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Behavioral differences in an over-invasion scenario: marbled vs. spiny-cheek crayfish

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Abstract. New species often invade ecosystems already dominated by previous invaders. Ornamental freshwater crayfish, particularly parthenogenetic marbled crayfish (*Procambarus virginalis*), increasingly establish in European water bodies where they interact with resident native and non-native species. Behavioral traits and behavioral syndromes can influence the outcome of these species interactions. The behavior of non-native crayfish is often studied in notorious invaders but rarely in new and emerging species, although those provide the best opportunity for management. Activity, aggressiveness, and boldness have repeatedly been associated with invasion success and species displacement. Further, crayfish can adapt their behavior after they have established in the new range. We investigated whether marbled crayfish can displace the widely established spiny-cheek crayfish (*Orconectes limosus*). Specifically, we compared their behavioral traits and evaluated whether these traits differ, using marbled crayfish populations from aquaria and the field and spiny-cheek crayfish from the field. We staged agonistic encounters, measured activity levels, and recorded the response to a simulated threat of both species and both origins (field and aquarium) in laboratory trials. We found that in agonistic encounters, marbled crayfish were on average more aggressive than spiny-cheek crayfish, even against larger opponents. Aggressiveness and activity were positively correlated, which is indicative for an aggression syndrome. Marbled crayfish from the field were less active than those from aquaria, but there was no difference in aggressiveness. Marbled crayfish often froze in response to a simulated threat, whereas spiny-cheek crayfish reacted either offensively or defensively. These results from the laboratory illustrate potentially important behavioral mechanisms behind crayfish over-invasions and show behavioral plasticity in a species where all known individuals are genetically identical. To better understand the invasion process in nature, the species' reproductive biology and interactions with other members of the community should be considered. We conclude that the recent success of marbled crayfish in establishing new populations could be influenced by their behavioral flexibility and their potential to competitively persist in the presence of established invasive crayfish.

Key words: aggression; behavioral flexibility; behavioral syndromes; behavioral variability; biological invasions; freshwater crayfish; shelter use; threat response.

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Introduction

Species invasions have already massively altered aquatic communities and are still increasing worldwide (Gallardo et al. 2016, Seebens et al. 2017). Consequently, more and more invasive species compete with already established, functionally similar invasive species, a process that has been termed over-invasion (Russell et al. 2014). In novel communities, such over-invasions and species introduction dates are of great relevance and allow for more detailed analyses than a simple, dichotomous distinction between native vs. non-native species that ignores species residence times (Dornelas et al. 2014).

The consequences of multiple species invasions are largely unknown (Hewitt and Huxel 2002), but the invasion outcome and interaction strength between invading and resident species can be assessed by behavioral differences and correlated suites of behavioral traits (i.e., behavioral syndromes; Chapple et al. 2012, Sih et al. 2012, Penk et al. 2017). Some behavioral traits such as activity, aggressiveness, and boldness have repeatedly been associated with invasion success (Weis 2010, Chapple et al. 2012). Furthermore, the ability to behaviorally adapt to a new environment, that is, behavioral flexibility, promotes invasion success (Wright et al. 2010). Naïve non-native species have to adapt to new prey, competitors, or predators by means of evolution or learning (Saul and Jeschke 2015, Wong and Candolin 2015). Comparative studies across invading species can help elucidate what makes some invaders more successful than others (van Kleunen et al. 2010).

Ornamental crayfish invasions

Particularly since the beginning of the 20th century, decapod crayfish invasions have resulted in a decline of native crayfish populations and severe changes to ecosystems, for example, in Europe (Holdich et al. 2009, Lodge et al. 2012). Nowadays, increasing numbers of new non-native crayfish species are imported by the pet trade from North America and Australasia to Europe, and some species have already been released in nature (Chucholl 2013, Chucholl and Wendler 2017). As more of these recently arrived species have started to establish populations, interactions with other invasive species will shape future crayfish distributions and novel species communities (Kouba et al. 2014). However, the propagule pressure of the new invaders and the incumbent advantage of the old invaders will be decisive for potential competitive displacement in these over-invasion scenarios (Lockwood et al. 2005, Russell et al. 2014). Crayfish from the pet trade have the disadvantage that they are naïve to prey, predators, or competitors when they are released from aquaria (Hazlett 1994, Martin 2014). For example, aquaria or other hatchery-reared fish are more vulnerable to predation than those that have experienced predation (Kellison et al. 2000, Yokota et al. 2007). Some studies looked at agonistic behaviors among competing old and new invasive crayfish species (Chucholl et al. 2008, Hudina et al. 2011, James et al. 2016), but broader behavioral comparisons are necessary to investigate the invasive potential of species before or at an early stage of invasion.

Invasive crayfish: behavioral differences and flexibility

Non-native crayfish are model organisms in invasion ecology and are also frequently used in behavioral studies (Gherardi et al. 2012, Lodge et al. 2012). Evidence suggests that highly invasive crayfish typically display stronger interspecific aggression toward resident congeners, in that way limiting access to critical resources for competitors (e.g., Gherardi and Cioni 2004, Klocker and Strayer 2004, Chucholl et al. 2008). Also, larger body and chela size are advantageous in these agonistic interactions (Garvey and Stein 1993, Vorburger and Ribi 1999). Invasive crayfish species are often more active (Bubb et al. 2006), perceive more predation cues (Hazlett et al. 2003), or avoid predation more effectively (Garvey et al. 1994) than native crayfish. Activity, aggressiveness, and boldness in crayfish are often correlated and thought to be part of an aggression syndrome (Pintor et al. 2008, 2009). These behavioral syndromes can be explained by state variables (such as growth) that often covary with sets of behaviors (Biro et al. 2014). Furthermore, invasive crayfish adapt behavioral traits after introduction in response to resident crayfish species and the community of invaded water bodies (Pintor et al. 2008, Hanshew and Garcia 2012). For example, native crayfish that had experience with an

invasive competitor were more aggressive toward the opponent than naïve native individuals (Hayes et al. 2009). Also, the presence of predators alters the activity of invasive and native crayfish (Hirvonen et al. 2007, Aquiloni et al. 2010). It has been shown that invasive crayfish and crabs can learn how to respond to newly emerging threats after invading new territories (Hazlett et al. 2002, Roudez et al. 2008). By looking at multiple behavioral traits and integrating behavioral flexibility and new concepts like behavioral syndromes (Gherardi et al. 2012), species displacements and ecological invasions might be better understood and managed.

