

Research Article

Investigating Calico Crayfish (*Faxonius immunis* Hagen, 1870) as a possible “sleeper” invasive species in northern Wisconsin, United States

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Abstract

“Sleeper” invaders are non-native populations that experience time-lags post-establishment before subsequent spread or negative impacts, challenging managers to differentiate harmless non-native species from invasive species. In lakes of northern Wisconsin, United States, Rusty Crayfish (*Faxonius rusticus* Girard, 1852) has dominated as an invasive species for decades, but this species has recently experienced population declines. Following these *F. rusticus* declines, we rediscovered in 2020 a population of non-native Calico Crayfish (*Faxonius immunis* Hagen, 1870) that had not been documented since the 1970s. Declining *F. rusticus* populations may create opportunities for *F. immunis* spread to other lakes and impacts as a sleeper invader. We conducted additional sampling in summer 2021 that suggests *F. immunis* remains isolated in only one lake within this watershed. We used mitochondrial DNA barcoding to confirm these crayfish were *F. immunis* and had not been misidentified as a congener. Next, we investigated whether biotic interactions with *F. rusticus* may have prevented *F. immunis* spread over the past several decades. We measured agonistic behaviors using *F. immunis* and *F. rusticus* pairs in the laboratory, and then modeled differences in aggression between species while controlling for size and reproductive form. We found that *F. rusticus* were consistently dominant over *F. immunis*, suggesting that competition with an established hyper-abundant invasive species may have restricted past spread by *F. immunis*. Managers and policy makers should consider whether precautionary actions against *F. immunis* are warranted while the population of this species remains small and localized, especially in the context of *F. rusticus* declines.

Key words: Barcoding, behavior, over-invasion, *Faxonius rusticus*, Rusty Crayfish, serial invasion

Introduction

Invasive species are non-native species that spread in a new region (Blackburn et al. 2011) and/or have negative impacts after establishment (Parker et al. 1999). This management-relevant distinction (Simberloff et al. 2011) can be complicated by some organisms experiencing long time-lags between establishment in a non-native region and subsequent spread or impacts (Crooks 2005; Spear et al. 2021). Spear et al. (2021) termed such invasive species “sleeper populations,” which can experience time-lagged spread or impacts due to a variety of abiotic or biotic factors, including environmental stochasticity, evolutionary change, or patterns of population growth (Sakai et al. 2001). For example, the invasive Spiny Water Flea (*Bythotrephes longimanus* Leydig, 1860) was present in Lake Mendota, Wisconsin, United States for 14 years before an uncommonly cold summer caused a delayed population boom to a high abundance state (Walsh et al. 2016). The Red Fire Ant (*Solenopsis invicta* Buren, 1972) was present in the United States for four decades as single-queen colonies before subsequent introductions of multiple-queen colonies initiated rapid spread and impacts of this invasive species (Porter and Savignano 1990; Krieger and Ross 2001). Population declines of well-established invasive species, whether due to human management (Zavaleta et al. 2001) or natural processes (Strayer et al. 2017), can also cause lagged spread or impacts of other invaders as a consequence of reduced predation or competition (Spear et al. 2021).

The Rusty Crayfish (*Faxonius rusticus* Girard, 1852) has been a prominent invasive species in lakes of northern Wisconsin for half a century (Capelli and Magnuson 1983; Olden et al. 2006). *Faxonius rusticus* became hyper-abundant relative to other crayfish species in these lakes (Hansen et al. 2013a), and consequently reduced the abundance and/or species richness of aquatic macrophytes, benthic macroinvertebrates, and some fish species (Wilson et al. 2004; McCarthy et al. 2006; Roth et al. 2007). Recently, a control and eradication effort reduced *F. rusticus* abundance in one lake in this region (Perales et al. 2021), whereas other lakes have experienced substantial *F. rusticus* population declines without human management intervention (Larson et al. 2019). These population declines might be caused by habitat modification by *F. rusticus* (Larson et al. 2019), climate factors like frequency or magnitude of drought (Perales et al. 2020), accumulation of pathogens or parasites over time since invasion (Sargent et al. 2014), or interactions with predatory fish communities (Roth et al. 2007). Population declines of *F. rusticus* provide opportunities for post-invasion community and ecosystem recovery (Hansen et al. 2013b; Szydlowski et al. 2023), but may also provide opportunities for serial or over-invasion (Russell et al. 2014; Karatayev et al. 2023) by other non-native crayfishes due to weakened biotic interactions with this dominant, abundant invader (Simberloff and Gibbons 2004; Strayer et al. 2017).

During routine population monitoring for *F. rusticus*, we captured three Calico Crayfish (*Faxonius immunis* Hagen, 1870) across 24 overnight traps in Wild Rice Lake, Vilas County, Wisconsin on August 28th, 2020 (Figure 1). These crayfish were vouchered as catalog #16809 at the Illinois Natural History Survey Crustacean Collection, Champaign, Illinois, United States. Capelli and Magnuson (1983) documented a similarly low-abundance population of *F. immunis* in Wild Rice Lake in the 1970s (0.2 male crayfish per trap) but did not detect this species in any of the other 66 lakes they sampled in Vilas County. This species was not detected in Wild Rice Lake or connected lakes in the Manitowish River watershed of Vilas County between Capelli and Magnuson (1983) and Larson et al. (2019). *Faxonius immunis* is native to southern Wisconsin, but the disjunct Wild Rice Lake population was 260 km from the nearest historical record at the time of Capelli and Magnuson (1983), leading these

authors to conclude that *F. immunis* had likely been introduced to Wild Rice Lake by humans. *Faxonius immunis* was similarly absent or undetected from the neighboring Upper Peninsula of Michigan, United States in the 1970s, but has been collected at two isolated locations in recent years (Smith et al. 2019). We suspect that *F. immunis* persisted at very low abundances in Wild Rice Lake over time, but may have been contained from spreading to other lakes due to biotic interactions with formerly hyper-abundant *F. rusticus* (Figure 1). *Faxonius immunis* has a large native range in eastern North America but has established non-native populations in both western North America (e.g., Newkirk et al. 2023) and Europe (e.g., Chucholl 2012). Non-native populations of *F. immunis* have been recently documented to exert strong per-capita predation effects on benthic macroinvertebrates (Chucholl and Chucholl 2021) and reduce the abundance and species richness of these organisms in small lakes (Herrmann et al. 2022), consistent with ecological impacts of other invasive crayfishes like *F. rusticus* (Wilson et al. 2004; McCarthy et al. 2006). Accordingly, we propose that *F. immunis* could become a sleeper invader in northern Wisconsin lakes if ongoing *F. rusticus* population declines facilitate future spread and impacts of this congener.

