

ORIGINAL ARTICLE

Impacts of native and invasive crayfish on three native and one invasive freshwater mussel species

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Funding information

Bayerisches Landesamt für Umwelt

Abstract

1. Freshwater mussels and crayfish provide important ecosystem functions and services. In both groups, global declines of native species are paralleled by invasions of non-native species. Knowledge on differences in predation susceptibility of native and invasive freshwater mussels exposed to native and invasive crayfish is essential for understanding their ecological interactions and for conservation management.
2. In this study, we compared the predation impact of the native European noble crayfish (*Astacus astacus*) and the invasive signal crayfish (*Pacifastacus leniusculus*) on three native (*Anodonta anatina*, *Anodonta cygnea*, and *Unio pictorum*) and one invasive (*Sinanodonta woodiana*) mussel species in controlled laboratory experiments. We repeated the same experiments with the same crayfish specimens to investigate a potential learning effect of crayfish and assessed the *in situ* impacts in a natural stream.
3. Mussel predation and damage caused by *P. leniusculus* was significantly higher than by *A. astacus*. Irrespective of the crayfish species, susceptibility was greater in native mussel species compared to invasive *S. woodiana*. Predation and damage was greatest in the thick-shelled *U. pictorum*, suggesting that other factors such as shell shape are more important in explaining susceptibility than shell thickness. A predation learning effect from previous co-exposure was evident, being most pronounced for *A. astacus*.
4. Even if our experimental findings could not be confirmed in the field, the experimental results suggest that crayfish may have a negative impact on mussels and this impact will likely increase with the ongoing spread of *P. leniusculus* replacing *A. astacus* populations in Europe. The co-occurrence of invasive *P. leniusculus* with the invasive *S. woodiana* could potentially exacerbate declines of endangered native mussel populations.

KEYWORDS

invasive species, *Pacifastacus leniusculus*, predation, *Sinanodonta woodiana*, unionids

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1 | INTRODUCTION

Freshwater mussels are considered important key faunal elements of freshwater systems that need to be considered in concepts of integrative freshwater biodiversity conservation (Geist, 2011). Their important ecosystem functions and services such as filtration of fine particles, bioturbation, nutrient cycling, and storage also are beneficial for other species (Boeker et al., 2016; Lummer et al., 2016; Vaughn, 2018; Vaughn & Hakenkamp, 2001). At the same time, freshwater mussel populations are globally in decline (see, e.g., Haag, 2012; Haag & Williams, 2014 for North America; Lopes-Lima et al., 2017 for Europe). At a European scale, none of the 16 species of native unionid bivalves can be considered secure throughout their range based on IUCN threat assessments (Lopes-Lima et al., 2017). In Germany, all of the seven indigenous freshwater mussels of the order “Unionoida” (Zieritz et al., 2012) are listed on the national red list of threatened species (Binot-Hafke et al., 2011) and protected by the federal species regulation. Most freshwater mussel populations are exposed to multiple threats such as habitat fragmentation and degradation, water pollution, and climate change (Dudgeon et al., 2006; Geist, 2010; Lopes-Lima et al., 2017; Lydeard et al., 2004; Regnier et al., 2009; Stoeckl et al., 2020; Strayer et al., 2004; Young & Williams, 1983). In addition to these globally important factors for decline, invasions by non-native species are suspected to contribute to the decline of mussel populations. Invasive mussel species, for example, tend to be more generalist in terms of the physicochemical habitat conditions as well as the spectrum of host fishes, and can therefore adapt more easily to non-optimal conditions compared to the more sensitive native ones (Bodis et al., 2016). In addition, there are several direct negative impacts of invasive mussel species on native ones. In the case of the Asian clam *Corbicula fluminea* (Müller, 1774), which often occurs in high densities, the relatively high filtration rate leads to a strong competition for food with native unionids (Cohen et al., 1984; Phelps, 1994; Strayer et al., 1999). This also applies to the invasive zebra mussel, *Dreissena polymorpha* (Pallas, 1771), which already has invaded many of the lakes and slow-flowing parts of rivers and streams in Europe (Van der Velde et al., 2010) and North America (Strayer, 2009). Its attachment to native unionids with its byssus threads reduces the physiological condition of the mussel (Sousa et al., 2011), and can lead to shell deformations, overturning, and death of the overgrown mussels (Ozgo et al., 2020).

In addition to competition for food, the Chinese pond mussel, *Sinanodonta woodiana* (Lea, 1834), an invasive species that was first brought from the Yangtze River basin in China to Europe in 1979 (Sarkany-Kiss, 1986), competes with native mussels during reproduction. *Sinanodonta woodiana* larvae are more persistent than larvae of native species, also tolerating warmer water temperatures (Benedict & Geist, 2021). Furthermore, *S. woodiana* has a broader range of suitable host fishes, higher infestation rates, and faster development than native species (Douda et al., 2012; Huber & Geist, 2019). This species can breed several times throughout the year while native unionid reproduction is limited to a shorter time span (Labecka & Czarnoleski, 2019). In addition, a cross-resistance of host fishes was

detected which reduces the reproduction success for native mussel species (Donrovich et al., 2017).

Besides competition, another important effect of non-native species on native ones is predation. As with freshwater mussels, crayfish can play important roles in the functioning of aquatic food webs, and there is a similar situation with native species being prioritised in conservation and invasive species being considered a major threat. Crayfish are omnivorous (Guan & Wiles, 1998; Gutiérrez-Yurrita et al., 1998; Mason, 1975), can occur in high densities, and can be potentially important predators on freshwater mussels (Machida & Akiyama, 2013; Meira et al., 2019; Sousa et al., 2019). Community shifts in crayfish populations due to die-offs of native species and dominance of non-native species may exacerbate this problem. The introduction of non-indigenous crayfish species from North America to Europe led to a massive spread of the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), since the 20th Century, followed by further declines of native species (Holdich, 2002). Invasive crayfish tend to have a competitive advantage over native ones as a result of faster growth (Paglianti & Gherardi, 2004), higher aggressivity (Söderbäck, 1991), greater dispersal ability (Bubb et al., 2006; Wutz & Geist, 2013), and exploitative or interference competition (Hudina et al., 2011). In addition, the North American crayfish are resistant to the crayfish plague, *Aphanomyces astaci* (Schikora, 1906), but can transmit this disease to crayfish native to Europe. This typically leads, apart from some rare exceptions (Martin-Torrijos et al., 2017), to a complete extinction of local native populations within only a few weeks (Vorbürger & Ribí, 1999). Of nine crayfish species that currently occur in Germany, only three are indigenous, excluding *Astacus leptodactylus* (Eschscholtz, 1823) which was introduced from Eastern Europe after arrival of the crayfish plague (Holdich et al., 2009; Kouba et al., 2014). Invasive crayfish, particularly *P. leniusculus*, now are the most widespread crayfish species in Europe (Holdich et al., 2009).

Previous studies already showed evidence for crayfish predation on unionids (Machida & Akiyama, 2013; Meira et al., 2019; Schmidt & Vandr , 2012; Sousa et al., 2019) and on dreissenids (Glon et al., 2017; zu Ermgassen & Aldridge, 2011). However, the following questions have not yet been answered: (a) Are invasive crayfish more problematic than native ones concerning predation on unionids? (b) Are crayfish able to learn using mussels as food?, and (c) Which mussel species and which characteristics (in particular shell thickness and shape) affect the susceptibility of mussels to be killed or damaged by crayfish?