Model organisms

Spiny-cheek crayfish (*Orconectes limosus*) and marbled crayfish (*Procambarus virginalis*) are examples for invaders with a high functional similarity. They can thus be used as comparator organisms sensu Penk et al. (2017): Comparing marbled crayfish to resident spiny-cheek crayfish allows assessing the invasive capacity of marbled crayfish. Furthermore, both species are included in the List of Invasive Alien Species of Union Concern (EU Regulation 1143/2014). They co-occur in some lakes in Germany, but differ in their invasion history and morphology (Chucholl and Pfeiffer 2010, Chucholl et al. 2012).

Spiny-cheek crayfish have been spread across Central Europe since the late 19th century, now being one of the most common European crayfish species (Kouba et al. 2014). They display sexual dimorphism with males having larger chelae than females (Souty-Grosset et al. 2006). In parts of their native range, spiny-cheek crayfish were outcompeted by other invasive species from the genus *Orconectes* (Klocker and Strayer 2004). The interactions of spiny-cheek crayfish with other crayfish in their invasive range, however, have rarely been studied (Musil et al. 2010).

The peculiar marbled crayfish are triploid descendants of the sexually reproducing slough crayfish (*Procambarus fallax*; Martin et al. 2010, Lyko 2017, Gutekunst et al. 2018). Marbled crayfish represent the only known decapod crustacean capable of apomictic parthenogenesis (Scholtz et al. 2003, Seitz et al. 2005). What makes marbled crayfish even more unique is the fact that no native population has been recorded so far (summarized in Chucholl et al. 2012 and citations therein). The obscure origin of marbled crayfish lies in the tanks of traders or breeders of crayfish, and neither behavior nor ecology of the species within invaded lakes is yet understood (Chucholl et al. 2012). The first naturalized marbled crayfish population (i.e., in the field) was reported near Freiburg, Germany, in 2003 (Marten et al. 2004). In recent years, sightings from the Netherlands, Italy, Slovakia, Sweden, and other German lakes followed (see Chucholl et al. 2012 for review). Since these populations stem from marbled crayfish previously reared in aquaria, they can be considered to have been naïve to interspecific competition and predators before they were released. The aquarium origin and the beginning establishment of isogenic populations in pre-invaded lakes provide a unique opportunity to study behavioral mechanisms of species displacement and behavioral flexibility in the natural environment.

Goals and hypotheses

We compared the behavior of marbled and spiny-cheek crayfish to assess competitive interaction strength, flexibility in behavior of an invader, and possible species displacement in crayfish (over-)invasions. Specifically, we assessed interspecific aggressiveness, activity, and boldness of the two focal species. In addition, we compared naïve, aquarium, and naturalized populations of marbled crayfish that are sympatric to spiny-cheek crayfish with each other to elucidate changes in behavior that result from naturalization. Finally, we looked for correlations between aggressiveness and activity, associated with aggression syndromes in individuals of both crayfish species.

We hypothesized that crayfish species differ in behavioral traits that are important for invasion success, for example, agonistic behavior. Resident spiny-cheek crayfish were expected to dominate marbled crayfish because their males have large chelae in contrast to the all-female marbled crayfish. The latter were thought to be more active than spiny-cheek crayfish and respond less appropriately to a threat since they originate from aquaria without natural selection regimes. Marbled crayfish should generally exhibit less variability in behavior since they are isogenic. We further hypothesized that after marbled crayfish came in contact with spiny-cheek crayfish

and predators in a natural environment, they will adapt their behavior. Marbled crayfish from invaded water bodies were expected to be more aggressive than aquarium crayfish to compete and coexist with spiny-cheek crayfish. Finally, marbled crayfish experiencing predation in the field should be less active and more responsive to threats than aquarium marbled crayfish.

Materials and Methods

Study sites

Spiny-cheek crayfish were collected in Lake Müggelsee in front of the institute (52°26′6″ N, 13°38′6″ E), Germany, with crayfish traps (type PIRAT, 610 × 315 × 250 mm, mesh width 40 × 10 mm, Rapurosvo, Parainen, Finland) between April 2015 and June 2016. The traps were baited with dog food or dead fish and were set overnight and checked on the next day. Aquarium stocks of marbled crayfish were provided by Peer Martin (Comparative Zoology, Humboldt University, Berlin, Germany). Additional marbled crayfish that live in sympatry with spiny-cheek crayfish were mostly hand-collected or, to a minor degree, caught by traps in the littoral zone from lakes (1) Moosweiher (48°01′51″ N, 7°48′17″ E) in Baden-Württemberg, Germany, and (2) Krumme Lanke (52°27′0″ N, 13°13′52″ E) in Berlin, Germany. Crayfish were transported in Styrofoam boxes filled with water 30 mm deep and macrophytes in excess.

Maintenance of test animals

All crayfish were sexed and measured manually with a sliding caliper to the nearest millimeter. The length was measured as carapace length (CL) from the tip of the rostrum to the posterior edge of the carapace. Tanks were set up on shelves in a climate chamber with a constant temperature at 17°C under a photoperiod of 14:10 h light:dark. All crayfish were kept in the laboratory for at least one month before being used in experiments. All aquarium marbled crayfish, naturalized marbled crayfish from Lake Krumme Lanke, and all spiny-cheek crayfish used for individual measurements (>3 replicates) in behavioral experiments were kept individually in tanks (300 × 200 × 200 mm) filtered by air-driven sponge filters. Naturalized marbled crayfish from Lake Moosweiher and additional spiny-cheek crayfish that have been used only as opponents in agonistic encounters were marked and housed in filtered single-species community tanks separated by sex (800 × 400 × 200 mm). All housing tanks were filled with 30 mm of fine gravel, and PVC pipes (150 mm, diameter 50 mm) were provided for shelter. Communal tanks were provided with a surplus of shelters (>2 per crayfish) to minimize aggression. To differentiate among the crayfish kept in communal tanks, we used the non-invasive, numerical marking system of Abrahamsson (1965) where crayfish were marked with a point-code on top of their carapace. The crayfish were marked with a white outdoor marker (Edding 8055, Ahrensburg, Germany). After molts, we waited for the exoskeleton to be hardened completely and measured the new length before remarking the animals. Tanks were cleaned once a week and around 75% of water was exchanged with fresh tap water. Individual crayfish were fed half a ring of commercial crayfish food (Crabs natural, sera, Heinsberg, Germany) daily. Dried and blanched oak leaves were provided ad libitum as additional food and environmental enrichment. After the end of the study, crayfish were used for further experiments on their prey choice and feeding mechanics.

