirs had been sampled using the standardized protocols during previous years (2004–2020), and either no crayfishes were observed, or none could be captured to be identified. During the October crayfish trapping attempt in Spotted Eagle Reservoir, water temperatures were cooler (< 8 °C) than is ideal for crayfish trapping (Barnett et al. 2017), and so again, crayfish may have been missed. Water leaving the hatchery flows through Spotted Eagle Reservoir and then on to the Tongue River, so the reservoir represents a critical location for detecting the potential spread of *P. simulans* beyond the hatchery.

Discovery of the nonnative *P. simulans* in a fish hatchery could provide several learning opportunities. A new hatchery biosecurity plan for the facility will be written and will include mitigating the spread of this species. Hatchery protocols and standard operating procedures have changed

dramatically in the last century in Montana, and every few years, ecological events motivate further improvements that benefit hatcheries in the long run. For example, when whirling disease (*Myxobolus cerebralis*) became a concern for hatcheries, many secured their water sources by only drawing spring water and enclosing the springs with buildings to keep pathogens out. But securing water sources has also benefitted hatcheries by excluding organisms (wildlife and humans) that could introduce invasive species into a hatchery's water supply. With invasive crayfishes, the introduction vectors we have been most focused on include aquarium dumps, bait releases/escapes, and releases of live crayfishes purchased in bulk for consumption. As a result of those vectors, we have been monitoring for several potential crayfish invaders—rusty crayfish (*F. rusticus*) (Girard 1852), *P. clarkii*, and marbled crayfish (*P. virginalis*) (Lyko, 2017)—because they have been introduced elsewhere by the vectors of highest concern and have been detected in nearby states. We were not looking for *P. simulans*. Finding *P. simulans* within a hatchery system will help FWP make beneficial changes in hatchery protocols and operations. Inspections and sampling at other hatcheries in Montana have not indicated the presence of this species in any other hatchery facility.

We plan to sample more intensively around Miles City in 2022 to assess the spread, if any, of *P. simulans*, particularly in areas hydrologically connected to the hatchery. Additionally, we are evaluating hatchery stocking records and prioritizing sampling for the species in areas where fish from the hatchery have been stocked. Finally, we are trying to determine the introductory pathway of the species. The crayfish may have been introduced with a fish transfer from the central US several decades ago, so we are reviewing fish transfer records from both FWP and the US Fish and Wildlife Service, the hatchery owner prior to 1983. We will also be investigating if there are other invasive occurrences of *P. simulans* and how they interact with native crayfish in those environments.

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Authors' contribution

SS: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation, ethics approval, funding provision, original draft.
 DS: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation, ethics approval, funding provision, review/editing.
 IB: genetics section investigation, data analysis and interpretation and writing, review/editing.
 SA: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation, ethics approval, funding provision, review/editing.

Declaration of interests

The use of trade names in this publication is for reader information and does not imply endorsement by the USDA or FWP of any product.

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