In order to answer these questions, the core objective of this study was to compare the predation pressure of two crayfish species, European noble crayfish, *Astacus astacus* (Linn , 1758) and invasive *P. leniusculus* on native and non-native European freshwater bivalves, including an assessment of their ability to learn from repeated exposure to mussels. More specifically, we: (a) compared the predation rates of *A. astacus* and *P. leniusculus* on three different native freshwater bivalves (*Anodonta anatina*, *Anodonta cygnea*, and *Unio pictorum*) and one invasive bivalve (*S. woodiana*); (b) assessed the ability of crayfish to learn using mussels as a source of food; and (c) assessed the shell damage

and mortality resulting from co-exposure of *A. astacus* and *P. leniusculus* with three freshwater bivalves (*A. anatina*, *A. cygnea*, and *Unio crassus*). We hypothesised that (a) mussels with more brittle and delicate shells such as *A. cygnea* (Killeen et al., 2004) would be most prone to damage by crayfish, irrespective of the crayfish species, and that the co-exposures would result in lowest predation, damage, and mortality in the thickest-shelled species *U. pictorum*, (b) the co-exposures would result in highest damages from co-exposure with invasive *P. leniusculus*, and (c) both species of crayfish would be able to learn to use freshwater mussels as a food source as evident from greater mussel damage in repeated exposure. Impacts of crayfish on the mussels were tested in a controlled laboratory experiment and also assessed in the wild where damage patterns of three species of mussels were compared at sites with occurrence of either one of the crayfish species.

2 | METHODS

2.1 | Animal origin and husbandry conditions

A. astacus were purchased from a regional breeder and directly transferred to a tank measuring 290 cm × 65 cm × 70 cm (length, width, height) filled with 10 cm of gravel and 600 L tap water (temperature 11.2°C ± 2.7 SD; water hardness 18.9°dH, electric conductivity adjusted to 20°C: 606.9 µS ± 57.0 SD; oxygen saturation 95%–100%) where crayfish were kept under a natural day/night regime. The tank was closed on the top with two wooden lids connected by a durable polyvinyl chloride (PVC) foil to avoid contamination with crayfish plague and to allow light to enter the interior. To avoid territorial struggles or cannibalism, we placed one dark brown PVC tube (7 cm × 25 cm) for each individual on the ground (Barim-Oz, 2018) and fed the crayfish every second day with fresh carrot slices. The water was changed twice a week. *P. leniusculus* specimens were caught in the river Moosach (48°23′38″N, 11°43′25″E) next to the laboratory using crayfish traps (type “pirate”, Engel Netze) with a mesh size of 11 mm × 45 mm baited with Frolic® dry dog food (Mars, Inc.) and kept under the same conditions as *A. astacus*.

Carapace length (CL) of all crayfish was measured from the apex of the rostrum to the mid-dorsal posterior edge of the carapace (±0.1 mm) with analogue callipers (following Wutz & Geist, 2013).

We obtained the three native bivalves (*A. anatina*, *A. cygnea*, and *U. pictorum*) and one invasive bivalve (*S. woodiana*) from commercial aquacultures 1 month before Part 1 of the experiment. We kept all mussel species (a total of 130 mussels) together in the same tank under similar conditions as the crayfish and fed them with 30 ml algae mixture (Shellfish Diet 1800®, Nannochloropsis 3600®, and tap water [2:1:1]; Varicon Aqua Solutions Ltd) every day.

2.2 | Study design

In order to investigate differences in predation of *A. astacus* and *P. leniusculus* on three native and one invasive mussel species, we

carried out co-exposure experiments under controlled laboratory conditions. We obtained the permission for these experiments from the District Government of Upper Bavaria (reference no. 2631. Vet_11-4-12).

In order to exclude a contamination of *A. astacus* with the crayfish plague, experiments were performed subsequently, starting with *A. astacus* followed by *P. leniusculus*. The aquaria, gravel, and the air supply were disinfected with Virkon® S for a minimum of 15 min and thoroughly washed with tap water afterwards. Before each experiment, the crayfish were acclimated in the aquaria for 10 days (Barim-Oz, 2018) with new carrot slices provided every other day. The water of the aquaria was aerated 48 hr before the crayfish were added and during the whole time of the experiment.

In each replicate of the experiments, we exposed one individual each of three native bivalve species (*A. anatina*, *A. cygnea* and *U. pictorum*) and one individual of an invasive mussel species (*S. woodiana*) of similar size classes (Table S1) together with one crayfish specimen in an aerated aquarium measuring 40 cm × 25 cm × 25 cm (length, width, height) filled with 5 cm fine gravel (4–8 mm) and 13 L tap water (temperature 16.5°C ± 0.9 SD; water hardness 18.9°dH; oxygen saturation 95%–100%) for 3 days (Figure 1a). This type of substratum was used consistently for all laboratory experiments based on previous experience of maintaining the selected mussel and crayfish species in the laboratory. Mussels were positioned in a way which mimicked their natural burrowing to minimise stress and provide a realistic exposure scenario. To ensure equal access possibilities of the crayfish to each of them, they were placed in rows with equal distance of individuals in random order. We provided a PVC tube (7 cm × 25 cm) as a shelter for the crayfish in each aquarium. Before each experiment, crayfish were not fed for 3 days following Meira et al. (2019).

2.3 | Co-exposure and predation

The co-exposure experiment aimed to compare the predation rates of *A. astacus* and *P. leniusculus* on the three native bivalves (*A. anatina*, *A. cygnea*, and *U. pictorum*) and one invasive bivalve (*S. woodiana*). This experiment was performed with *A. astacus* and *P. leniusculus* and with eight replicates per crayfish species. We used four crayfish specimens with similar size of each male (carapace length 5.09 cm ± 0.37 SD for *A. astacus* and 4.92 cm ± 0.23 SD for *P. leniusculus*) and female (carapace length 4.98 cm ± 0.09 SD for *A. astacus* and 5.25 cm ± 0.27 SD for *P. leniusculus*).

2.4 | Learning effect

Previous contact of the crayfish with mussels before the experiment could be excluded. The hatchery from which we obtained *A. astacus* does not have mussels in their facility and there are no mussel populations in the stream of origin of *P. leniusculus*. This allowed testing of a possible learning effect of the crayfish to use