The protocol and procedures employed were ethically reviewed and approved by the Landesamt für Gesundheit und Soziales (LAGeSo), Berlin, Germany. All experiments were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Setup and standard procedure

All experiments were conducted in the climate chamber to reduce handling and guarantee minimal disturbance from outside. Two tanks measuring 400 × 400 × 200 mm were set up in the chamber, each filmed by two cameras (one vertically above the tank and another one at the side of the tank). All experiments were recorded or photographed with network cameras (Dinion HP 1080p, Bosch, Stuttgart, Germany) capable of recording under infrared illumination. Videos and photographs were recorded and saved with VLC player (version 2.2.1.0). Each setup was covered with an opaque, black plastic tarpaulin to further minimize disturbances.

All crayfish used for the experiments were in good condition (no obvious diseases, all appendages present and intact). Each crayfish was used only for one experimental trial per day. Intermolt individuals of both male and female sex (22–50 mm CL) were used in experiments. Females carrying eggs or larvae were excluded from experiments up to at least one week after the release of the brood. Test animals were randomly chosen among available crayfish with a pair of ten-sided dice.

In each experiment, the tanks were filled with 20 mm of fine gravel and 150 mm of tap water of 15°C temperature. Crayfish were released into experimental tanks and allowed to acclimatize for 30 min prior to the experiment. After each trial, the tank was completely drained before setting up another experiment to avoid a potential bias by remaining pheromones in the water (Breithaupt 2011).

Allometry

Since larger chelae can be advantageous in agonistic encounters, we measured chela length of the right cheliped (in mm) for a random set of crayfish from three groups: male spiny-cheek crayfish (N = 52), female spiny-cheek crayfish (N = 28), and marbled crayfish (N = 81) with CLs between 20 and 50 mm. We fitted linear regression models (command lm()) in R version 3.4.0 (R Core Team 2017) to predict chela size depending on CL in each of these groups. To test for differences in intercept and slope in the three regression lines, we fitted three models for each pair of two of the three groups accounting for CL, the group, and their interaction.

Aggressiveness

We tested agonistic behavior against size-matched opponents (±1 mm CL) in interspecific encounters of individual spiny-cheek crayfish males (N = 12), spiny-cheek crayfish females (N = 7), aquarium-reared marbled crayfish (N = 14), and naturalized marbled crayfish (N = 13). Three encounters were staged for each individual against three different opponents. The availability of matching pairs was reduced by egg-bearing females and molting individuals and resulted in uneven numbers of replicates. To better discriminate species and size effects, we later staged confrontations of the same individuals with smaller (<4 ± 2 mm CL [mean ± standard deviation, SD]; N = 15 for spiny-cheek crayfish, N = 13 for marbled crayfish) and larger opponents (>4 ± 2 mm CL [mean ± SD]; N = 16 for spiny-cheek crayfish, N = 14 for marbled crayfish; modified from Vorburger and Ribi 1999).

Experimental tanks were separated into two sides with a removable opaque divider (PVC). The corners were rounded with plastic glass to avoid that submissive animals become trapped. For each trial, one crayfish was transferred into each compartment. After acclimatization, the divider was lifted and the encounter recorded on video to later assess and score each interaction. The experiments were conducted in the dark when crayfish are most active and illuminated by infrared headlights (Holdich and Black 2007, Luna et al. 2009).

Each confrontation was recorded with both cameras. The recording time was set at 35 min. The first 30 min after opening the divider was analyzed for agonistic behavior, and 5 min was added as buffering time. If fewer than five interactions took place within the 30 min, the buffering time was checked for more interactions. If there were still fewer than five interactions including the buffering time, the experiment was repeated with another opponent for each crayfish on another day.

To quantify interaction strength during the confrontations, the observed behavior was scored with the system developed by Atema and Voigt (1995; Table 1). For every five-seconds, each member of the pair was assigned an aggression score. The scoring system was modified by giving ignoring, which was not originally included in the system, the score 0. Ignoring was observed when crayfish were within one body length of one another or had physical contact, but did not show any visible response (i.e., taxis) toward the opponent's presence (e.g., crawling along the aquarium pane, crawling over or under the body of the opponent). The opponent could show another agonistic behavior at the same time and was scored, respectively. The term separate includes all situations where the individuals were apart for more than one body length and no score was applied. When more than one agonistic behavior was shown within five-seconds, higher scores outranked lower (positive) scores

Table 1. Definitions of agonistic behaviors observed in crayfish and their designated score (modified from Atema and Voigt 1995).

Score	Behavior	Definition
−2	Fleeing	Walking away (rapidly), walking backward (rapidly), tail-flipping (rapid contraction of the abdomen)
−1	Avoidance	Walking away (slowly), walking backward (slowly), turning away
0	Ignoring	Indifference toward each other within less than one body length, or even in contact
1	No physical contact (initiation)	Facing, approaching, turning toward, following
2	No physical contact (threat display)	High on legs, claw open, meral spread, claw forward, antenna point
3	Physical contact (claws not used to grasp)	Antenna touching, claw touching, claw tapping, claw pushing, antenna whipping, claw boxing, claw scissoring
4	Physical contact (claws used to grasp)	claw lock
5	Unrestrained use of claws	Claw snapping, claw ripping
n/a	separate	Opponents one body length or more apart

(0–5). Score −2 outranked −1 and both flight behaviors (scores −1 and −2) outranked score 0 or positive scores. The interactions ended with one crayfish fleeing or separating itself from the counterpart by more than one body length.

For every individual and confrontation, we counted the total number of each observed score (for all five-second intervals) during the 30 min of confrontation for each crayfish. To see relative frequencies of certain scores among the groups, a standardized count was calculated by adding up the scores for each group and dividing it by the number of tested individuals. For every individual and confrontation, we calculated an aggression score by multiplying each score with the number of observations and adding them up for all behaviors (Karavanich and Atema 1998). We then adjusted the aggression score by dividing it by the number of interactions (5-s intervals) that were observed during 30 min (adj. AS). We did this adjustment to obtain a better measure of average aggression level since the time spent interacting with the other crayfish differed largely between trials. A negative or low aggression score represents a submissive individual or the loser of the encounter, whereas a high value indicates an aggressive individual or the winner of the encounter.

We performed analyses using linear mixed-effects models to detect agonistic score differences between the groups or species with individual as random factor (command lmer() from package lme4 (Bates et al. 2014)). As fixed effects, we used species, CL at the time of the fight (molting and therefore growth can occur between days of the experimental period), origin (aquarium or naturalized, only applicable to marbled crayfish), and sex (only applicable to spiny-cheek crayfish). All possible combinations of fixed effects and interactions between fixed effects were calculated—except between species, origin, and sex as these are confounded. Models were ranked by Akaike's information criterion (AIC) and Akaike's model weight. Marginal (fixed factors only) and conditional (fixed factors and random factor) R^2 values for the best model were calculated using the MuMIn package (Bartón 2013).