We report here a series of studies on the rediscovered *F. immunis* population in Vilas County, Wisconsin. First, because the majority of sampling for crayfish in this region has occurred in lakes (Capelli and Magnuson 1983; Larson et al. 2019; but see Olden et al. 2011), we sampled streams and rivers throughout the Manitowish River watershed in summer 2021 to investigate whether *F. immunis* might be more prevalent than realized in under-sampled lotic ecosystems. This sampling included baited trapping of the outlet flowage between Wild Rice Lake and the downstream Manitowish Chain of Lakes to investigate whether *F. immunis* was already spreading. Next, we used mitochondrial DNA (mtDNA) barcoding to confirm identifications of *F. immunis* from Wild Rice Lake, as juvenile or female individuals of this crayfish could be misidentified as a congener like the Virile Crayfish (*Faxonius virilis* Hagen, 1870). Finally, we compared agonistic interactions between *F. rusticus* and *F. immunis* in the laboratory to determine whether differences in aggression between these species may have contributed to historical and contemporary distributions of *F. immunis* in our study region. Crayfish communities of northern Wisconsin lakes are structured by behavioral dominance and associated competitive outcomes for limited shelter (e.g., rock or cobble substrate) from fish predators (Garvey et al. 1994; Hill and Lodge 1994). Within its native range, *F. immunis* can be competitively excluded from some habitats by *F. virilis* (Bovbjerg 1970), and accordingly we suspect that competition with the even more dominant *F. rusticus* has prevented *F. immunis* spread out of Wild Rice Lake into adjacent waters (see also Smith et al. 2019). Yet if *F. rusticus* population declines in this region persist (Figure 1), *F. immunis* may have opportunities to spread, become more prevalent, and impact freshwater ecosystems in the future.

Methods

Crayfish monitoring

Lakes of Vilas County have been monitored for crayfish populations since Capelli and Magnuson (1983) using a standardized baited trapping protocol (Larson et al. 2019; Perales et al. 2021). Wire-mesh Gee minnow traps with 3–4 cm openings are baited with approximately 120 g of beef liver and set overnight at 1–3 m depths. Trapping effort varies depending on lake size or habitat heterogeneity, but crayfish monitoring programs in the county have returned to georeferenced locations within the same lakes over time (Larson et al. 2019; Perales et al. 2021; Figure 1). Crayfish population

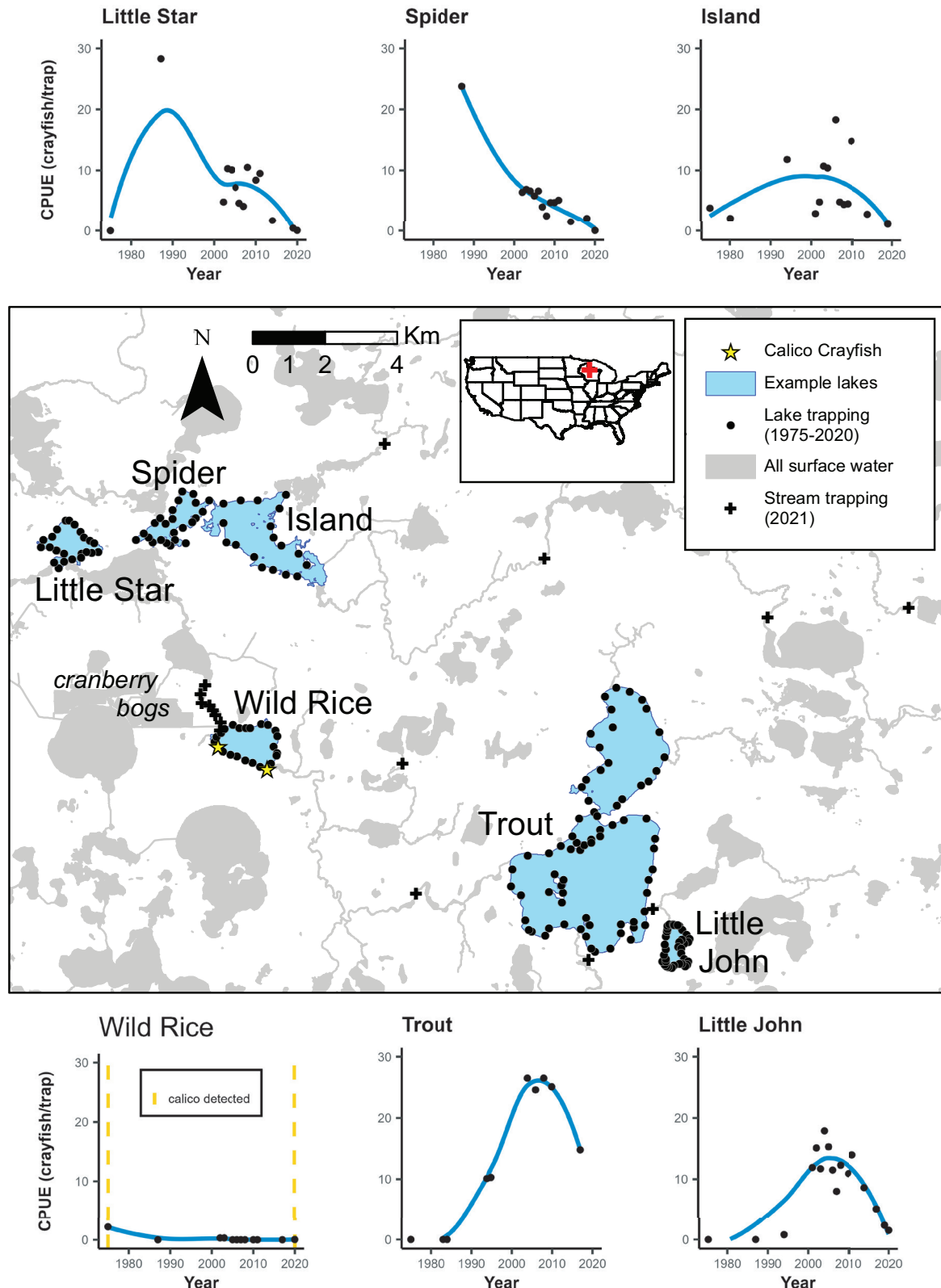


Figure 1. Calico Crayfish (*Faxonius immunis*) were collected in summer 2020 at two locations (stars) in Wild Rice Lake of the Manowish River watershed, Vilas County, Wisconsin, United States (inset map). Population declines of Rusty Crayfish (*Faxonius rusticus*) in six regularly monitored lakes of the watershed (blue) from Capelli and Magnuson (1983) through Larson et al. (2019) are presented as plots of male catch per unit effort (CPUE) fitted with locally estimated scatterplot smoothing. Crayfish trapping locations in lakes (circles) from Larson et al. (2019), as well as trapping in streams and rivers of the Manowish River watershed in summer 2021 (crosses; Table 1), are mapped. Infrequent, low abundance (CPUE < 1) collections of the Northern Clearwater Crayfish (*Faxonius propinquus*) and Virile Crayfish (*Faxonius virilis*) for these six lakes are available in Larson et al. (2019) supporting information.