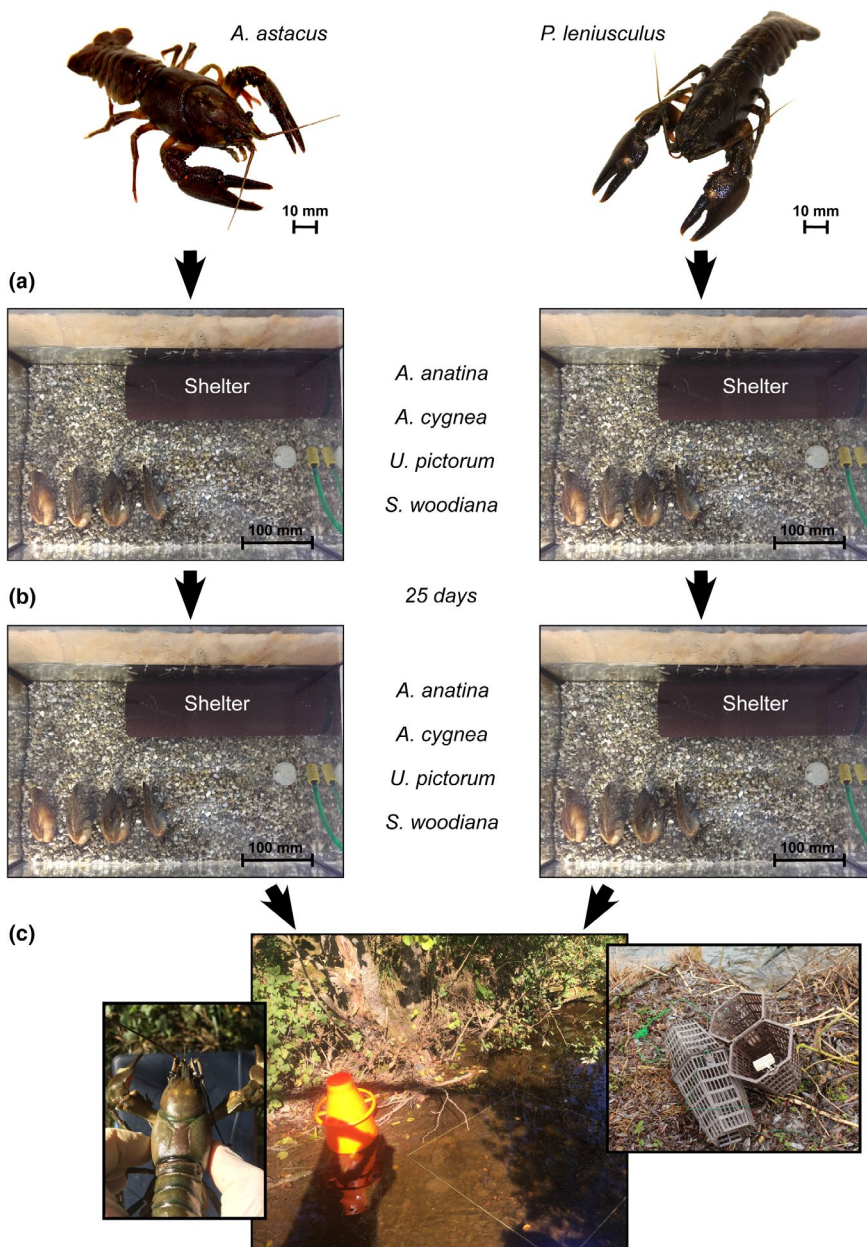


FIGURE 1 Schematic illustration of the study design showing (a) the co-exposure experiment placing one crayfish specimen together with one specimen each of three native (*A. anatina*, *A. cygnea*, and *U. pictorum*) and one invasive (*S. woodiana*) mussel species in each aquarium with eight replicates per crayfish species (*A. astacus* and *P. leniusculus*), (b) the repeated exposure experiment with the same crayfish specimens after 25 days and (c) the field validation of the experimental results

mussels as prey. Thus, the same experiment was repeated after holding the crayfish together with five mussels of each species (8–9 cm), also providing them with five dead mussels to increase attraction for 25 days (Figure 1b). During this co-exposure, mussels were fed with 30 ml algae mixture (Shellfish Diet 1800[®], Nannochloropsis 3600[®], and tap water [2:1:1]; Varicon Aqua Solutions Ltd) and crayfish with carrot slices every other day. We observed crayfish feeding on all of the dead mussels, but we could not detect severe damage to the living mussels. To recognise each crayfish, we individually marked the crayfish carapaces with nail polish and frequently checked the tank for freshly moulted crayfish.

Carapace length changed between the experiments because some of the crayfish moulted. In the second experiments, the crayfish had carapace lengths of $5.16 \text{ cm} \pm 0.09 \text{ SD}$ for female and

$5.17 \text{ cm} \pm 0.28 \text{ SD}$ for male *A. astacus*, and $5.25 \text{ cm} \pm 0.26 \text{ SD}$ for female and $5.17 \text{ cm} \pm 0.34 \text{ SD}$ for male *P. leniusculus*.

We used eight new specimens of similar size classes of each mussel species per crayfish species (Table S1; Figure 1b), except for four completely intact individuals of *A. cygnea* we re-used in the second experiment with *A. astacus*.

2.5 | Field impact assessment

In order to assess the shell damage and mortality resulting from *in situ* co-exposure of *A. astacus* and *P. leniusculus* with three freshwater bivalves (*A. anatina*, *A. cygnea*, and *U. crassus*), we conducted field investigations (Figure 1c) in the stream Mooshamer Weiherbach ($47^{\circ}53'25''\text{N}$, $11^{\circ}31'0''\text{E}$) in October 2018. The stream belongs to

the upper Danube drainage system and has a similar water chemistry to the water in the laboratory experiment (for details, see Richter et al., 2016). Sampling frames (1 × 1 m) were placed onto the streambed every 250 m of the stream within a stretch of 4.5 km after an outflow of a pond. We collected all visible and buried mussels down to a substrate depth of 10 cm within these frames, determined their species, and measured their length, width and height. We also examined all mussels carefully for predation marks. To confirm the presence of crayfish, we performed two capture events within 7 days. For this purpose, we laid out crayfish fyke traps (type "pirate", Engel Netze) with dimensions 61 cm × 31.5 cm × 25 cm (length, width, height) and a mesh size of 11 mm × 45 mm baited with Frolic® dry dog food (Mars, Inc.) at each mussel sampling point for 24 hr. All trapped crayfish were sexed and determined to species level, and the carapace length was measured. Afterwards we released all *A. astacus* at the point they were caught. Caught *P. leniusculus* were not released.

2.6 | Assessment of mussel damage

After each experiment, mortality and shell damage were assessed. For the assessment of mussel damage patterns, shells were positioned on a reference grid and individually photographed with a digital single-lens reflex camera (Canon EOS 650D). All images of valves with predation marks were georeferenced by polynomial transformation (polynomial 3) of the open source geographic information system software Quantum GIS v3.4.5 (QGIS Development Team, 2019) using nearest neighbour as resampling method and a custom coordinate reference system with a transverse Mercator projection, a latitude and longitude origin of 0 with a coordinate value at x and y origin of 0, a scale factor of 1, WGS84 as the ellipsoid used and the units in metres. For georeferencing, we distributed 12 control points on the reference grid around the valves. For a systematic analysis, each shell side was subdivided into four areas: posterior-dorsal (PD), posterior-ventral (PV), anterior-ventral (AV), and anterior-dorsal (AD). To distinguish between posterior and anterior, we used the axis perpendicular to the hinge and running through the umbo, and to distinguish between dorsal and ventral, we used the axis running from posterior to anterior in 1/2 of the height (Figure 2).

We counted all marks and measured each length from the outermost, undamaged edge of one side to the outermost, undamaged edge of the other side of the mark along the natural edge of the shell and the depth of all marks from the natural edge of the shell to the deepest part of the notch (Figure 2) using ArcGIS v10.7.1 (ESRI, 2019).

2.7 | Statistical analyses

We calculated the total damage rates (%) as the proportion of each mussel species and each quadrant of each mussel species being

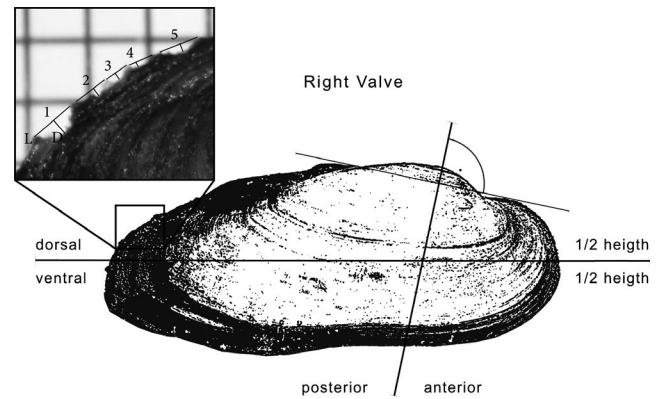


FIGURE 2 Analysis of predation marks with shells divided into four sections, posterior-dorsal (PD), posterior-ventral (PV), anterior-ventral (AV), and anterior-dorsal (AD). The length (L) and the maximum depth (D) of each mark were measured. Numbers show the count of the marks

damaged in the pool of specimens assessed. To assess the damage patterns, we summarised the number and length of shell predation marks and calculated the maximum shell injury depth for each mussel used in the experiments (Figure 2).