Activity

We tested the activity (time spent outside of the shelter) of individual spiny-cheek crayfish males (N = 11), spiny-cheek crayfish females (N = 5), aquarium-reared marbled crayfish (N = 13), and naturalized marbled crayfish (N = 14). Each individual crayfish was tested in three trials. The experimental tanks were filled with gravel 20 mm deep and completely divided by half with an opaque divider (PVC). A PVC pipe (l = 150 mm, diameter 50 mm) in each compartment was provided as shelter. A crayfish was transferred into each compartment. After acclimatization, photographs were taken in the dark under infrared light every 30 min for 6 h, starting 30 min after artificial nightfall. Photographs were later checked for the position of the crayfish in the tank. Crayfish were considered to be outside the shelter when all of the carapace and the pereopods were visible outside the PVC pipe, as viewed from above. We summed up the number of observations outside

the shelter and the number of observations inside the shelter for each trail.

We then applied a generalized linear mixed-effects model (GLMM) for binary responses with R to detect differences in time spent outside and inside the shelter among spiny-cheek crayfish males, spiny-cheek crayfish females, aquarium-reared marbled crayfish, and naturalized marbled crayfish (command glmer; package lme4). The individual was included in the model as random factor. Similar to the aggression scores, we calculated all possible combinations of fixed effects and interactions between fixed effects—except between species, origin, and sex as these are confounded. Models were ranked by AIC and Akaike's model weight, and we calculated marginal and conditional R^2 values for the best model using the MuMIn package (Bartón 2013).

Threat response

The response to a simulated threat as a measure of boldness was tested for spiny-cheek crayfish males (N = 15), spiny-cheek crayfish females (N = 15), aquarium-reared marbled crayfish (N = 13), and naturalized marbled crayfish (N = 19). Individual crayfish were placed in the experimental tank with 20 mm of sand as substrate and allowed to acclimatize. The crayfish were then approached from the upper front, using an angle of ~45°, by the hand of the experimenter in a steady but brisk movement. Threat responses were recorded under dim light conditions from above the tank. Each individual crayfish was tested three times but only once per day. The experiment followed the approach by Pintor et al. (2008), but with a modification since most crayfish in preliminary trials did not show a response to the hand if its movement was stopped above the surface. Thus, the movement of the hand was extended into the water, aiming for the front of the crayfish until a contact would occur. The hand was put through a hole in the tarpaulin when the crayfish was in a suitable position. Before the crayfish was approached, it needed to be at least one body length away from the aquarium pane, so it would not be constrained when displaying a flight reaction. Hands were washed with warm water after each trial to avoid a potential bias by remaining pheromones (Breithaupt 2011).

The first, initial response of the crayfish to the hand was assessed. Crayfish responded either by tail-flipping, that is, shooting backward (flight; score −1); stop moving and ducking (freezing; score 0); or by showing a threat display, that is, lifting their claws (fight; score 1). The scores of the three trials were summed up, and a general response score was given to each individual crayfish. A negative sum resulted in a general flight response, a positive score resulted in a fight response, and a sum of 0 was classified as freeze. We tested for differences between the groups with a chi-square test in R (command chisq.test) with 100,000 bootstrap simulations. We also compared all combinations of groups of crayfish and corrected for multiple testing using the Bonferroni-Holm method. Additionally, all groups of crayfish were checked for potential effects of CL using Spearman rank correlations.

Results

Allometry

Carapace length was a significant predictor of chela length for crayfish from all groups (Appendix S1: Fig. S1). The average chela length was significantly smaller, and the slope was less steep for marbled than for spiny-cheek crayfish males (linear regression, $t = 8.75$, $P < 0.001$ and $t = -13.49$, $P < 0.001$). There were also significant differences in chela length and slope of the regression lines between spiny-cheek crayfish females and males (linear regression, $t = 4.60$, $P < 0.001$ and $t = -7.16$, $P < 0.001$). There was no significant difference in chela size between marbled crayfish and spiny-cheek crayfish females or slope of regression lines (linear regression, $t = 1.55$, $P = 0.12$ and $t = -1.3$, $P = 0.20$).

Aggression

All linear mixed-effects models were sorted according to their delta-AIC value and AIC weights. Models with an AIC weight above 0.05 are presented in Table 2 (see Appendix S1: Table S1 for all models). The best model includes species and individual CL (size) as predictors (marginal $R^2 = 0.16$; conditional $R^2 = 0.31$). All other models with a model weight above 0.05 also include species and size plus either origin, sex, or interaction terms. Species and size thus seem to be the most important predictors for aggressiveness, whereas other factors are less important. Carapace length was positively

Table 2. Linear mixed-effects models of adjusted aggression score (adj. AS) analysis.

Model (fixed effects)	delta-AIC	AIC weight
– species(SC) + size	0	0.221
species(SC) + size – species(SC):size	1.3	0.116
– species(SC) + size + origin(aq)	1.7	0.095
– species(SC) + size – sex(m)	2	0.082
– species(SC) + size + sex(m) – size: sex(m)	2.5	0.063
species(SC) + size + origin(aq) – species(SC):size	2.9	0.053

Notes: Listed are the best models according to Akaike's model weight (Akaike's information criterion [AIC] weight). Indicated positive or negative effects of variables relate to the values of these variables given in brackets (m, male; SC, spiny-cheek crayfish; aq, aquarium origin); these are compared to female marbled crayfish from the field as reference. All models include the individual (IND) as random factor (Adj. AS ~ intercept + fixed effects + (1|IND)).

correlated with adj. AS. We considered individual as random factor in the analyses but found no statistical effect on aggression. In initial exploratory analyses, we also looked for an effect of the day of experiment (1st, 2nd, or 3rd) but did not find such an effect.

The adj. AS of marbled crayfish was on average 0.67 ± 0.16 standard error (SE) higher than the adj. AS of spiny-cheek crayfish (Fig. 1). The results of the mixed-effects models that neither (1) sex nor (2) origin is important predictor of aggression are also illustrated in Fig. 1: adj. AS of (1) male and female spiny-cheek crayfish, and (2) aquarium-reared and naturalized marbled crayfish were similar.

Aggression encounters rarely escalated (scores 4 and 5 were rare; Appendix S1: Fig. S2) and were mostly resolved by claw pushing or boxing (score 3). Marbled crayfish rarely initiated fights with a threat display or responded equally to spiny-cheek threat displays (score 2). Furthermore, marbled crayfish often ignored their opponent (score 0). Higher negative scores indicated that spiny-cheek crayfish lost more encounters than marbled crayfish.

