RESEARCH ARTICLE

Potential impacts of invasive crayfish on native benthic fish: shelter use and agonistic behaviour

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Abstract

Spinycheek crayfish (*Faxonius limosus*) and signal crayfish (*Pacifastacus leniusculus*) are successful North American invasive crayfish species distributed throughout Europe. Both species compete with native benthic fish for shelter. In a laboratory approach, we assessed competition for shelter and antagonistic interactions between these invasive crayfish species and the native benthic fish species, stone loach (*Barbatula barbatula*) and bullhead (*Cottus gobio*). This allows for studying the potential impacts of invasive crayfish on native benthic fish. Spinycheek crayfish and signal crayfish were able to gain control of the shelter and could successfully displace both benthic fish species. For stone loach, the presence of crayfish significantly decreased their shelter use and caused several behavioural changes such as reduced activity and increased hiding behaviour outside the shelter. Although the shelter use by bullheads was not reduced, they displayed similar behavioural changes, if less intense. Invasive crayfish species showed remarkable combative interactions against both species of benthic fishes, evidenced by the high number of aggressive interactions, especially concerning stone loach. Our results highlight the pronounced dominance of invasive crayfish over benthic fish in terms of shelter competition and aggressive interactions under laboratory conditions, which consequently might promote the latter's exposure to predation.

Keywords

Introduction

Introducing non-native species into a new habitat exhibits wide-ranging ecological impacts on native taxa, and thereby can globally threaten biodiversity to the entire ecosystem functioning (Carbonell et al. 2017; Flood et al. 2020; Mathers et al. 2020). Invaders are considered the second cause of biodiversity loss after the loss of habitat, particularly in freshwater ecosystems (Mack et al. 2000; Sala et al. 2000; Simon and Townsend 2003; Thomaz et al. 2015) because non-native species can displace native species, resulting in local extinctions (Vitousek et al. 1997; Mooney and Cleland 2001). Invasive species are responsible for the extinction of 34 species and partly responsible for 91 extinctions, causing severe changes in the structure and functioning of the invaded ecosystem (Parker et al. 1999; Clavero and García-Berthou 2005; Walther et al. 2009; Blackburn et al. 2011; Teixeira and Creed 2020).

Crayfish are among the most widely imported exotic freshwater taxa which are introduced to the environment by bait-bucket releases, intentional introduction to support fisheries, or release after educational use (Hobbs et al. 1989; Gherardi 2010). Non-native crayfish act as ecosystem engineers and represent a major threat to freshwater biodiversity, causing potential consequences across all levels of the ecosystem. Crayfish are omnivores, therefore they can affect both primary and secondary producers (Lodge et al. 2000; Perry et al. 2001). Population declines and extinctions of native species are among the most alarming outcomes of crayfish invasion (Lodge et al. 2000).

In direct contests between two competitors, morphological and physiological traits determining the potential to win these contests are defined as an individual's resource-holding potential (RHP) (Parker 1974). RHP of non-native crayfish as well as robustness in agonistic interactions restrict the access of native competitors to food resources (Gherardi and Cioni 2004; Larson and Magoulick 2009) and shelter (Gherardi and Cioni 2004; Nakata and Goshima 2006). Such exclusion from the use of limited resources can negatively impact the growth rates of native species (Pintor et al. 2008; Gherardi et al. 2011) and promote their vulnerability to predators (Garvey and Stein 1993; Mather and Stein 1993), thereby driving their decline (Capelli and Munjal 1982; Usio et al. 2001). Crayfish predation drives declines in diversity and abundances of native invertebrates (Mccarthy et al. 2006; Correia and Anastacio 2008; Mathers et al. 2018; Mathers et al. 2020) and reduces amphibian populations through direct predation on their eggs and larvae (Gamradt and Kats 1996; Gamradt et al. 1997). Accordingly, crayfish invasions resulted in fish declines through direct predation, shelter competition, and indirect competition for preys (Reynolds 2011). For several benthic species including crayfish, shelter availability is a critical factor for survival, allowing them to avoid predation by terrestrial mammals, birds, fish, or other crayfish species (Olsen 1973; Eggleston and Lipcius 1992; Hill and Lodge 1994; Lodge 1994; Englund 1999; Englund and Krupa 2000; Usio and Townsend 2000; Heinsohn et al. 2003; Almany 2004).

Two successful and important North American crayfish species invading Europe are the spinycheek crayfish (*Faxonius limosus*) and signal crayfish (*Pacifastacus leniusculus*).