In order to test the effect of the two different crayfish species and to test for the learning effect of the crayfish, we conducted three zero-inflated generalised linear mixed models using the function `glmmTMB` in the package `glmmTMB` (v0.2.3; Brooks et al., 2017) in R (v3.5.0; R Core Team, 2020). The models differed in the response variable, using length of predation mark per quadrant in Model 1, maximum depth of predation mark per quadrant in Model 2, and number of predation mark per quadrant in Model 3. In the models, the variance distribution was a Poisson regression function with a log link for the number, and a Gaussian regression function for length and depth of predation marks. Since we used four mussel specimens of four different species with one crayfish and eight replicates per crayfish species for the co-exposure (experiment Part 1) and we repeated the co-exposure with the same crayfish individuals (experiment Part 2), we included mussel species, crayfish species, experimental parts, and quadrant as fixed factors. We also included interactions between crayfish species and mussel species, mussel species and quadrant, as well as between crayfish species and experimental parts in each of these models. The factors tank (replicate) and mussel specimen were included as random effects to adjust for the repeated use of a tank as well as the four reused mussel specimens. Significance levels of fixed effects were assessed with Wald χ^2 tests using the function `ANOVA` within the package `car` (v3.0-7; Fox & Weisberg, 2019). Pairwise comparisons between treatment levels of fixed effects were calculated using the function `emmeans` in R/`emmeans` (v1.4.3.01, Lenth, 2019). The *p*-values were adjusted using the Bonferroni method. We visually evaluated the residuals of the fitted models in terms of normality of errors and homogeneity of variances using diagnostic plots in R/`DHARMa` (v0.2.7; Hartig, 2020). For all statistical analyses, significance levels were set to $p < 0.05$.

3 | RESULTS

Across all mussel species, predation injuries during the experiment were detected in 58.6% of all specimens, with the thick-shelled *U. pictorum* being most affected (81.3%). For all mussel species pooled, this value was lower in experiment Part 1 without previous contact (48.4%) than in Part 2 (68.8%). Mortality was observed only in *A. anatina*, of which five specimens died (one in Part 1 with *A. astacus*, three in Part 2 with *A. astacus* and one in Part 2 with *P. leniusculus*). No further mortality was observed within an additional period of 7 days after the experimental parts.

3.1 | Predation in co-exposure

In the co-exposure, each shell had an average of 10.6 ± 15.9 SD marks per specimen with a mean length of $33.4 \text{ mm} \pm 53.7$ SD and a maximum depth of $0.5 \text{ mm} \pm 0.6$ SD per mussel. Most of the predation marks were observed in *A. cygnea* (16.8 ± 23.8 SD) with a length of $55.5 \text{ mm} \pm 82.0$ SD and a maximum depth of $0.6 \text{ mm} \pm 0.9$ SD, fewest in the invasive *S. woodiana* (4.3 ± 11.8 SD) with a length of only $13.9 \text{ mm} \pm 40.7$ SD and a maximum depth of only $0.2 \text{ mm} \pm 0.4$ SD (Table 1).

Significant differences in lengths of predation marks were observed only in the factor quadrant (Model 1; $\chi^2 = 41.36$; $p < 0.001$) and for the interaction between mussel species and quadrant (Model 1; $\chi^2 = 19.3$; $p < 0.05$). Model 1 showed no preference of *A. astacus* or *P. leniusculus* for mussel species.

Considering depths of the marks, significant differences were detected for the factors mussel species (Model 2; $\chi^2 = 23.3$; $p < 0.001$), crayfish species (Model 2; $\chi^2 = 20.84$; $p < 0.001$), experimental parts (Model 2; $\chi^2 = 13.9$; $p < 0.001$), and quadrant (Model 2; $\chi^2 = 133.76$; $p < 0.001$), as well as for the interactions between crayfish species

and mussel species (Model 2; $\chi^2 = 14.3$; $p < 0.01$), crayfish species and experimental parts (Model 2; $\chi^2 = 6.4$; $p < 0.05$), and mussel species and quadrant (Model 2; $\chi^2 = 39.5$; $p < 0.001$) (Table 2). Furthermore, pairwise comparison showed significant differences between *S. woodiana* and *A. cygnea* (Model 2; $p < 0.01$) as well as between *S. woodiana* and *U. pictorum* (Model 2; $p < 0.001$) for the depth of marks.

For *A. astacus*, the pairwise comparison only revealed significant differences in depth of the marks between *S. woodiana* and *A. anatina* (Model 2; $p < 0.05$). In the experiment with *P. leniusculus*, marks differed significantly in depth between *S. woodiana* and *A. cygnea* (Model 2; $p < 0.05$), *S. woodiana* and *U. pictorum* (Model 2; $p < 0.01$), as well as between *A. anatina* and *A. cygnea* (Model 2; $p < 0.01$).

Number of marks were significantly different in mussel species (Model 3; $\chi^2 = 23.72$; $p < 0.001$), crayfish species (Model 3; $\chi^2 = 16.36$; $p < 0.001$), experimental parts (Model 3; $\chi^2 = 8.49$; $p < 0.01$), quadrant (Model 3; $\chi^2 = 123.36$; $p < 0.001$), and in the interaction between crayfish species and experimental parts (Model 3; $\chi^2 = 12.17$; $p < 0.001$), as well as between mussel species and quadrant (Model 3; $\chi^2 = 47.12$; $p < 0.001$).

Pairwise comparisons showed significant differences in number of predation marks between *S. woodiana* and the other three mussel species *A. anatina* (Model 3; $p < 0.001$), *A. cygnea* (Model 3; $p < 0.01$), and *U. pictorum* (Model 3; $p < 0.001$). In *A. anatina* and *U. pictorum*, most of the predation marks were found in the posterior-dorsal quadrant of the shell (35.1% vs. 50.0%), but for *A. cygnea* and *S. woodiana* the distribution was anterior-ventral (42.0% vs. 68.1%).

A. astacus caused a mean of 3.2 ± 7.6 SD marks per mussel with an average length of $8.5 \text{ mm} \pm 19.8$ SD and a maximum depth of $0.2 \text{ mm} \pm 0.4$ SD per mussel. By contrast, *P. leniusculus* caused more than five times more marks ($18.1 \text{ mm} \pm 18.4$ SD) with an average length of $58.3 \text{ mm} \pm 64.7$ SD and a maximum depth of $0.8 \text{ mm} \pm 0.7$ SD per mussel. For *A. astacus*, pairwise comparisons revealed

TABLE 1 Percentage of damaged mussels (*A. anatina*, *A. cygnea*, *U. pictorum*, and *S. woodiana*), average number of predation marks per specimen and percentage of marks within the four quadrants (anterior-ventral, anterior-dorsal, posterior-ventral, and posterior-dorsal) after first (Part 1) and second (Part 2) co-exposure

	Mussel species	% damaged mussels	% predation marks in quadrants				Average number of predation marks
			Anterior-ventral	Anterior-dorsal	Posterior-ventral	Posterior-dorsal	
Part 1	<i>A. anatina</i>	68.8	32.1	4.2	28.7	35.1	8.9 ± 9.1 SD
	<i>A. cygnea</i>	43.8	42.0	6.7	22.3	29.0	16.8 ± 23.8 SD
	<i>U. pictorum</i>	62.5	15.0	4.5	30.5	50.0	12.5 ± 13.3 SD
	<i>S. woodiana</i>	18.8	68.1	1.4	23.2	7.2	4.3 ± 11.8 SD
Part 2	<i>A. anatina</i>	68.8	26.7	5.4	28.8	39.2	15.0 ± 17.2 SD
	<i>A. cygnea</i>	62.5	40.2	2.9	23.4	33.6	15.2 ± 19.6 SD
	<i>U. pictorum</i>	100.0	23.9	5.6	31.8	38.6	22.2 ± 12.8 SD
	<i>S. woodiana</i>	43.8	39.7	0.0	39.7	20.5	4.9 ± 6.6 SD
Change	<i>A. anatina</i>	+0.0	-4.1	+3.3	-2.0	+2.8	+6.1
	<i>A. cygnea</i>	+18.8	-1.8	-3.8	+1.1	+4.6	-1.6
	<i>U. pictorum</i>	+37.5	+8.9	+1.1	+1.3	-11.4	+9.7
	<i>S. woodiana</i>	+25.0	-28.4	-1.4	+16.6	+13.3	+0.6