monitoring generally occurs in late summer or early fall, after male crayfish have molted to a reproductively active form (form I) of the male gonopods. Male catch-per-unit effort (CPUE) is used as a measure of relative abundance because trap catch is biased towards males, but this measure of relative abundance corresponds well with other estimates of crayfish abundance or biomass in these lakes (Olsen et al. 1991).

Vilas County crayfish monitoring has historically prioritized lentic ecosystems in this lake-rich region, but crayfish also occur in streams and rivers (Olden et al. 2011). Crayfish species often differ in their habitat use, including between lotic and lentic ecosystems (Smith et al. 2019; O'Shaughnessey et al. 2021). Within its native range, *F. immunis* often occurs in ephemeral wetlands and intermittent streams (Bovbjerg 1970). As such, occasional *F. immunis* presence in Wild Rice Lake might represent dispersal or straying of individuals from the adjacent Trout River into the lake. We sought to determine whether *F. immunis* might be more prevalent in streams and rivers of the Manitowish River watershed than past lake-focused sampling has detected. During August 2021, we used the same trap design and bait as lake monitoring (above) to sample eight wadeable stream and river sites of the Manitowish River watershed with an effort of three traps per night (Figure 1; Table 1). In addition, we investigated whether *F. immunis* is dispersing downstream out of Wild Rice Lake into the adjacent Manitowish Chain of Lakes by trapping the Trout River flowage below the lake with nine total traps (Figure 1; Table 1). All crayfish collected by baited trapping in streams or rivers were identified to species and sexed.

Table 1. Results of summer 2021 baited trapping for crayfish in streams and rivers of the Manitowish River watershed, Vilas County, Wisconsin (Figure 1). Sites are presented in the order they were sampled. Date is for recovery of traps after overnight set. Latitude and longitude are WGS84. Results are given as number of individuals by sex (female = F, male = M) collected for each crayfish species across all traps at the site.

Site	Date	Latitude, Longitude	Traps (#)	Result
Allequash Creek	8/19/2021	46.0238, -89.6528	3	<i>F. rusticus</i> (4 F, 4 M)
Mann Creek	8/19/2021	46.0111, -89.6760	3	<i>F. rusticus</i> (1 M)
Trout River	8/19/2021	46.0278, -89.7377	3	No crayfish
Gresham Creek	8/19/2021	46.0603, -89.7423	3	No crayfish
Nixon Creek	8/20/2021	46.0986, -89.5608	3	No crayfish
White Sand Creek	8/20/2021	46.0964, -89.6112	3	No crayfish
Manitowish River	8/20/2021	46.1113, -89.6911	3	<i>F. virilis</i> (1 M)
Rice Creek	8/20/2021	46.1400, -89.7484	3	<i>F. rusticus</i> (1 F), <i>F. virilis</i> (1 F)
Trout River	8/21/2021	46.8076, -89.8076	1	No crayfish
Trout River	8/21/2021	46.0708, -89.8073	1	No crayfish
Trout River	8/21/2021	46.0724, -89.8092	1	No Crayfish
Trout River	8/21/2021	46.0737, -89.8102	1	<i>F. virilis</i> (1 F)
Trout River	8/21/2021	46.0748, -89.8114	1	No crayfish
Trout River	8/21/2021	46.0754, -89.8142	1	No crayfish
Trout River	8/21/2021	46.0777, -89.8145	1	No crayfish
Trout River	8/21/2021	46.0798, -89.8130	1	<i>F. virilis</i> (1 F)
Trout River	8/21/2021	46.0798, -89.8130	1	<i>F. virilis</i> (1 F)

Molecular barcoding

Identification errors are possible between *F. immunis* and similar congeners like *F. virilis*, particularly when only female or juvenile crayfish are collected because taxonomic keys for cambarid crayfishes rely on form I male gonopods. Accordingly, we used mtDNA barcoding to confirm identifications of *F. immunis* individuals

used in behavioral trials with *F. rusticus* (below), including all individuals collected from Wild Rice Lake during the summer of 2021. We also sequenced two *F. virilis* individuals collected from the Manitowish River watershed in summer 2021 to confirm their identifications. We prioritized female or juvenile *F. virilis* individuals with life colors or morphology most similar to *F. immunis* for sequencing.

We froze crayfish used for molecular sequencing in a -20 °C freezer, and subsequently dissected muscle tissue from the frozen crayfish abdomens, which was then preserved in 99% ethanol. We extracted DNA from muscle tissue using a DNeasy Blood and Tissue Kit following the protocol for animal tissues (Qiagen, Hilden, Germany). We then used Folmer et al. (1994) primers to amplify the cytochrome c oxidase subunit I (COI) mtDNA gene region on a PTC-100 thermal cycler (MJ Research, Waltham, Massachusetts, United States). Amplification was confirmed by gel electrophoresis and cleaned using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sanger sequencing was conducted at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign (Urbana, Illinois). All mtDNA COI sequences were visualized and proofread using Geneious Prime 2020.2.2. Sequences were then aligned with the Clustal Omega method and default settings in Geneious Prime to obtain the final 535 base pair COI sequence (Kearse et al. 2012).

We used Geneious Prime 2020.2.2 (Kearse et al. 2012) to construct a bootstrapped (2000 iterations) neighbor joining tree based on all of our *F. immunis* sequences from summer 2021, as well as all available *F. immunis* COI sequences from GenBank, representative sequences from the three congener crayfishes known from Vilas County, and an outgroup. Only four *F. immunis* sequences were available on GenBank: [AY701220.1](#) from Illinois (Taylor and Knouft 2006), [DQ882095.1](#) from Ontario, Canada (Costa et al. 2007), and [JF438005.1](#) and [JF438006.1](#) from Europe (Filipová et al. 2011). We used two GenBank sequences each for the three *Faxonius* crayfishes previously known from Vilas County: *F. rusticus* ([AY701249.1](#), [KX238168.1](#)), *F. virilis* ([AF474365.1](#), [KU603541.1](#)), and the Northern Clearwater Crayfish (*Faxonius propinquus* Girard, 1852) ([AF474357.1](#), [DQ889165.1](#)). We anchored the neighbor joining tree at two sequences of the Red Swamp Crayfish (*Procambarus clarkii* Girard, 1852) used as outgroup ([MK026671.1](#), [MK026674.1](#)).