Spinycheek crayfish was first introduced by the end of the nineteenth century, while signal crayfish was first introduced to Europe in the 1960s (Holdich 2002). Both species share some typical life-history traits of invaders such as fast growth, high fecundity, and early maturation (Souty-Grosset et al. 2006), which contribute to their invasion success. They also displayed a high reliance on shelters, potentially competing with the native benthic fish within European streams.

To better assess the potential consequences of crayfish invasion, this study was devoted to highlighting the competition for shelter between the invasive crayfish species, spinycheek crayfish and signal crayfish, and the native benthic fish species, *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead). Stone loach, bullhead, and crayfish species are similar in their ecological tolerances and habitat requirements. They are primarily nocturnal organisms that hide within shelters of rock crevices in streams and rivers (Miller 1984; Hill and Lodge 1994; Musil et al. 2010; Grabowska et al. 2016; Błońska et al. 2017). Due to the obvious spatial and temporal overlap in their shelter needs, it is anticipated that intense competition for shelter will occur, particularly during daylight hours. We, therefore, tested whether invasive crayfish would dominate benthic fish species concerning shelter use and if they express aggressive behaviour, thereby leading to behavioural changes in the benthic fish.

Materials and methods

Sampling and keeping

Crayfish and benthic fish specimens needed for laboratory behavioural studies were sampled between May 2018 and September 2019. We collected spinycheek crayfish from the right tributary (Nidda) in Schotten (50°28'N, 9°6'E) and the River Moselle near Koblenz, Germany (50°21'N, 7°36'E), while signal crayfish were sampled from the hyporhithral zone of the small river (Wied) near Neustadt (50°35'N, 7°26'E). All these sites are identified as invasion core areas. Stone loaches and bullheads were collected from a small gravel-bed Nister river (50°43'N, 7°44'E), where crayfish were observed but not abundant. All animals were active and used only once during the experimental procedures. Crayfish with missing or regenerating chelipeds or displayed moulting signs or had incomplete hardening were not considered for the experimental procedures. Crayfish were gathered using plastic rounded crayfish traps (60L × 26W × 24H cm) equipped with fish baits and dried dog food. These traps were set up along the shore overnight (ca. 0.5 m to 1 m depth). Benthic fish were collected by electrofishing (DK300, Brettschneider, Germany) with 350V DC and 4A. All animals were transported to the laboratory at Koblenz University. Crayfish were transported in a big dark container (66 L) lined with wet straw to avoid aggressive contact while benthic fish were transported in a 500 L container with aerated stream water. In the laboratory, 40 spinycheek crayfish and 40 signal crayfish were housed in plastic boxes (60 × 40 × 20 cm) in stocks, not more than three animals in one box (separated

by sex and species). Having benthic fishes collected one species at a time, 30 benthic fish were housed in two glass tanks (120 × 50 × 50 cm). All tanks and boxes were filled with dechlorinated tap water, contained a layer of gravel, and were equipped with PVC tube shelters (5 cm diameter and 15 cm length) more than the number of animals to avoid competition for shelter. All animals were kept under controlled conditions (light regime 16:8 L:D, water temperature 20.7 °C to 22.4 °C, pH 8.7–9.0). Benthic fish were fed frozen chironomid larvae whereas crayfish were fed on crabs' food JBL Novo Crabs tablets twice per week (food supplements with similar ingredients from other providers should be useful as well). Before the start of the experiments, all animals were acclimatised to lab conditions for seven days at least before starting the experiment. Body mass, standard length of benthic fishes, crayfish carapace length, and crayfish sex were recorded before each experiment (Table 1).

Behavioural experiments

In the shelter competition experiment, we performed 60 experiments with each benthic fish species (stone loach or bullhead), 20 trials with spinycheek crayfish and benthic fish species, and 20 trials with signal crayfish and benthic fish species. Furthermore, 20 control trials were performed with benthic fish only to observe their shelter use. For better observation, we built up 6 transparent glass aquaria (65 cm × 50 cm × 50 cm) with the same conditions in the acclimatisation tanks, providing only a single shelter in each trial (Fig. 1). All behaviours regarding the shelter and against the opponent were videotaped for 14h (6h during daytime, and 8h during night-time) using Raspberry Pi3 Model B. During the dark period, aquaria were lighted by infrared LED spots (Synergy 21, 10W SECURITY LINE, 850 nm). Each experimental trial started with introducing one species of benthic fish to the experimental arena and allowing them to acclimatise for 10h. Thereafter, one crayfish species was added to the experimental arena for 2h before starting the experiment to acclimatise.