TABLE 2 Results of zero-inflated generalised linear mixed models investigating differences in mean length, maximum depth, and mean number of predation marks per shell quadrant between four mussel species, two crayfish species, two experimental parts, and four quadrants, as well as the interactions of crayfish species with mussel species, crayfish species with the experimental parts, and mussel species with the quadrants

Response variable	Source	η^2	df	p
Length $R^2 = 0.95$	Mussel species	—	—	n.s.
	Crayfish species	—	—	n.s.
	Experimental parts	—	—	n.s.
	Quadrant	41.36	3	<0.001
	Crayfish species*mussel species	—	—	n.s.
	Crayfish species*experimental parts	—	—	n.s.
	Mussel species*quadrant	19.29	9	<0.05
Depth $R^2 = 0.58$	Mussel species	23.33	3	<0.001
	Crayfish species	20.84	1	<0.001
	Experimental parts	13.85	1	<0.001
	Quadrant	133.76	3	<0.001
	Crayfish species*mussel species	14.25	3	<0.01
	Crayfish species*experimental parts	6.35	1	<0.05
	Mussel species*quadrant	39.51	9	<0.001
Number $R^2 = 0.76$	Mussel species	23.72	3	<0.001
	Crayfish species	16.36	1	<0.001
	Experimental parts	8.49	1	<0.01
	Quadrant	123.34	3	<0.001
	Crayfish species*mussel species	—	—	n.s.
	Crayfish species*experimental parts	12.17	1	<0.001
	Mussel species*quadrant	47.12	9	<0.001

significant differences in number of marks between *S. woodiana* and *A. anatina* (Model 3; $p < 0.001$) as well as between *S. woodiana* and *U. pictorum* (Model 3; $p < 0.001$); and for *P. leniusculus* between *S. woodiana* and *A. anatina* (Model 3; $p < 0.05$), between *S. woodiana* and *A. cygnea* (Model 3; $p < 0.01$), as well as between *S. woodiana* and *U. pictorum* (Model 3; $p < 0.001$).

Thus, *A. astacus* mostly damaged *A. anatina* with 6.0 ± 6.8 SD and *A. cygnea* with 4.5 ± 12.7 SD marks per specimen, whereas *P. leniusculus* mostly damaged *A. cygnea* with 29.1 ± 26.5 SD and *U. pictorum* with 22.6 ± 11.0 SD marks per specimen (Figure 3; Table S2).

3.2 | Learning effect

The repeated exposure revealed a greater crayfish impact on the mussels as evident from significantly deeper shell marks (Model 2; $p < 0.001$; mean $0.7 \text{ mm} \pm 0.6$ SD) and from a higher number of marks (Model 3; $p < 0.001$; mean 14.3 ± 15.8 SD) compared to the first exposure. Concerning depth of the marks, pairwise comparison of mussel species were significantly different between *S. woodiana* and *A. cygnea* (Model 2; $p < 0.01$), and between *S. woodiana* and *U. pictorum* (Model 2; $p < 0.001$) for the second co-exposure. Between the experimental parts, pairwise comparison revealed significant differences in the depth of marks (Model

2; $p < 0.001$) caused by *A. astacus*, but not for marks caused by *P. leniusculus*.

In line with experiment Part 1, significant differences were detected in number of predation marks between *S. woodiana* and the other three mussel species *A. anatina* (Model 3; $p < 0.001$), *A. cygnea* (Model 3; $p < 0.01$) and *U. pictorum* (Model 3; $p < 0.001$). Most of the predation marks were observed in *U. pictorum* (22.2 ± 12.8 SD) with a length of $86.1 \text{ mm} \pm 41.1$ SD and a maximum depth of $1.0 \text{ mm} \pm 0.3$ SD, and fewest marks were seen in *S. woodiana* (4.9 ± 6.6 SD) with a length of $23.4 \text{ mm} \pm 38.4$ SD and a maximum depth of $0.4 \text{ mm} \pm 0.5$ SD (Table 1).

Between the experimental parts, pairwise comparison showed significant differences in the number of marks (Model 3; $p < 0.001$) caused by *A. astacus*, but again not for marks caused by *P. leniusculus*. In Part 2 of the experiment of *A. astacus*, the mean number of marks as well as their length per mussel specimen strongly increased in *U. pictorum* (+16.0 marks per specimen; +73.3 mm) and in *A. anatina* (+8.8 marks per specimen; +49.1 mm) compared to Part 1. In Part 2 of the experiment with *P. leniusculus*, this increase was much lower in *U. pictorum* (+3.4 marks per specimen; +14.8 mm) and in *A. anatina* (+3.4 marks per specimen; +45.7 mm). In *A. cygnea* (−4.1; −18.0 mm) and *S. woodiana* (−3.5; −11.4 mm), both the number and the length of predation marks, decreased in contrast to Part 1 of the experiment with *P. leniusculus* (Figure 4). Based on the mean number of marks, both crayfish species mostly

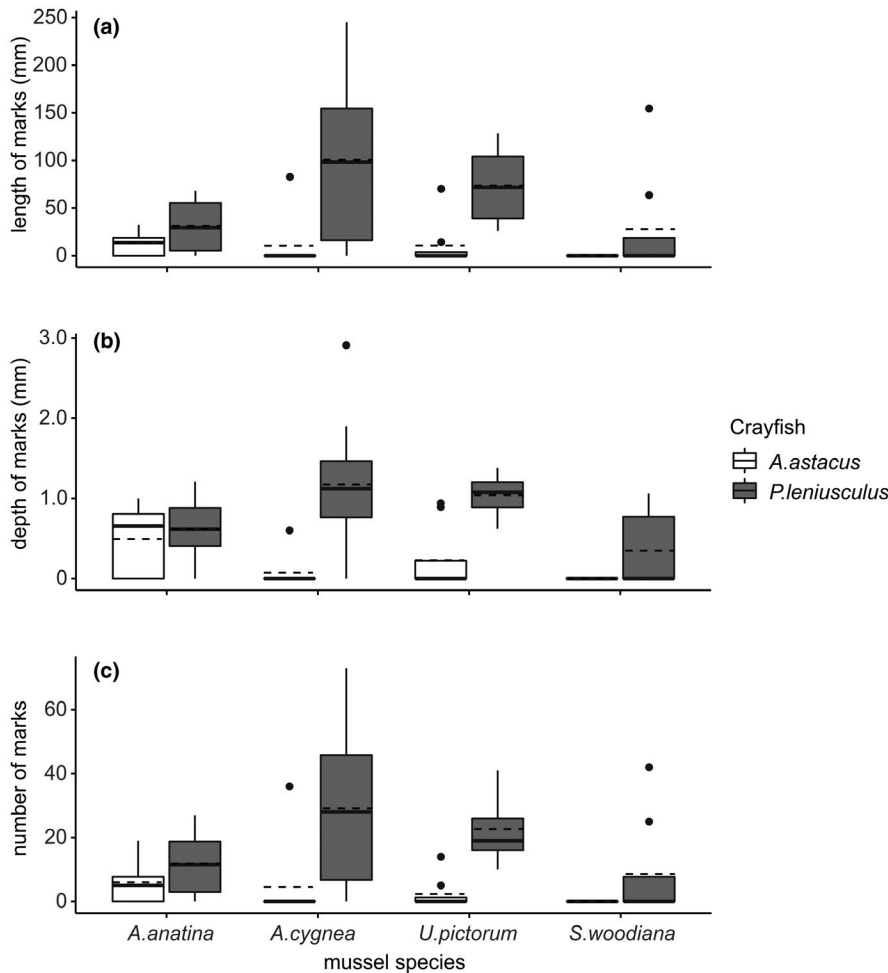


FIGURE 3 Boxplots of (a) the length, (b) the depth, and (c) the number of the predation marks of each mussel species per crayfish species of the co-exposure experiment. The horizontal lines indicate median values, dashed lines the mean values, boxes the 25th to 75th percentiles, whiskers the lowest and highest values within 1.5 times the values observed in the percentile boxes and black dots single cases exceeding 1.5 times the values observed in the percentile boxes

preferred *U. pictorum* in Part 2 of the experiment, with the number of marks being four to five times greater in this species compared to *S. woodiana* where the lowest number of marks was found (Figure 5; Table S2).