Behavioral assays

Because *F. immunis* were so rare in Wild Rice Lake in Capelli and Magnuson (1983) and our 2020 sampling (0.13 male CPUE), we first conducted a pilot study with *F. immunis* and *F. rusticus* collected in Illinois to increase our replication for understanding behavioral interactions between these species. Further, we conducted the same behavioral assays between *F. immunis* and *F. rusticus* populations collected from both Illinois and Wisconsin allowing us to evaluate consistency in behavioral interactions between these species across regions, habitat types, and invasion histories (Glon et al. 2018; Reisinger et al. 2020). In Illinois, we collected *F. immunis* from Douglas Creek, Champaign County (40.0770, -88.2106) on July 18th and July 23rd, 2021 using our baited trapping protocol (above). The second sampling event was used to better size match *F. immunis* individuals to *F. rusticus*. Douglas Creek is an intermittent stream within the native range of *F. immunis* where this species was believed to occur in allopatry without other crayfish species. We collected *F. rusticus* from the Little Calumet River, Cook County (41.6518, -87.6600) on July 22nd, 2021 by seining. The Little Calumet River is a heavily modified navigation canal within the non-native range of *F. rusticus*. We collected

no other crayfish species from the Little Calumet River during our sampling, and *F. immunis* has not been documented from this river (O'Shaughnessey et al. 2021). As such, both *F. immunis* and *F. rusticus* individuals used in the Illinois study were believed to be behaviorally naïve to one another. Crayfish were transported to a laboratory at the University of Illinois at Urbana-Champaign in individual tackle box compartments filled with water to prevent interactions with other crayfish. We used 11 *F. immunis* individuals for behavioral assays with a mean total carapace length of 20.6 mm (range 16.2–30.4 mm), and 11 *F. rusticus* individuals with a mean total carapace length of 22.0 mm (range 16.9–29.7 mm). Total carapace length was measured for all crayfish in this study from the tip of the rostrum to the terminus of the carapace using digital calipers with 0.1 mm accuracy. All *F. immunis* and *F. rusticus* individuals used in the Illinois study were form II males.

At the University of Illinois at Urbana-Champaign, crayfish were kept in individual 3.79 L aquariums at ambient room temperature (22–27 °C) with a 12:12 light:dark cycle. Aquariums were filled with dechlorinated tap water and cycled for one week before the addition of crayfish. Aeration and filtration were provided by foam biofilters, and aquariums also contained two unglazed ceramic tiles (10.16 cm × 10.16 cm) and a PVC pipe for shelter. We analyzed water quality from aquariums biweekly with an API Freshwater Master Test Kit (Mars Fishcare North America, Chalfont, Pennsylvania, United States). pH ranged from 7.6–8.4 and ammonia, nitrite, and nitrate levels were all low. We changed one liter of water in each tank weekly. We fed crayfish an alternating diet of half an algae wafer (Hikari Tropical Algae Wafers, Himeji, Japan) or three to four small crab pellets (Hikari Crab Cuisine, Himeji, Japan) every other day.

In Wisconsin, we collected *F. immunis* from Wild Rice Lake between August 11th and 13th, 2022. We set 49 traps over three nights, collecting only eight *F. immunis* from one location on the southern shoreline (46.0591, -89.7909) near the inflow of the Trout River. We collected two female, five male form II, and one male form I *F. immunis*, which had a mean total carapace length of 19.0 mm (range: 12.8–31.0 mm). *Faxonius rusticus* are occasionally collected from Wild Rice Lake at very low abundances, and consequently *F. immunis* from this lake would be expected to have some behavioral experience with *F. rusticus*. We collected *F. rusticus* from traps throughout Boulder Lake (46.1253, -89.6604), which were recovered on August 12th, 2021 as part of routine crayfish monitoring in Vilas County (Larson et al. 2019). For behavioral assays, we used eight *F. rusticus* from Boulder Lake, including two female, two male form II, and four male form I crayfish. *Faxonius rusticus* used in Wisconsin behavioral assays had a mean total carapace length of 24.0 mm (range: 20.5–30.5 mm). Because no *F. immunis* have previously been collected from Boulder Lake (Capelli and Magnuson 1983; Larson et al. 2019), we assume that these *F. rusticus* individuals were behaviorally naïve to the other species. Using methods consistent with the Illinois study, we transported crayfish to a laboratory at the University of Wisconsin Trout Lake Station. At Trout Lake Station, crayfish were kept in individual 1.18 L containers with a natural light:dark cycle provided by windows. We equipped containers with an aeration stone and a PVC pipe (7.5 cm long × 4.5 cm diameter) for shelter. We filled containers using Trout Lake water, which was changed out completely every other day. Water quality was not monitored during the Trout Lake Station trials. Wisconsin crayfish were fed on the same alternating food schedule as the Illinois crayfish.

In both Illinois and Wisconsin, crayfish acclimated in the lab for five to seven days prior to the start of behavioral assays to remove effects of previous social interactions (Zulandt Schneider et al. 2001). Behavioral trials began on July 29th, 2021

and concluded on August 7th, 2021 in Illinois and began on August 17th, 2021 and concluded on August 21st, 2021 in Wisconsin. Each individual crayfish was used in three replicate trials against different individuals of the opposing species, which were deliberately stratified to produce a variety of size matches and size differences between species. Replicate trials for individual crayfish were always separated by 48 hours, and no crayfish was fed in the 24 hours before a behavioral assay. All behavioral trials in both Illinois and Wisconsin occurred between 12:00 and 17:00, and were conducted in the same plastic bins (5.68 L, 32.5 cm × 20.0 cm) filled with fresh lab water prior to each assay. These bins were initially divided into two halves by a clear plexiglass divider. We placed an individual crayfish of each species on opposite sides of the arena and allowed them to acclimate for 15 minutes. The divider was removed and the two crayfish allowed to interact for 15 minutes. An opaque plastic sheet with a slit cut in it was used to obscure the observer from the crayfish. Occurrences of behaviors for each individual were recorded according to a previously described ethogram (Bruski and Dunham 1987): -2 (fast retreat), -1 (slow retreat), 0 (no visible interaction), 1 (approach without threat, i.e., meral spread), 2 (approach with threat), 3 (boxing or pushing with closed chelae), 4 (grabbing or tearing with open chelae), and 5 (full-out fighting with interlocked chelae). All behavioral observations were made by the lead author.