For 14h, time spent inside the shelter by crayfish and benthic fish species was recorded during day and nighttime. Shelter occupation was considered when more than 50% of the animal's body was inside the shelter. Shelter status (occupied or not) was observed before the individual entered the shelter. If one species occupied the shelter and the other one entered the shelter, the reaction of the inhabitant (stayed inside, moved away, or evicted from the shelter) was recorded. The reaction of benthic fish (moving or not) to an approaching crayfish outside the shelter was also recorded. Meanwhile, the contest between the experimental species represented by crayfish aggressive movements and the retreating behaviour of the benthic fish species was also recorded. We noted: (1) the frequency of aggressive actions performed, (2) the type of aggressive and retreating behaviours observed which was then used to generate a behavioural intensity score (see Table 2), and (3) the duration of all contests during the observation period. Animals' behaviour was recorded every 30 min for 14h. An aggression score was assigned to each frame, using the score index from (Gherardi et al. 2013; Dalosto et al. 2015; Lopez et al. 2019) adapted to our species (Table 2).
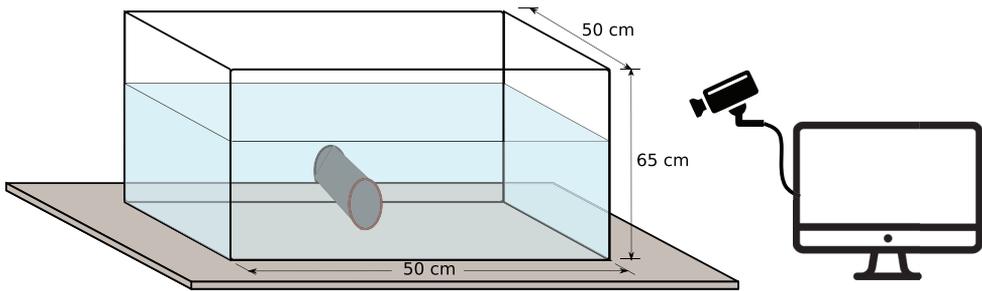
Figure 1. Model representing the experimental tank setup, showing the shelter position in the middle of the tank and the camera for recording the animal behaviour in front of the tank which is connected to a PC to observe and download the videos after recording.

Table 1. Mean (±SD) morphometric values of body mass and standard length (SL) of the native benthic fish stone loach (*Barbatula barbatula*) and bullhead (*Cottus gobio*) as well as body mass, carapace length (CL), and the number of males and females used in the experiment (*N* = 20 pairs) of the invasive crayfish species spinycheek crayfish (*Faxonius limosus*) and signal crayfish (*Pacifastacus leniusculus*).

	Native benthic fish		Invasive crayfish				
Experiment	Body mass (g)	SL (cm)	Species	Body mass (g)	CL (cm)	Male	Female
Stone Control	2.40±0.59	5.4±0.56					
Stone loach + Spinycheek	3.10±1.10	5.6±0.58	Spinycheek	21.9±6.10	4.18±0.48	6	14
Stone loach + Signal	3.00±0.80	5.6±0.70	Signal	28.1±4.85	4.70±0.50	7	13
Bullhead Control	5.25±2.33	5.5±0.90					
Bullhead + Spinycheek	5.75±1.94	6.0±0.65	Spinycheek	29.4±7.96	4.60±0.61	11	9
Bullhead + Signal	7.00±2.70	6.3±0.94	Signal	32.0±6.60	4.70±0.46	14	6

Table 2. Ethogram of the behaviours observed in this study and the associated intensity score based on previous assessments of crayfish contests (Gherardi et al. 2013; Dalosto et al. 2015).

	Behavior	Description	Score
	Non-aggressive interactions	An approach without any agonistic reactions	0
Aggressive interactions	Antenna wave	The antennae of crayfish are whipped rapidly over the opponent.	+1
	Cheliped touching	Aggression with closed chelae: touching and pushing the opponent.	+2
	Cheliped half raise	The spreading and half raising of the chelipeds while facing an opponent.	+3
	Cheliped full raise	The spreading and full raising of the chelipeds while facing an opponent.	+4
	Grappling and pull	Intense combat: animals performing several agonistic acts simultaneously, trying to grab and pull the opponent's body. Kill the opponent.	+5