3.3 | Field impact assessment

During our field investigation, we found a total of 196 mussel specimens in eight of 18 sampling frames (R10–R11 and R13–R18). With 127 individuals, most of these mussels were *U. crassus*, but we also found 31 *A. anatina* and 38 *A. cygnea* (Table 3). The presence of crayfish was confirmed at 16 of the 18 locations, whereby only the native *A. astacus* occurred at places with mussels. The invasive *P. leniusculus* was found only in the lower parts of the Mooshamer Weiherbach (R02–R06) where no living mussels are found. None of the found mussels showed any predation marks.

4 | DISCUSSION

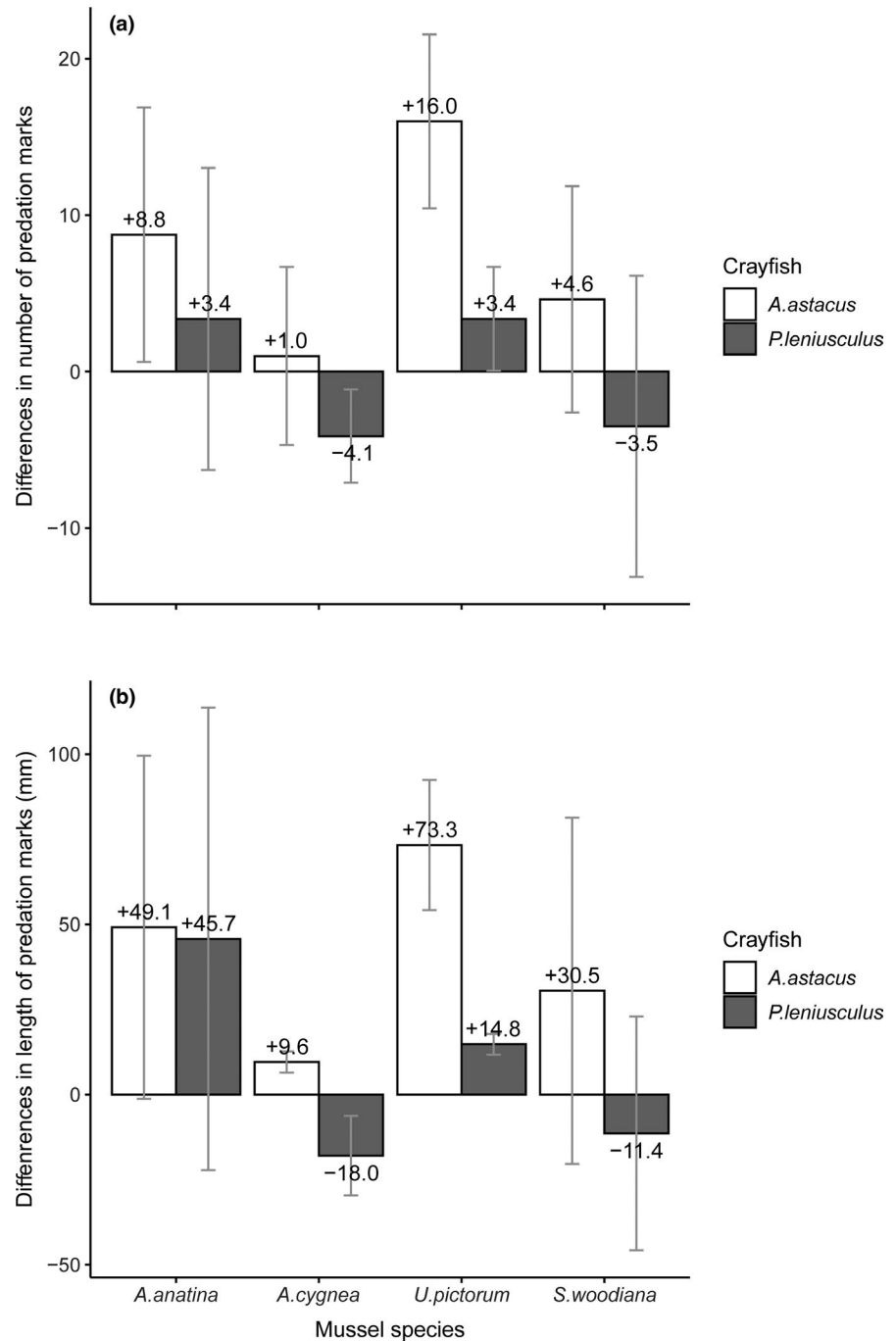
The findings of this study show that both the native *A. astacus* and the invasive *P. leniusculus* are able to learn how to prey on freshwater

bivalves. Contrary to expectations, the more thick-shelled *U. pictorum* had higher damage rates compared to mussel species with thinner shells, indicating that other factors such as shell shape seem to be important in determining susceptibility. The lowest observed damage occurring in the non-native *S. woodiana* combined with the greater mussel predation damage caused by the invasive crayfish species suggests that synergistic interactions of both non-native species may lead to multiple stressors critical for native freshwater mussel populations of conservation concern, yet no such effect could be confirmed in the wild.

4.1 | Effects of native and invasive crayfish

Across all of our laboratory experimental parts and all used mussel species, shell damage caused by crayfish could be observed. This was expected from previous studies that report mussel predation effects by crayfish (Glon et al., 2017; Klocker & Strayer, 2004; Machida & Akiyama, 2013; Meira et al., 2019; Perry et al., 1997; Schmidt & Vandré, 2012; Sousa et al., 2019). To the best of our knowledge, no other study had yet applied a cross-experimental design with native and invasive crayfish as well as native and invasive unionids, allowing a direct comparison of predation effects.

FIGURE 4 Differences in the mean (a) number and (b) length of predation marks per mussel species between parts 1 and 2 of the experiments with *A. astacus* and *P. leniusculus*



As evident from the observed mussel damage in the co-exposure, *P. leniusculus* has greater effects than the native *A. astacus*. In general, *P. leniusculus* is known for its greater activity (Wutz & Geist, 2013), faster growth, and more aggressive behaviour (Söderbäck, 1991) compared to the native *A. astacus*, probably further exacerbating its impact on both native crayfish and mussel populations. Several impacts of *P. leniusculus* on freshwater biodiversity are known. For example, invasive crayfish such as *P. leniusculus* have negative effects on benthic invertebrates (Usio et al., 2009) and hence on insectivorous fish (Guan & Wiles, 1998). Their ecological impacts are greater compared to native crayfish (James et al., 2015). According to Nyström et al. (1996),

P. leniusculus also is more efficient in grazing compared to the native *A. astacus*, which can result in strong decreases of macrophyte biomass. Gherardi (2007) even suspects that predation and competitive behaviour of non-indigenous crayfish species also can change interactions within communities resulting in trophic cascade effects. In addition to the direct effects on mussels, invasive crayfish such as *P. leniusculus* are geomorphic agents that mobilise sediment (Johnson et al., 2011) which can cause considerable damage to river banks via their burrowing (Sibley, 2000). The resulting increased sediment erosion can have negative impacts on water quality (Faller et al., 2016) and overall mussel habitat quality (Geist & Auerswald, 2007), negatively affecting mussel populations.