We compared aggression between *F. immunis* and *F. rusticus* using the difference in aggression scores between individuals from each behavioral pair, while simultaneously controlling for other covariates like size or form difference, identity of individual crayfish, and region or source of populations (Illinois and Wisconsin). We first calculated the behavioral score difference for each interacting pair of crayfish by subtracting the mean *F. rusticus* behavioral score from the mean *F. immunis* behavioral score. Negative behavioral score differences indicate *F. rusticus* dominance, whereas positive behavioral score differences indicate *F. immunis* dominance. We similarly calculated size differences for pairs by subtracting *F. rusticus* total carapace length from *F. immunis* total carapace length. Negative values indicate that the *F. rusticus* individual was larger, whereas positive values indicate that the *F. immunis* individual was larger. To calculate form difference, we ranked forms by aggression, where form I and form II males are more aggressive than female crayfish but form I males are more aggressive than form II males (Bovbjerg 1956; Figler et al. 2005). Therefore, the ranking scale was as follows: form I male (2), form II male (1), and female (0). After assigning a form-associated aggression rank to individual crayfish, form difference was calculated for each interacting crayfish pair by subtracting the *F. rusticus* form rank from the *F. immunis* form rank. Positive form difference values were anticipated to correspond with more aggressive individuals, whereas negative form difference values were anticipated to correspond with less aggressive individuals. All crayfish used in the Illinois data set were form II males, and accordingly the form differences were zero for these pairs.

We first analyzed our data with a linear mixed-model where behavioral score difference was the response variable, size and form difference were fixed effects, location (Illinois or Wisconsin) was also a fixed effect, and the identity of individual *F. immunis* and *F. rusticus* crayfish included in the pairs represented two random effects to account for repeated observations on the same organisms. Location as a fixed effect was entered as a binary scale with Illinois coded as 1 and Wisconsin coded as 0. We also ran location-specific models for Illinois and Wisconsin separately with the same structure but omitting form difference as a fixed effect for the Illinois model. We ran all linear mixed-models in the lme4 package (Bates et al. 2015) in R (R Core Team 2019) with Satterthwaite's method for approximating degrees of freedom and evaluated normality of model residuals using QQ plots.

Results

We did not detect *F. immunis* from the 24 traps set throughout eight different stream and river sites in the Manitowish River watershed during summer 2021 (Table 1). Similarly, we did not detect *F. immunis* from nine traps set in the Trout River flowage downstream of Wild Rice Lake (Figure 1). We only collected eight *F. immunis* from 49 traps set over a total of three nights in Wild Rice Lake (0.12 male CPUE, 0.16 total CPUE), and all *F. immunis* from this lake were collected in close proximity on the southern shoreline (46.0591, -89.7909). Mitochondrial DNA barcoding confirmed all eight of these crayfish as *F. immunis* (Figure 2), and two ambiguous individuals collected from elsewhere in the watershed (White Sand Lake at 46.0892, -89.5931) were identified as *F. virilis* by mtDNA barcoding. *Faxonius immunis* collected from Wild Rice Lake were most similar to the mtDNA COI sequence of this species from Ontario, Canada (GenBank accession [DQ882095.1](#)). Surprisingly, only seven of the 11 crayfish used in the Illinois study were identified as *F. immunis* by mtDNA, with the remaining four individuals identified as *F. virilis*. We removed these four individuals and all of their interaction pairs with *F. rusticus* from linear mixed-models of crayfish behavior prior to analyses. *Faxonius immunis* collected from their native range at Douglas Creek, Illinois were more diverse than *F. immunis* from Wild Rice Lake, Wisconsin, with four individuals resembling the previous mtDNA COI sequence from Illinois (Genbank accession [AY701220.1](#)) and three other sequences being distinct (Figure 2). European *F. immunis* were distinct, but monophyletic, to the crayfishes used in our study (GenBank accessions [JF438005.1](#) and [JF438006.1](#)). The new *F. virilis* mtDNA sequences generated by our study are excluded from Figure 2 to emphasize *F. immunis* results.

Faxonius rusticus was significantly more aggressive than *F. immunis* from our combined location model, as evidenced by an intercept that was significantly lower than zero (Intercept = -1.375, df = 14.202, $t = -5.817$, $P < 0.001$). However, we found no significant effect of size difference (Coefficient = 0.050, df = 37.242, $t = 1.539$, $P = 0.132$), form difference (Coefficient = 0.230, df = 39.520, $t = 0.924$, $P = 0.361$), or location (Coefficient = 0.022, df = 14.273, $t = 0.092$, $P = 0.928$) on crayfish behavior. The two location-specific models were highly similar to each other (Figure 3), with *F. rusticus* significantly more aggressive than *F. immunis* in both Illinois (Intercept = -1.352, df = 4.966, $t = -3.582$, $P = 0.016$) and Wisconsin (Intercept = -1.352, df = 10.580, $t = -3.987$, $P = 0.002$). We found no significant effect of size difference on crayfish behavior in either Illinois (Effect = 0.052, df = 18.906, $t = 0.993$, $P = 0.333$) or Wisconsin (Effect = 0.041, df = 10.162, $t = 1.118$, $P = 0.289$), and no significant effect of form difference on crayfish behavior in Wisconsin (Effect = 0.421, df = 12.179, $t = 1.637$, $P = 0.127$). *Faxonius rusticus* was more aggressive than *F. immunis* even when size-matched or in most cases where *F. immunis* was the larger individual in the pair (Figure 3).