Data analysis

During video analysis, different behaviours were noted: (1) shelter occupation (measured as time spent inside the shelter), (2) shelter status (occupied or empty), and (3) agonistic behaviour. To do so, we developed a multi-object tracking algorithm to track the movement of the fish and the crayfish inside the experimental tank in the recorded videos. This algorithm was inspired by the procedures used by (Amen et al. 2020). The

basic idea of the algorithm was to compare each frame of the video with a background image, where no moving objects exist, to identify the pixels which undergo a colour change. Those pixels describe the exact location of the moving objects in the respective frame. The video-tracking algorithm was coded in Matlab (R2018b, MathWorks Inc., Natick, MA, USA). This routine was consistently validated for each video by comparing the actual locations with the predicted locations of the fish and the crayfish for randomly selected frames (1000 frames) to confirm its accuracy. For each video, we calculated a hit-rate value based on the following equation:

$$q = \frac{100}{n} \sum_{i=1}^{n} Ni \tag{1}$$

where q is the hit-rate in percent, n is the number of frames (1000 frames), and N is the hit value (1 for a correct prediction and 0 for an incorrect one). We accepted the automated process only for q values higher than 95%. Otherwise, we manually set the fish and the crayfish locations in each frame of the videos which scored q values less than 95%. We further processed the outputs of this routine, i.e., the generated spatio-temporal database of the moving objects (fish and crayfish), to observe the shelter occupation time, covered distance, and activity of the fish and the crayfish inside the experimental tank. For visualization, a spatial heatmap was used to show how the species locations are clustered or vary over space in the experimental tank. The colour variation represents the intensity of species locations in a 2-D form. Heatmaps were used also to measure the habitat proximity relative to the shelter, as a focal patch, to infer the potential for animal movement close to the shelter. The index of habitat proximity, H_x, is calculated, following (Winfree et al. 2005), using the weighted mean patch area for all the patches in the heatmap as follows:

$$H_x = \frac{\sum_{i=1}^{n} A_i e^{-d_i/D}}{\sum_{i=1}^{n} e^{-d_i/D}} \tag{2}$$

where A_i is the area of patch i, d_i is the distance between the shelter and patch i considered overall patches in the system, and D is a mobility constant scaled to the fish (diagonal of the tank). High H_x values indicate the high proximity of a fish to the shelter and vice versa.

The activity of the fish is calculated, following (MacLean et al. 1982), using the activity index (AI) to characterize the covered distance and speed of the fish. The maximum expected distance (d_m) and speed (s_m) were used to scale the distance and speed of the fish. We rewrote the original AI equation suggested by (MacLean et al. 1982) in the integral form so that it matches the spatiotemporal database. Therefore, AI was calculated as follows:

$$AI = \int_{t=t_1}^{t=t_2}(d/d_m)(s/s_m)^{0.5}dt \qquad (3)$$

where t_1 and t_2 are the starting and ending times of the required period of fish activity. To calculate all these quantities, we used a Matlab post-processing script, which was designed specifically for this study.

To assess the impact of crayfish species on benthic fish species, we compared shelter occupation time as an independent variable between experimental groups using R software (R 4.2.1). In this study, shelter occupation time was non-normal distributed, over-dispersed (variance of the distribution greater than mean), and contained excessive zeroes. Consequently, a zero-inflated regression model (with Poisson errors and negative binomial errors) was performed by applying the "zeroinfl" function in the "pscl" package (Jackman 2012). To assess the difference in shelter status (occupied or unoccupied) between benthic fish and crayfish species prior to entering the shelter, we employed the McNemar Test.

To determine which benthic fish species faced more aggressive behaviour from the two crayfish species, and which crayfish species exhibited the most aggressive actions towards benthic fish, we transformed our non-normal distributed data in terms of aggressive movement scores and durations. Data from spinycheek crayfish and signal crayfish towards stone loach and bullhead were transformed using arcsine-square root. Afterwards, two-way ANOVA was performed to compare the score and the duration of aggressive movements in the different groups. Accordingly, two-way ANOVA was used to evaluate the influence of crayfish sexes on the score of aggressive movements towards stone loach and bullhead. Since the transformation to achieve the assumptions of normal distribution and homogeneity of variances was not possible, the Mann-Whitney test was used to compare the frequency of aggressive acts from both crayfish species (Table 2). In addition, a spearman correlation was computed to determine the relationship between the frequency of aggressive movements, crayfish carapace length, chela length, crayfish body mass, benthic fish body mass, and benthic fish standard length in each benthic fish group. These statistical analyses were performed in SPSS version 26.