In agonistic encounters against smaller opponents from either sex, marbled crayfish differed significantly from spiny-cheek crayfish and won all interactions, whereas spiny-cheek crayfish lost most interactions (Fisher's exact test, df = 25, $P < 0.001$; Fig. 2). Against larger opponents, marbled crayfish similarly won 64% of

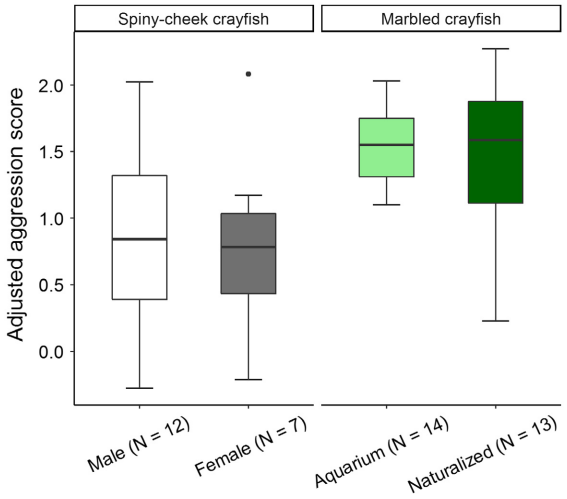

Fig. 1. Adjusted aggression scores in pairwise interspecific interactions among spiny-cheek crayfish males (open boxplot), spiny-cheek crayfish females (gray boxplot), and marbled crayfish from aquaria (light green boxplot) and naturalized populations (dark green boxplot).

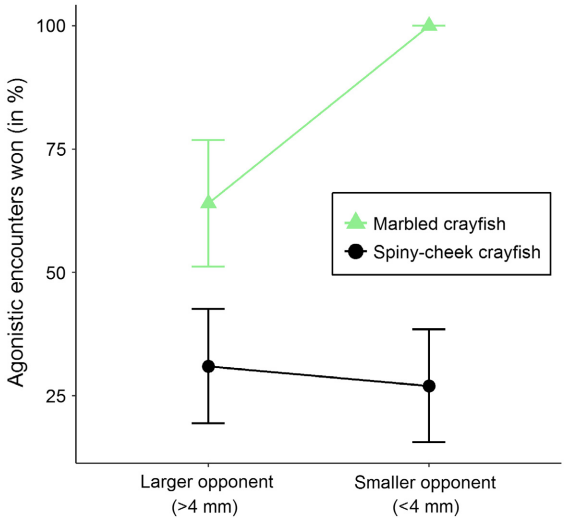

Fig. 2. Agonistic encounters won (in percent ± standard error) by marbled crayfish (triangles, light green) and spiny-cheek crayfish (both sexes; circles, black) with opponents of unequal size of the other species. The left side shows the outcomes against larger opponents (spiny-cheek crayfish, N = 16; marbled crayfish, N = 14) and the right side against smaller opponents (spiny-cheek crayfish, N = 15; marbled crayfish, N = 13).

encounters and spiny-cheek only 31%, but this difference was not statistically significant (Fisher's exact test, df = 28, $P = 0.14$).

Activity

The best model (marginal $R^2 = 0.10$; conditional $R^2 = 0.21$) uses origin and size as predictors: Aquarium marbled crayfish were more active than all other groups (Table 3, Fig. 3; see Appendix S1: Table S2 for all models). Activity was negatively correlated with size for all crayfish. Spiny-cheek crayfish males, spiny-cheek crayfish females, and naturalized marbled crayfish spent more time inside than outside the shelter. All models using other predictors along origin were weaker than the one with origin and size as the sole predictors, and models not accounting for origin were negligible in explanatory power (AIC weights <0.001; Table 3). In conclusion, shelter use did not differ markedly among sex or species, but the rearing environment (origin) and size were meaningful predictors of activity. We considered individuals as random factor, but these had no effect on shelter use. In initial exploratory analyses, we also looked for an effect of the day of experiment but did not find one.

Aggression syndrome

We tested for correlations between activity and aggression. Therefore, we used the means of individual aggression scores and ratios of time spent outside or inside the shelter for all individuals where we had at least three observations for aggression and activity. We calculated separate linear regressions for spiny-cheek crayfish, aquarium marbled crayfish, and naturalized marbled crayfish. Individuals of both sexes of spiny-cheek crayfish have been combined in the analysis since we did not find differences in activity and aggression (see above). Naturalized marbled crayfish and marbled crayfish from aquaria have been tested separately; as they differed in activity (see above).

One aquarium marbled crayfish had only two aggression scores because an interspecific mating took place during the third experiment; thus, the observation was excluded. Also, one naturalized marbled crayfish had only two observations for activity because it was cannibalized during molting before the third experiment could be conducted.

We found that in naturalized marbled crayfish, mean adj. AS (aggression) was positively correlated with the ratio of time spent outside or inside a shelter (activity; Fig. 4). A similar trend was observed for aquarium-reared marbled crayfish, whereas no such correlation was found for spiny-cheek crayfish.

Threat response

Crayfish groups significantly differed in their threat response (Pearson's chi-square test with

Table 3. Generalized linear mixed-effects model results of activity analysis.

Model (fixed effects)	delta-AIC	AIC weight
origin(aq) − size	0.0	0.161
origin(aq)	0.4	0.131
origin(aq) − size − origin(aq):size	1.4	0.079
origin(aq) + sex(m) − size − sex(m):size	1.5	0.077
origin(aq) + sex(m) − size − sex(m):size − origin(aq):size	1.8	0.066
origin(aq) + sex(m) − size	1.9	0.062
origin(aq) − size − species(SC)	2.0	0.060
origin(aq) + sex(m)	2.3	0.051

Notes: Listed are the best models with decreasing Akaike's model weight. Indicated positive or negative effects of variables relate to the values of these variables given in brackets (m, male; SC, spiny-cheek crayfish; aq, aquarium origin); these are compared to female marbled crayfish from the field as reference. All models include the individual (IND) as random factor (ratio of time spent outside/inside the shelter ~ intercept + fixed effects + (1|IND)). AIC, Akaike's information criterion.