Discussion

Our study confirmed an *F. immunis* population in Wild Rice Lake, Vilas County, Wisconsin, which had not been documented since the 1970s (Capelli and Magnuson 1983) and could potentially spread to become an invasive species in the future (Spear et al. 2021). *Faxonius immunis* currently occurs at very low abundances in Wild Rice Lake and has not been documented elsewhere in the Manitowish River watershed, whether by regular crayfish sampling in lakes (Larson et al. 2019) or our stream and river sampling in summer 2021. We believe that

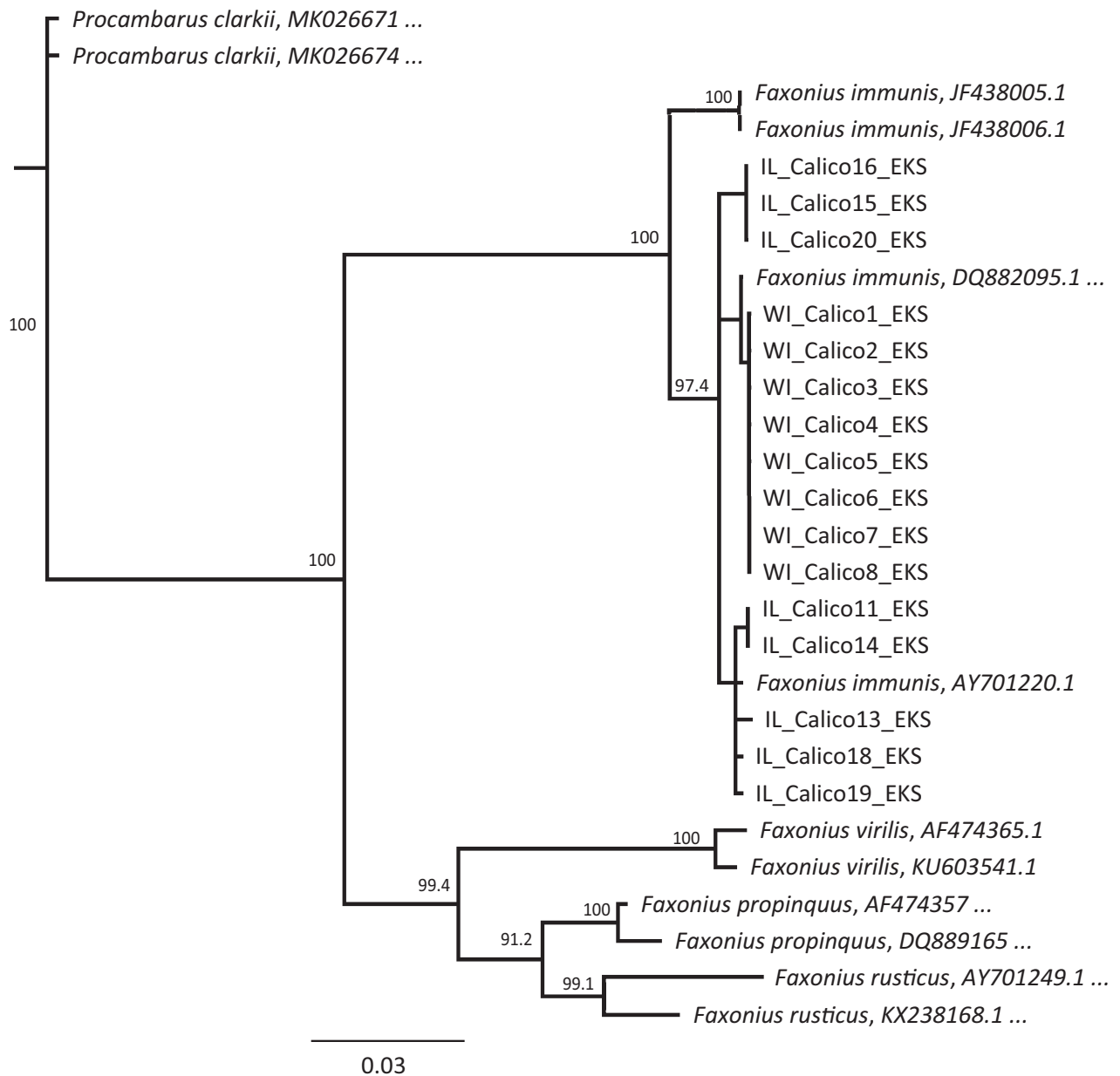


Figure 2. Neighbor joining phylogenetic tree with 2000 bootstrap replicates including sequences of Calico Crayfish (*Faxonius immunis*) collected from Illinois (IL) or Wisconsin (WI). We include four additional *F. immunis* sequences from GenBank, as well as two sequences each of Virile Crayfish (*Faxonius virilis*), Northern Clearwater Crayfish (*Faxonius propinquus*) and Rusty Crayfish (*Faxonius rusticus*) as three known *Faxonius* crayfish species from Vilas County, Wisconsin. We used two sequences of Red Swamp Crayfish (*Procambarus clarkii*) as an outgroup. Nodes on the tree represent bootstrap support, and GenBank accession numbers are given at node tips. GenBank accessions for new *F. immunis* sequences are [OQ759592–OQ759606](#).

this *F. immunis* population has been prevented from spreading out of Wild Rice Lake due to competition with invasive, hyper-abundant *F. rusticus* in neighboring lakes over the past several decades, particularly as *F. immunis* was previously documented as a poor competitor relative to congeners (Bovbjerg 1970; Smith et al. 2019). Laboratory behavioral assays supported *F. rusticus* as consistently dominant over *F. immunis* across source population, body size, and sex or form differences. Accordingly, *F. immunis* may be unlikely to emerge as a sleeper invader in this region per Spear et al. (2021). However, ongoing population declines of *F. rusticus* (Larson et al. 2019) increase uncertainty about species composition and potential ecological interactions of crayfish communities in this region in the future.