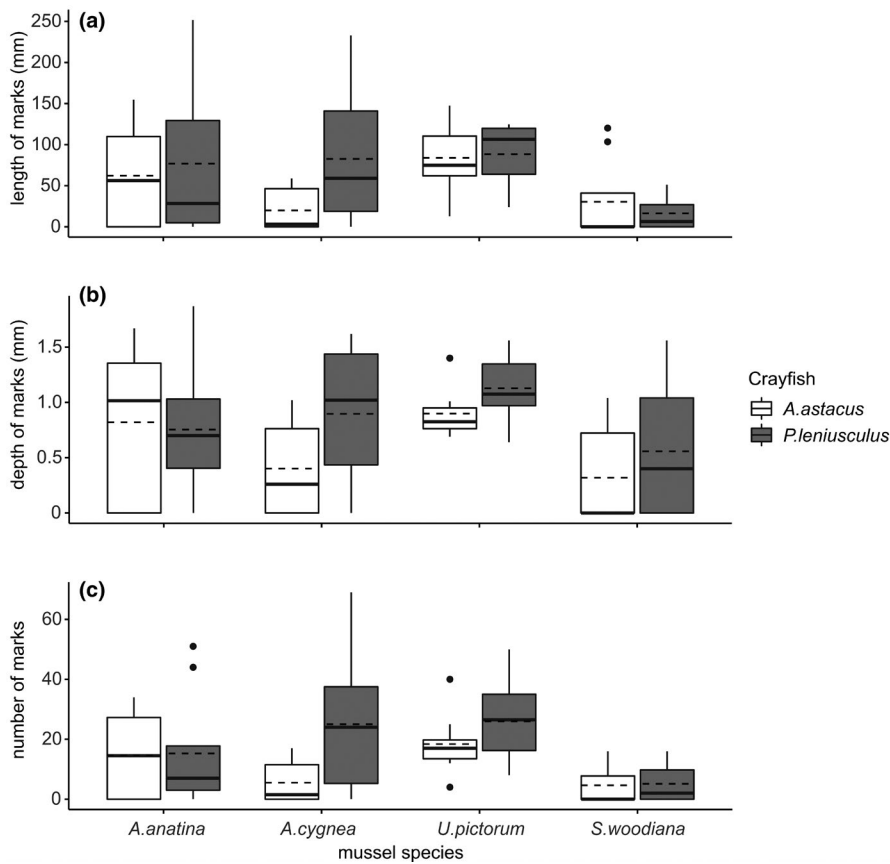


FIGURE 5 Boxplots of (a) the length, (b) the depth, and (c) the number of the predation marks of each mussel species per crayfish species of the learning experiment. The horizontal lines indicate median values, dashed lines the mean values, boxes the 25th and 75th percentiles, whiskers the lowest and highest values within 1.5 times the values observed in the percentile boxes and black dots single cases exceeding 1.5 times the values observed in the percentile boxes

Location	<i>Unio crassus</i>	<i>Anodonta anatina</i>	<i>Anodonta cygnea</i>	<i>Astacus astacus</i>	<i>Pacifastacus leniusculus</i>
R18	9	12	28	2	—
R17	35	14	9	8	—
R16	14	3	1	8	—
R15	2	2	—	2	—
R14	51	—	—	14	—
R13	14	—	—	17	—
R12	—	—	—	5	—
R11	1	—	—	10	—
R10	1	—	—	7	—
R09	—	—	—	4	—
R08	—	—	—	—	—
R07	—	—	—	3	—
R06	—	—	—	—	9
R05	—	—	—	—	4
R04	—	—	—	—	4
R03	—	—	—	—	3
R02	—	—	—	—	8
R01	—	—	—	—	—
Total	127	31	38	80	28

Note: R18 was the uppermost point after the outflow of the Mooshamer Weiher pond and R01 was the lowest point on the east side of Ascholding village.

TABLE 3 Number of mussels and crayfish per species at the different locations in the Mooshamer Weiherbach

4.2 | Effects on mussel species

Our hypothesis that predation effects would be greatest in mussel species with thinnest and most delicate shells (i.e., *A. anatina* and *A. cygnea*) has to be rejected. Over all experimental parts, the thick-shelled *U. pictorum* was most strongly affected in terms of number of damaged specimens and the invasive *S. woodiana* was hardly affected, with *A. anatina* and *A. cygnea* in an intermediate position.

However, direct mortality was observed in only five of 128 specimens used during the experiment and only in *A. anatina*. In addition, it could be observed during the experiment that crayfish lifted several specimens of *A. anatina* out of the sediment and moved them into the tubes. Even if this suggests that the lower weight of the mussels plays an important role in being attractive for crayfish, these results should be interpreted with caution since no predation marks could be found in two of the empty dead shells and mortality of *A. anatina* was also observed in mussels without treatment. Besides direct mortality over the short time of the experiment, other effects such as excavating and “playing” with mussels resulted in shell clamping, and consequently reduced filtration and energy intake as well as translocation to less favourable sites (e.g., inside crayfish shelters), even if this does not necessarily play a major role under natural conditions. Nevertheless, this could lead to a weakening of the mussels which will – together with the slight damages all around the shells that result in mussels no longer being able to close completely and being exposed unprotected to external influences – probably decrease fitness and increase the risk for mortality.

For our experimental design, we chose a conservative approach and used only adult mussels of similar size classes for inter-species comparison since shell size seems to influence predation susceptibility. zu Ermgassen and Aldridge (2011) showed that *P. leniusculus* increasingly harmed smaller specimens of *D. polymorpha* with a size range of 7–12 mm. A likely explanation is that mussels reach their size refuge very quickly and begin to be unattractive as food for crayfish already at small sizes compared to adult unionids. However, this species stays much smaller in comparison to the unionids used in our study and juvenile unionids stay burrowed in the substrate and thus are not easy to find or even accessible for crayfish in natural conditions (Ozgo et al., 2021). In addition, Olden et al. (2009) found in a study of signal crayfish predation on non-native mystery snails (*Bellamya chinensis*) a u-shaped relationship between snail size and prey value to the crayfish. Small snails are valuable because they are easier to handle, whereas very large snails are still valuable because the reward for breaking into their shell is high. Intermediate sized snails were the least valuable, because they are both hard to handle and consume but contain less food than the largest snails. Similar results were obtained by Machida and Akiyama (2013) who showed in experiments with two margaritiferid species from Japan and *P. leniusculus* that mortality resulting from predation occurred only in juvenile specimens (10.09–19.37 mm), but the damage rate was higher in larger mussels (>50 mm), whereas Sousa et al. (2019) found higher damage rates and mortality in smaller specimens of *Margaritifera margaritifera* (22.0–39.7 mm) caused by *P. leniusculus*.