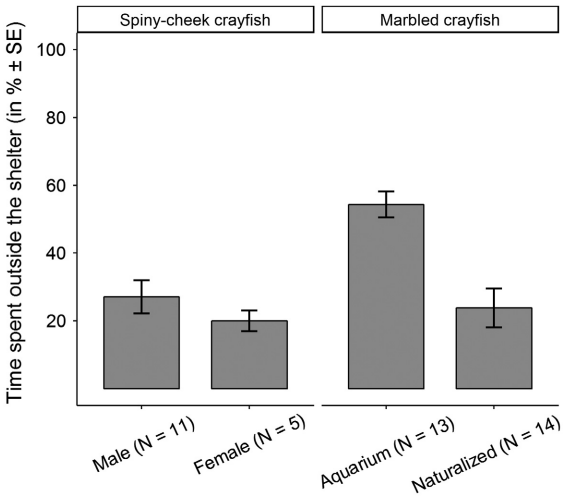

Fig. 3. Percentage of time spent outside the shelter (± standard error) over 6 h for spiny-cheek crayfish males, spiny-cheek crayfish females, and marbled crayfish from aquarium and naturalized populations.

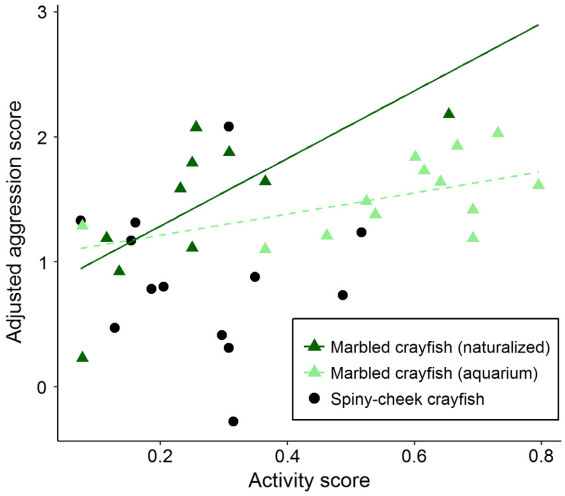

Fig. 4. Correlations between mean activity score (ratio of time spent outside/inside the shelter; ACT) and mean adjusted aggression score (adj. AS) of spiny-cheek and marbled crayfish individuals across experiments. Regression lines: naturalized marbled crayfish, Adj. AS ~0.75 + 2.7·ACT (linear regression, t = 3.09, P = 0.015, adj. R^2 = 0.49); aquarium marbled crayfish, Adj. AS ~1.05 + 0.85·ACT (linear regression, t = 2.1, P = 0.06, adj. R^2 = 0.22). No line is shown for spiny-cheek crayfish, as no trend was observed, Adj. AS: ~0.97 – 0.39·ACT; linear regression: t = −0.295, P = 0.77, adj. R^2 = 0.08).

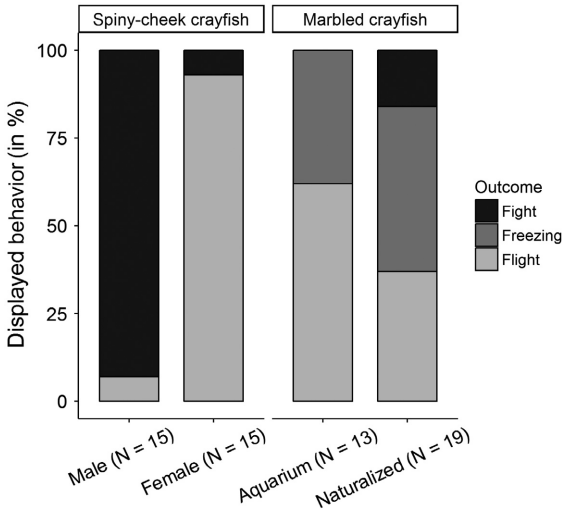

Fig. 5. The percentage of displayed behaviors in response to a simulated threat for spiny-cheek crayfish of either sex and marbled crayfish from aquarium and naturalized populations. The behaviors displayed encompass aggressive behavior (dark gray bars), freezing (gray bars), or flight behavior (light gray bars).

simulated *P*-value, based on 100,000 replicates, χ^2 = 54.91, $P < 0.001$, Fig. 5, Table 4). The CL was not related to threat response in any of the groups (spiny-cheek crayfish [male], $r_S = -0.046$, $P = 0.87$; spiny-cheek crayfish [female], $r_S = 0.149$, $P = 0.60$; marbled crayfish [aquarium], $r_S = -0.321$, $P = 0.29$; marbled crayfish [naturalized], $r_S = 0.217$, $P = 0.40$). Remarkably, marbled crayfish frequently stopped and ducked in response to the approaching threat (37% or 47% for aquarium or naturalized marbled crayfish, respectively), whereas spiny-cheek crayfish did not show such behavior. Male spiny-cheek crayfish mainly responded with aggression (93% of all trials), whereas females mostly displayed flight behavior (93%). If not freezing in response to a threat, marbled crayfish most often fled from the threat (62% or 37% for aquarium or naturalized marbled crayfish, respectively). Aquarium marbled crayfish did not fight, whereas naturalized marbled crayfish showed fight behavior in about 15% of the trials.

Table 4. Chi-square statistics (χ^2) of all pairwise comparisons between groups of crayfish and among all groups tested for their threat response.

Comparison	χ^2	*P*
Spiny-cheek crayfish (female) vs. spiny-cheek crayfish (male)	19.29	<0.001
Marbled crayfish (naturalized) vs. marbled crayfish (aquarium)	3.20	0.21
Spiny-cheek crayfish vs. marbled crayfish (species)	21.96	<0.001
Marbled crayfish (aquarium) vs. all naturalized crayfish (origin)	7.20	0.055
All-female crayfish vs. male spiny-cheek crayfish (sex)	35.81	<0.001
Comparison between all groups	50.44	<0.001

Note: *P*-values are corrected for multiple testing using the Bonferroni-Holm method.

Discussion

Differences between species and implications

Our results illustrate some of the key behavioral characteristics in invasion success of crayfish. In the over-invasion scenario we investigated, the recently invading marbled crayfish were able to dominate resident spiny-cheek crayfish of either sex in agonistic encounters even if their opponents were larger and had larger claws.

Aggressiveness has been one of the main behavioral traits associated with species displacement in crayfish (Capelli and Munjal 1982, Usio et al. 2001). So far, interspecific aggression has only been tested for juveniles of marbled crayfish interacting with red swamp crayfish (*Procambarus clarkii*), which were similarly aggressive (Jimenez and Faulkes 2011). As we demonstrated here, the lack of sexual dimorphism does not constrain the ability of marbled crayfish to win agonistic encounters against another species. Aggressive dominance also translates to superiority in competition over shelters, an important resource for crayfish that relieves them from predation pressure (Gherardi and Daniels 2004, Moore 2007). Thus, we assume that preferred resources of spiny-cheek crayfish like shelters would be frequently occupied by invading marbled crayfish where both species co-occur.