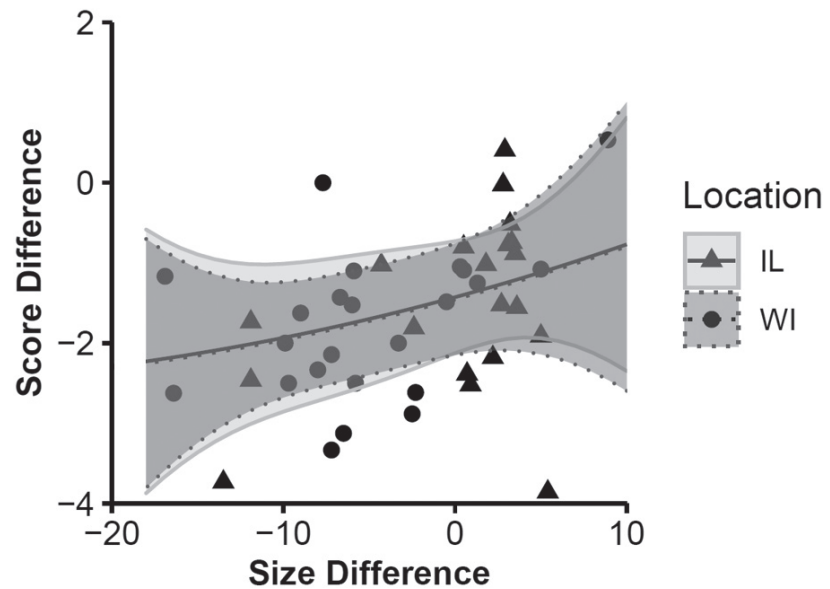


Figure 3. Linear mixed models with 95% confidence intervals between size difference and behavioral score difference of Rusty Crayfish (*Faxonius rusticus*) and Calico Crayfish (*Faxonius immunis*) pairs from location-specific models of our two study regions, Illinois (IL) and Wisconsin (WI). Negative size difference indicates *F. rusticus* was larger than *F. immunis*. Negative score difference indicates *F. rusticus* behaved more aggressively than *F. immunis*. Both models include random effects for individual crayfish used in pairs to account for repeated observations on the same organisms, and the Wisconsin model includes a non-significant fixed effect for reproductive form difference that is not plotted.

We found *F. rusticus* to be consistently dominant over *F. immunis* despite using individuals from different regions (Illinois or Wisconsin), invasion histories (native or non-native populations), and methods of collection (trapped or seined). This contradicts some recent research that has found region of origin and invasion history of populations to affect behavioral outcomes between crayfish species pairs (Glon et al. 2018; Reisinger et al. 2020), but supports past work indicating that *F. immunis* is a poor competitor relative to congeners (Bovbjerg 1970; but see Chucholl et al. 2008). For example, *F. rusticus* was dominant over *F. immunis* even when *F. immunis* had a moderate size advantage and despite some mismatches by sex or male reproductive form that affect crayfish behavioral interactions (Bovbjerg 1956; Figler et al. 2005). Although *F. immunis* is an emerging invasive species in Europe (Chucholl and Chucholl 2021; Herrmann et al. 2022), its spread and ecological effects in northern Wisconsin have likely been limited by biotic interactions with *F. rusticus* and other organisms (e.g., competitive dominance by *F. rusticus* may expose *F. immunis* to increased fish predation due to limited shelter access; Garvey et al. 1994; Hill and Lodge 1994). To reduce the intensity of these biotic interactions (Creed 2006), *F. immunis* often uses habitats like ephemeral wetlands or intermittent streams rather than permanent waterbodies (Bovbjerg 1970; Smith et al. 2019; Newkirk et al. 2023). As such, the distribution of *F. immunis* in our study region could be under-estimated due to a monitoring emphasis for crayfish on large, permanent lakes with both crayfish competitors and fish predators (Larson et al. 2019; Perales et al. 2021).

We sampled streams and rivers of the Manitowish River watershed in summer of 2021 to survey for *F. immunis* populations outside of lake ecosystems commonly monitored in Vilas County, Wisconsin. We did not detect *F. immunis* from any of these stream or river sites, including relatively intensive trapping of the Trout River flowage downstream of Wild Rice Lake. This suggests that *F. immunis* is not spreading out of Wild Rice Lake into downstream ecosystems at present, although

more widespread, intensive sampling focused on habitats neglected for crayfish monitoring in Wisconsin may reveal other *F. immunis* populations (Olden et al. 2011; Newkirk et al. 2023). Like Capelli and Magnuson (1983), we interpret *F. immunis* as an isolated, non-native population in Wild Rice Lake given its remote location relative to *F. immunis* populations in southern Wisconsin (Creaser 1932; Hobbs and Jass 1988). *Faxonius immunis* was similarly absent or undetected by systematic crayfish sampling in the nearby Upper Peninsula of Michigan in the 1970s, but has been documented from two locations within the past decade (Smith et al. 2019). Phylogeographic or landscape genomics studies might be used to investigate whether *F. immunis* is a non-native species in northern Wisconsin and the Upper Peninsula of Michigan or instead a rare, under-sampled native species (Larson et al. 2012; Schmidt et al. 2023). This seems unlikely, as *F. immunis* is not difficult to detect by common methods, like baited trapping, relative to congeners within its native range (O’Shaughnessey et al. 2021; Smith et al. 2019; Newkirk et al. 2023). Regardless, future molecular studies could provide insights into *F. immunis* distributions and ecological interactions, including the possibility that strong effects of invasive *F. immunis* in Europe may be unique to this highly diverged lineage or possible cryptic species (Filipová et al. 2011).

Species identification challenges could also account for some uncertainty around *F. immunis* distributions in northern Wisconsin and elsewhere, as evidenced by our misidentification of four *F. virilis* individuals from Douglas Creek, Illinois as *F. immunis* prior to mtDNA barcoding. Crayfishes of the family Cambaridae should be identified using male form I gonopods (e.g., Taylor et al. 2015). In the seasonal absence of reproductively active male crayfish, biologists often use life colors or other morphology to make species identifications. *Faxonius immunis* generally differs from *F. virilis* by a pale, continuous dorsal stripe extending the length of the carapace and abdomen as well as a deep incision at the base of the moveable finger of the claw (Taylor et al. 2015). Other identification sources (e.g., Chucholl et al. 2008), including a crayfish identification guide from the Wisconsin Department of Natural Resources (Roesler 2019), propose tufts of hairs or setae at the base of the moveable finger of the claw as diagnostic of *F. immunis*. However, two crayfish we sequence-confirmed as *F. virilis* from outside of Wild Rice Lake in Vilas County, Wisconsin also had hairs at the base of the moveable finger of the claw, which had motivated our mtDNA barcoding of these individuals. These crayfish otherwise resembled *F. virilis* by life color and morphology. Conversations with several crayfish taxonomists suggested that hairs on the claws should not be used for *F. immunis* identifications, as *F. virilis* can routinely share this morphological trait (Christopher A. Taylor, Illinois Natural History Survey and Bronwyn W. Williams, North Carolina Museum of Natural Sciences; personal communications). Molecular sequencing may assist identification and monitoring of *F. immunis* and other crayfish populations, although we caution that mtDNA barcoding cannot detect hybrids that may be common among some crayfishes of the genus *Faxonius* (Rozansky et al. 2021; Merovich et al. 2022). We do not at present suspect *F. immunis* of hybridization with either *F. rusticus* or *F. virilis* given their phylogenetic distance (Figure 2), whereas the more closely related *F. rusticus* and *F. propinquus* are well-documented to hybridize in lakes of northern Wisconsin (Perry et al. 2001).