Over all experimental parts, a clear difference in predation preference was evident. Whilst the number of marks is likely an indicator for the intensity and number of crayfish attacks proportional to disturbance, the depth of marks is likely more strongly linked to the injury intensity. In line with our hypothesis, Part 1 of the experiment (without previous contact to mussels) revealed a tendency of thin-shelled *A. anatina* and *A. cygnea* being preferentially preyed upon by *A. astacus* and *P. leniusculus*, respectively. However, in Part 2 both crayfish species mostly preferred the most thick-shelled *U. pictorum*, suggesting our original hypothesis has to be rejected. This is in contrast to the results of Meira et al. (2019) where signal crayfish preferred thin-shelled *A. anatina* over thick-shelled *Potomida littoralis* and *U. delphinus* and invasive species *C. fluminea*. The authors attributed this to the thinner and more fragile shells that facilitates both easier handling and less energetically cost-intensive predation. However, both results are difficult to compare since Meira et al. (2019) used two different mussel species present in southern Europe and a wider range of shell sizes (e.g., 35–126 mm for *A. anatina*; 44–78 mm for *P. littoralis* and 36–97 for *U. delphinus*). Based on our results of experiment Part 1 with *A. astacus*, it can be assumed that the thinner shells and thus the lower weight makes these mussels more attractive for unexperienced crayfish. However, the observed damage patterns of our repeated exposure experiment with both crayfish species as well as our behavioural observations suggest that it is not the thickness of the shells that determines their susceptibility to crayfish predation, but that this is rather a matter of how the crayfish manage to fix the shells and can hold on to them to get access. In this respect, the more pointed shape of the shells as in *U. pictorum* allows easier access for crayfish mandibles. This also may explain the differences in sectional damage as, for example, in the posterior-dorsal part in *U. pictorum*. In *A. anatina* most of the damage also was found in the posterior-dorsal part which could be explained by the triangular shape of the hinge. Still, much damage also was found in the originally buried anterior-ventral part of this species. It was observed during the experimental parts that the crayfish used the first and second pairs of walking legs to excavate the mussels and to turn them to find a spot accessible for their mandibles. Perry et al. (1997) also have made similar observations in laboratory experiments with *Faxonius* spp. crayfish and zebra mussels.

Hence, our findings also can explain the low damage rates of the invasive *S. woodiana*. This species has a rounder shape and thus provides fewer angles to be fixed and attacked by the crayfish. Considering, however, that size can affect predation susceptibility, *S. woodiana* also may have a considerable advantage over native ones as a consequence of its much greater maximum size and its faster growth.

Whilst our experimental approach allowed a standardised comparison between crayfish and mussel species, these findings cannot be transferred directly to field situations, as evident for example from the field validation at the Mooshamer Weiherbach. This may be explained by differences between the controlled laboratory exposure and realistic field settings including differences related to food choice, ambient environmental conditions,

and the greater variation of mussel sizes at the field site, as well as the absence of *P. leniusculus* at sites with mussel occurrence. Under natural conditions, mussel species show great differences in their burrowing behaviour (Ožgo et al., 2021; Zieritz et al., 2014) and thus may be less accessible to crayfish compared to aquaria-based exposures with limited substrate depth. In addition, invasive freshwater crayfish are omnivorous and often show opportunistic feeding behaviour (Guan & Wiles, 1998; Lewis, 2002; Nyström et al., 1996). Therefore, if enough alternative and better accessible food sources are available, the effects on mussels may not be that large, since excavation may be too time-consuming and energetically wasteful for crayfish (Klocker & Strayer, 2004). Furthermore, it is not common that both native and invasive crayfish species occur in the same system, as was found in the Mooshamer Weiherbach with only *A. astacus* in the upper and *P. leniusculus* in the lower parts of a stream. All of these factors individually or in synergy can explain why we did not find damaged mussel specimens. Nevertheless, there already is evidence for crayfish predation on another species of freshwater mussel which is more exposed to open water conditions in its adult stage in natural conditions (Schmidt & Vandr , 2012).

Even though we cannot directly link shell damage in our results with population-level effects in the wild, our experimental findings still clearly suggest that invasive crayfish can exert significantly greater effects on freshwater mussel communities than co-evolved native species of crayfish. Moreover, since invasion of signal crayfish mostly results in a timely die-out of native crayfish (Vorburger & Ribi, 1999), signal crayfish rarely face direct competition with native crayfish. This mechanism provides the opportunity for a fast establishment and expansion of *P. leniusculus*. In addition, signal crayfish can reach high densities of 0.4 (Wutz & Geist, 2013) to 2.2 (Guan, 2000) specimens per m². In line with our results, this could rapidly lead to an increased predation pressure on native mussels.

4.3 | Effects of the spread of *P. leniusculus*

Given the fact that *P. leniusculus* can reach higher densities than the native crayfish species (Guan & Wiles, 1996), the expected increase in invasive crayfish densities as well as their distribution expansion (Kouba et al., 2014) will increase predation pressure since crayfish have a major impact on the benthic food web (Reynolds et al., 2013).

In addition, if signal crayfish colonise new habitats that were unsuitable for native crayfish, this will introduce a new stressor for mussels resulting in potentially irreversible species diversity shifts (Hobbs et al., 1989). Our results of the co-exposures suggest that with *P. leniusculus* appearing in new habitats, this could create high predation pressure on mussel populations that had never been in contact with crayfish. Moreover, *A. astacus* may be introduced into new refuges to conserve this species. However, James et al. (2015) propose that native and non-native crayfish are ecologically similar

and accordingly native crayfish should not be translocated to ark sites, since they may cause impacts to benthic communities. Even if our results show a higher impact from invasive crayfish, the results of the learning part of the experiment indicate that a translocation of native crayfish also could be a threat for native mussel species after *A. astacus* learned to use mussels as prey.

However, all this should be interpreted with caution. Even though we observed clear mussel damage patterns directly linked to crayfish exposure, our experiment also shows that the mortality of adult mussels was relatively low over this short period of time, suggesting that most adult mussels survive even prolonged exposure to crayfish in a confined space. Nevertheless, predation effects should not be ignored, particularly since they might be much higher on juvenile mussels. Also, considering the lifespan of mussels, for example over 28 years for *Anodonta* (Aldridge, 1999), a cumulative predation and damage effect over time, which begins with the early life stages, can be expected.

Given that the invasive *S. woodiana* was significantly less affected in both experimental parts, and that *P. leniusculus* caused more damage than the native *A. astacus*, an occurrence of both invasive species could enhance the competitive ability of *S. woodiana*. Considering the known competitive advantages of *S. woodiana* over native mussel species, it can be assumed that an introduction of this species will already be disadvantageous for native mussel populations in absence of crayfish predation. In particular, possible outcompeting effects already have been shown to be related to reproduction success: the greater larval survival (Benedict & Geist, 2021), larger number of suitable hosts, higher excystment rates, and faster development of *S. woodiana* (Douda et al., 2012; Huber & Geist, 2019), as well as the greater glochidia output and the ability for breeding multiple times a year (Labecka & Czarnoleski, 2019; Labecka & Domagala, 2018), increase the reproduction success of *S. woodiana*. In contrast to the reproduction success of *S. woodiana*, the reduced transformation success rates of *A. anatina* on host fish previously infested with *S. woodiana* (Donrovich et al., 2017) reduce the recruitment of native mussel populations. In addition, its wider tolerance towards changing environmental conditions (e.g., temperature) also could give this species an advantage during ongoing climate change (Bielen et al., 2016; Corsi et al., 2007; Douda et al., 2012).

If both *P. leniusculus* as well as *S. woodiana* invade the same system, the high impact in native species but the low impact of *P. leniusculus* on *S. woodiana* could potentially lead to multiple stressors for native mussels as has already been reported, inter alia, in the upper Danube River (Brandner et al., 2013) and in a laboratory experiment with invasive round goby and amphipod on native gammarids (Beggel et al., 2016). This may result in increased problems for, and local extinction of native mussel populations, as well as the facilitated spread of invasive species.

ACKNOWLEDGMENTS

We are grateful for the financial support of this study by the Bavarian Environmental Protection Agency. We would also like to thank A. Benedict for her assistance with photo analysis, H. Buxbaum for his

active support during fieldwork, and M. Mueller and R. Wild for their statistical support. AD and JG jointly contributed to all steps of this work. Open access funding enabled and organized by ProjektDEAL.

ETHICAL APPROVAL

All procedures were ethically reviewed and approved by the District Government of Upper Bavaria (reference number: 2631.Vet_11-4-12). Experiments were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

Data are available from the authors upon reasonable request.

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How to cite this article: Dobler, A. H., & Geist, J. (2022).

Impacts of native and invasive crayfish on three native and one invasive freshwater mussel species. *Freshwater Biology*, 67, 389–403. <https://doi.org/10.1111/fwb.13849>