In former invasions of North American species across Europe, interspecific competition between crayfish was mostly no relevant determinant of invasion success because the crayfish plague (*Aphanomyces astaci*, Leptolegniaceae) often completely eradicated native competitors before or shortly after introduction of non-native crayfish (Gherardi and Holdich 1999). The die-off of potential competitors and its high tolerance toward poor habitat quality probably had a major effect on the former success of spiny-cheek crayfish. There is surprisingly little work on the competitive ability of spiny-cheek crayfish in contrast to other major invasive crayfish in Europe like the red swamp crayfish or the signal crayfish (*Pacifastacus leniusculus*). In the few available studies on spiny-cheek crayfish aggression, they were usually inferior in agonistic encounters (either in their native range against an invader or against another invader in their introduced range (Klocker and Strayer 2004, Hudina et al. 2011). It should be noted that the average aggression level of the tested spiny-cheek crayfish might be even lower since they were caught with traps, which can select for aggressive individuals (Ogle and Kret 2008).

In the invasion scenarios we are facing today, crayfish plague-resistant species over-invade other plague-resistant species, and the traits and interactions with the community will become paramount for distribution and impacts of crayfish (Russell et al. 2014, James et al. 2016). In general, species distributions and impacts in novel communities and ecosystems can probably be better understood when considering the time of introduction of species rather than simply dividing species into native and non-native ones. The latter, dichotomous classification is often based on a reference year, for example, 1492 which is sometimes rounded to 1500 (DAISIE 2009): Species present before this year are considered native, and species introduced thereafter are considered non-native. A finer approach considering the time of introduction and the eco-evolutionary experience of introduced and resident species (Saul and Jeschke 2015) seems to be a promising way forward.

Behavioral syndromes

We also explored the flexibility and the correlations among behaviors (i.e., behavioral syndromes). We observed two traits in particular that are beneficial during the introduction and spread of species: Higher aggression jointly with higher activity was observed in marbled as compared to spiny-cheek crayfish. Positive correlations of aggressiveness and activity have been referred to as so-called aggression syndromes in invasive species (Sih et al. 2004, Pintor et al. 2009). Our results suggest that marbled crayfish exhibit such an aggression syndrome which can lead to more agonistic encounters, but may also be positively related to attacks on prey, that is, increased foraging rate (Sih et al. 2004, Sih and Bell 2008, Pintor et al. 2009). Both would facilitate species displacement through either interspecific aggression or competition for resources.

Thus far, marbled crayfish have not outcompeted spiny-cheek crayfish in water bodies where both species co-occur (Chucholl and Pfeiffer 2010). Trade-offs associated with the aggression syndrome, for example, higher intraspecific aggression, might limit the success of marbled crayfish. Elevated intraspecific aggression levels might, for example, constrain marbled crayfish densities. We did not include intraspecific aggression in our study design, but from our observations in communal tanks, we suspect intraspecific aggression to be low. High genetic relatedness has been shown to lower intraspecific aggression in insects (Carazo et al. 2014, Jandt et al. 2014), but marbled crayfish also form dominance hierarchies (Luna et al. 2009).

Marbled crayfish might also suffer higher predation rates despite similar activity levels because their antipredator behavior (i.e., threat response) is not appropriate or their morphology makes them easier to attack. After handling both species for years, we have the impression that spiny-cheek crayfish have a thinner carapace and they have, as their name implies, spines in contrast to marbled crayfish. To our knowledge, data on exoskeleton thickness are not available in the literature for either species. We also noted that, if lifted up, spiny-cheek crayfish pull their legs together beneath the carapace and the abdomen to form a spiny ball that is difficult to swallow for gape-limited predators like fish. The importance of the aggression syndrome for population dynamics and invasion success should therefore be examined in relation to predators foraging on marbled crayfish (Pintor et al. 2009).

Species displacement in crayfish can take decades, as a long-term study on a Finnish lake has demonstrated (Westman et al. 2002). Higher reproduction rates, activity, and aggressive behavior were suspected to promote the displacement of noble crayfish (*Astacus astacus*) by plague-free signal crayfish in the Finnish lake, but the mechanisms of displacement have remained unclear.

Individual differences in behavior are often linked to variation in life-history parameters and morphology. Biro et al. (2014) found that individual differences in life-history and behavior of common yabby (*Cherax destructor*) express very early in life, and variation might arise primarily from genetic or permanent environmental effects. The limited genetic diversity of marbled crayfish, however, should not allow for significant effects on variability in behavior. Permanent environmental effects such as maternal effects, epigenetic effects, and other effects that influence development already before hatching can be the cause for this variation (Dochtermann et al. 2015). For example, clonal Amazon mollies (*Poecilia formosa*) consistently showed individual variation in behavior among isogenic individuals reared in isolation, and social experience during ontogeny had no effect on individual behavioral variation (Bierbach et al. 2017). In marbled crayfish, developmental variation probably explains much of the variation in coloration, growth, lifespan, reproduction, number of sense organs, and behavior, even when they are reared under identical conditions (Vogt et al. 2008). The emergence of personality and its genetic basis are yet barely understood, and studies on isogenic marbled crayfish might help deepen our understanding.

Will these behaviors promote marbled crayfish invasions?

We presented evidence that marbled crayfish are more aggressive and active competitors than spiny-cheek crayfish. Risk assessments confirm that marbled crayfish have many traits promoting high invasiveness (Twardochleb et al. 2013, Chucholl and Wendler 2017). For example, marbled crayfish cope well with low water temperatures despite their origin in warm-water aquaria (Veselý et al. 2015). High aggression and activity together with high potential population growth rates make marbled crayfish exemplary for a fast pace-of-life species (Réale et al. 2010). Marbled crayfish have a higher reproductive potential than most other crayfish, as they lay more clutches and are not bound to mating seasons due to parthenogenesis (Scholtz et al. 2003, Souty-Grosset et al. 2006). By parthenogenetic reproduction, marbled crayfish overcome many challenges that invasive species face after introduction. For example, small founder populations of marbled crayfish should not be impaired by failing to recognize conspecifics or mate choice (Chapple et al. 2012). A single marbled crayfish is sufficient to establish a population. However, parthenogenetic reproduction also reduces adaptability to cope with parasites or changes in the environment.