Our study could be interpreted to support *F. immunis* as an innocuous non-native species, rather than a harmful invasive species, in Wild Rice Lake. Invasive species spread in new regions (Blackburn et al. 2011); by contrast, *F. immunis* has not apparently spread over the half century since its discovery in Wild Rice Lake by Capelli and Magnuson (1983). Invasive species often achieve hyper-abundance resulting in negative ecological impacts (Parker et al. 1999; Hansen et al. 2013a);

by contrast, *F. immunis* has remained rare in Wild Rice Lake over time (Larson et al. 2019; Figure 1). One explanation for the lack of success of *F. immunis* in the Manitowish River watershed may be poor competitive ability against the widespread, abundant *F. rusticus*. Even if *F. rusticus* population declines in this region continue, *F. immunis* may be unlikely to become an invasive species. For example, *F. rusticus* has been present but rare in Wild Rice Lake for decades (Larson et al. 2019), and this lake evidences minimal community or ecosystem impacts of *F. rusticus* (Szydlowski et al. 2023). *Faxonius immunis* has been unable to take advantage of these low abundances and weak ecological effects of *F. rusticus* in Wild Rice Lake by increasing its population size or spreading. Factors that have limited more robust population growth of *F. immunis* within Wild Rice Lake over the past half century are unknown. Further, populations of the native crayfish *F. virilis* may recover as *F. rusticus* declines (Perales et al. 2021), and *F. virilis* is also competitively dominant over *F. immunis* (Bovbjerg 1970). Lastly, the still-uncertain causes of population declines for *F. rusticus*, like pathogens or fish predation, may also be rendering these lakes less suitable for subsequent crayfish invaders (Sargent et al. 2014; Roth et al. 2007). A minority of non-native species are able to spread or cause negative impacts as invasive species (Williamson 1996; Jeschke 2014), and managers and policy-makers use this distinction to prioritize which organisms require prevention, control, or eradication efforts (Lodge et al. 2016). In our study region, managers and policy-makers may be well-justified in identifying *F. immunis* as a species that is unlikely to become invasive in the future.

Alternatively, time lags between the introduction and establishment of a non-native species and subsequent spread or negative impacts as an invasive species can complicate management decisions and policy priorities (Sakai et al. 2001; Crooks 2005). Long-present non-native species may become invasive following major abiotic or biotic changes (Spear et al. 2021). We propose that population declines of *F. rusticus* may create opportunities for serial or over-invasion by subsequent crayfish species (Russell et al. 2014; Karatayev et al. 2023), including *F. immunis* as a possible sleeper invader in the future (Spear et al. 2021). For example, invasive status in another region is often an accurate predictor of invasion risk in a focal region (Gordon et al. 2008). *Faxonius immunis* is invasive in Europe, where it competes effectively against other invasive crayfish of the genus *Faxonius* (Chucholl et al. 2008) and has community and ecosystem effects on benthic macroinvertebrates comparable to *F. rusticus* (Chucholl and Chucholl 2021; Herrmann et al. 2022). We also suggest that future crayfish invaders in northern Wisconsin may not necessarily resemble *F. rusticus* by habitat preference, life history, or ecological function. Manoomin or Wild Rice (*Zizania palustris* L.) is an example of a species that could be vulnerable to a novel crayfish invader in lakes of Vilas County. *Zizania palustris* is a culturally significant resource to Native American tribes in the Great Lakes region but has experienced recent declines (Matson et al. 2021). *Faxonius rusticus* has been investigated as a risk to *Z. palustris* through direct consumption or habitat modification (Wenner 2017), but this crayfish generally requires firm substrates distinct from the softer substrates where *Z. palustris* grows (Hill and Lodge 1994). By comparison, *F. immunis* tolerates soft substrates and low dissolved oxygen concentrations typical of some wetlands (Bovbjerg 1970; Smith et al. 2019), and was collected in our study from Wild Rice Lake adjacent to *Z. palustris* habitat. Failure to compete effectively with *F. rusticus* may not exclude a subsequent crayfish invader from having novel impacts on other ecosystem processes or aquatic community members like *Z. palustris*. A risk-averse management strategy might choose to control or eradicate *F. immunis* from Wild Rice Lake while its population remains small and localized (Vander Zanden et al. 2010; Lodge et al. 2016).

Lakes of northern Wisconsin have experienced a sequence of natural, post-glacial colonization and human-assisted invasion by crayfishes that will likely continue following declines of *F. rusticus* populations. Creaser (1932) found only *F. virilis* and the semi-terrestrial burrowing crayfish *Lacunicambarus nebrascensis* (Girard, 1852) from our study region in the early 20th century. By Capelli and Magnuson (1983), *F. propinquus* and *F. rusticus* had sequentially invaded these lakes due to human introductions, with *F. rusticus* ultimately displacing *F. propinquus* by a combination of competition, differential vulnerability to fish predation, and hybridization (Garvey et al. 1994; Hill and Lodge 1994; Perry et al. 2001). As *F. rusticus* experiences ongoing population declines (Larson et al. 2019; Szydlowski et al. 2023), other crayfish species may arrive by tracking their preferred environmental conditions under climate change (Hellmann et al. 2008) or as a consequence of continuing human introductions (Seebens et al. 2017). *Faxonius immunis* offers managers and policy makers a thought exercise for how to meet these future crayfish arrivals to north temperate lakes. For example, the resist-accept-direct (RAD) framework offers alternative approaches for how to address both climate-tracking species (Feiner et al. 2022; Rahel 2022) and biological invasions (Dunham et al. 2022). *Faxonius immunis*, for example, could be resisted by an eradication effort (Hansen et al. 2013b), accepted with no management intervention, or directed away from sensitive habitat for *Z. palustris* by barrier design or maintenance (Coward et al. 2018). Managers and policy makers should apply the well-documented lessons of *F. rusticus* invasions in northern Wisconsin lakes in preparing responses to future crayfish invasions, while also considering that future crayfish invaders may not necessarily resemble *F. rusticus* given the considerable ecological diversity of this taxonomic group (Taylor et al. 2019).

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Ethics and permits

Crayfish were collected under fishing licenses consistent with Illinois and Wisconsin regulations.

Author's Contribution

EKS: Funding Acquisition, Conceptualization, Investigation, Data Curation, Formal Analysis, Writing - Original Draft, Writing - Review and Editing; JHH: Methodology, Data Curation, Formal Analysis, Writing - Review and Editing; DKS: Conceptualization, Investigation, Writing - Review and Editing; ERL: Funding Acquisition, Conceptualization, Investigation, Writing - Review and Editing, Supervision

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