Predation by native predators, for example, might limit the spread of marbled crayfish. The response to threat or boldness that we observed in marbled crayfish differs from many other crayfish species. We expected marbled crayfish to respond inappropriately to a threat because organisms from the pet trade should be naïve to threats. Fight-or-flight behavior is most often observed in crayfish as appropriate responses to predation threats (Stein and Magnuson 1976). However, marbled crayfish ducked or seemed to freeze before the approaching hand. We tried to minimize contacts with the crayfish during cleaning or feeding and never approached them upfront, but their aquarium legacy might have made them more used to handling. A comparable antipredator behavior was found in New Zealand big-handed crabs (*Heterozius rotundifrons*) that

remained immobile when an enemy approached them (Hazlett and Mclay 2005). Marbled crayfish have the eponymous marbled pattern and might rely more on their camouflage, like it was reported for invasive green crabs (*Carcinus maenas*; Lohrer and Whitlatch 2002). The camouflage made marbled crayfish less conspicuous than spiny-cheek crayfish when we caught them in the lakes. We also observed freezing when we approached marbled crayfish in the lakes in a brisk and steady movement, but they still tail-flipped when the movement was more sudden.

Also, chemical stimuli might have been more important for marbled crayfish to elicit tail flips. For example, northern-clearwater crayfish (*Orconectes propinquus*) showed a stronger tail-flip behavior when chemical and tactile cues were presented simultaneously (Bouwma and Hazlett 2001). We can only speculate whether natural enemies like birds or fish are faced in an effective way. Active predators that can detect the crayfish might prey more heavily on marbled crayfish than passive predators that rely on movement of their prey. Studying predator–prey interactions with natural enemies would help to shed light on these questions and could explain population dynamics in invaded lakes.

In the direct agonistic interactions, marbled crayfish sometimes did not react to the threat display of spiny-cheek crayfish and simply ignored them. Ignoring behavior of competitors or predators is rarely included in behavioral studies on crayfish (Bergman and Moore 2003). However, ignoring was found to be pronounced in marbled crayfish. It might be related to problems in sensing signals of the opponent. Chemical communication via the urine plays an important role in intraspecific recognition and social dominance in crayfish (Breithaupt 2011); agonistic interactions last longer when chemical cues are absent (Zulandt Schneider et al. 2001). Both species are part of the same family (Cambaridae), but they are relatively distantly related (Martin et al. 2010). We speculate that marbled crayfish cannot recognize signals of the opponent and engage more strongly in agonistic interactions.

Aquarium vs. naturalized populations of marbled crayfish

We compared a naturalized and an aquarium population of marbled crayfish to look for changes in behavior. In contrast to our predictions, no differences were found in aggressiveness, and small differences in boldness toward a threat. As marbled crayfish are generally more aggressive than spiny-cheek crayfish, they do not have to elevate aggressiveness in sympatry to better compete in agonistic encounters. By contrast, resident spiny-cheek crayfish that live in sympatry with marbled crayfish might have adapted their aggressiveness. For example, native populations of virile crayfish (*Orconectes virilis*) have been shown to be more aggressive when they had prior experience with invading rusty crayfish (*Orconectes rusticus*; Hayes et al. 2009). Additional trials with sympatric spiny-cheek crayfish, which we did not test, might show more elevated aggression levels in these populations. Hayes et al. (2009) asked whether behavioral flexibility or evolution of genotypes in naïve and experienced populations underlie this difference. Marbled crayfish are genetically uniform due to parthenogenesis (see Martin et al. 2007), and behavioral differences should therefore be mostly linked to behavioral flexibility or learning, respectively. However, the correlation of activity and aggressiveness was not decoupled by adapting a lower activity in naturalized marbled crayfish, but instead persisted on a different level. Some individuals seem to be generally more aggressive and active than others, but are still flexible enough to adapt their activity to different environments.

Naturalized marbled crayfish had a lower activity level than aquarium animals and mimicked the activity levels of spiny-cheek crayfish. Rearing conditions in early juvenile stages might have had an influence on their activity. However, the marbled crayfish from the aquarium were reared under similar, stable laboratory conditions. In the critical phase after introduction of a new species, flexibility in behavior is crucial for survival and helps to overcome the problem of small propagule size (Sagata and Lester 2009). Invaders often lack experience in ecological interactions with competitors, prey, and predators (Saul and Jeschke 2015). Invasive signal crayfish, for example, reduced shelter use and increased their foraging activity despite the presence of predator cues (Hirvonen et al. 2007). Behavioral flexibility can counteract potentially maladaptive responses (Wright et al. 2010). The lower activity

in naturalized marbled crayfish could be a response to predation. For example, European eels (*Anguilla anguilla*) reduced foraging activity in invasive red swamp crayfish (Aquiloni et al. 2010). In a recent study on rusty crayfish, Reisinger et al. (2017) found that prior experience had a strong effect on activity (i.e., time spent walking or feeding) in the presence of predatory smallmouth bass, but not when predators were absent (*Micropterus dolomieu*). However, they also found that crayfish raised with predatory fish exhibited reduced activity levels in general. We found activity to be lower in experienced individuals even in the absence of predators, which can be attributed to a high capacity of flexible behavior and a notable memory capacity. Invasive crayfish and crabs are behaviorally flexible and able to learn and memorize new predation cues quickly (Hazlett et al. 2002, Roudez et al. 2008). Leaving the shelter to forage is very risky in an environment with predators. Naturalized individuals that have experienced predation seem to have adapted their activity and memorized predation threat also under safe laboratory conditions.

Conclusions

Ecological consequences of over-invasions, specifically the interactions of invaders with other invaders in the community, are largely unknown (Russell et al. 2014). The recent success of marbled crayfish in establishing new populations might be influenced by their superiority in agonistic encounters and their behavioral flexibility. Marbled crayfish seem to be very adaptive and have the potential to competitively exclude or coexist with the most common invasive crayfish in Central Europe when competing for limited resources. Furthermore, experience with natural conditions can reduce activity of invasive crayfish. Marbled crayfish that originated in the aquarium trade showed that they adapt their behavior to the new environment. This trade-off between foraging and defense might, however, limit the impact of marbled crayfish. Behavioral syndromes in marbled crayfish can occur despite genetic uniformity and thus should stem from permanent environmental effects. Our results from the laboratory explain important behavioral mechanisms behind crayfish over-invasions and reveal large behavioral variability in an isogenic crayfish. To predict invasion success and assess ecological risks in nature, the species' reproductive biology, feeding behavior, and predator–prey relationships in the community should be considered. Marbled crayfish (and spiny-cheek crayfish) have been listed in the new EU regulation on invasive alien species (No 1143/2014). This regulation lays the foundation for the prevention of further spread and future introductions of non-native crayfish.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2385/full