Results

Shelter occupation and habitat use

The presence of invasive crayfish species significantly affected the shelter occupation time of both benthic fish species. Stone loaches reduced the time spent within the shelter in response to both crayfish species. This reduction was more intense in the case of spinycheek crayfish during the daytime, resulting in a significant interaction term (Table 3, Fig. 2a, b). In the case of bullheads, the time spent in the shelter was
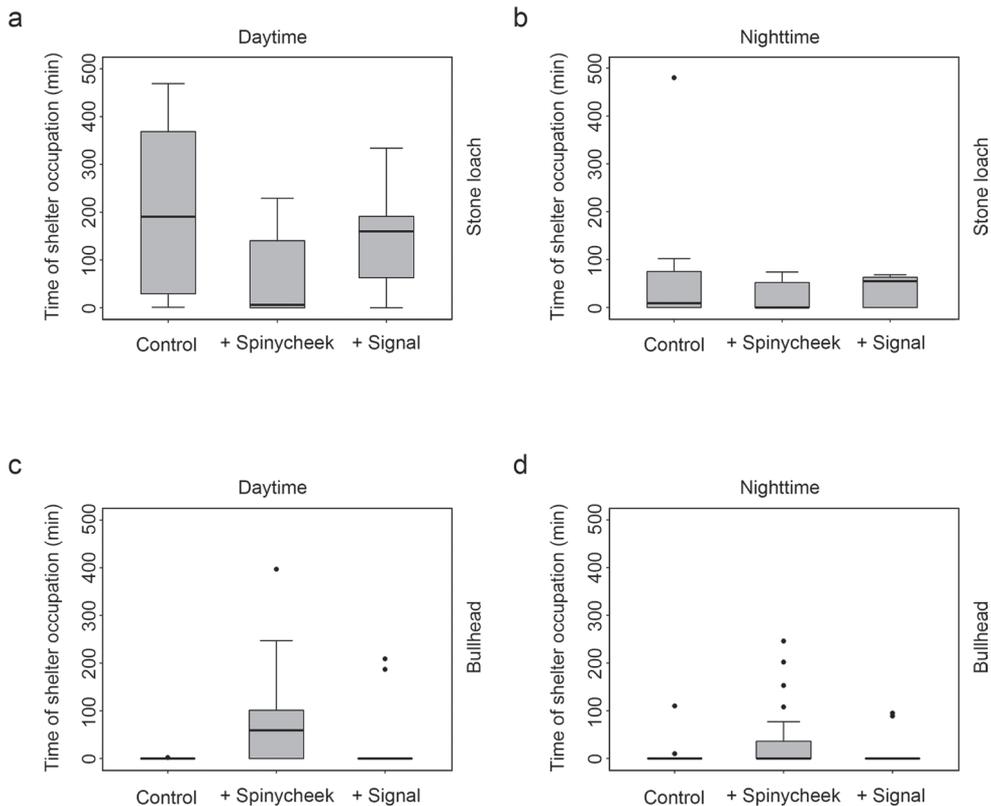
Figure 2. Box plots of shelter occupation time of the benthic fish species stone loach (**a, b**) and bullhead (**c, d**), kept either alone (Control; *n* = 20) or in the presence of spinycheek crayfish (+Spinyceek; n = 20) or signal crayfish (+Signal; *n* = 20) during the day and night times (line: median, box: 25,75% percentiles, whiskers: 5%,95% percentile, dots: outliers).

significantly prolonged, but only in the presence of spinycheek crayfish (Table 3, Fig. 2c, d). The entrance into an already occupied shelter was observed more often for crayfish (20 up to 40 times, Fig. 3) than for benthic fish (3.6 up to 17.2 times), as demonstrated by the significant difference in the proportion of shelter status before occupation between crayfish species and benthic fish species (*p* = 0.001, McNemar test). Notably, both benthic fish and crayfish shared the shelter just once (1× stone loach with spinycheek crayfish, 1× bullhead with spinycheek crayfish). On both occasions, the benthic fish escaped quickly from the shelter. Both benthic fish species were more often evicted from the shelter by signal crayfish (stone loach: 62%, bullhead: 55%) than by spinycheek crayfish (stone loach: 37%, bullhead: 45%).

Both crayfish species spent more time inside the shelter during the daytime than the nighttime. Therefore, the stone loach stayed for a long time, more or less stationary, in certain spots outside the shelter. This behaviour increased in the presence of both crayfish species during day and night (Table 4, Fig. 4a). Likewise, bullheads settled
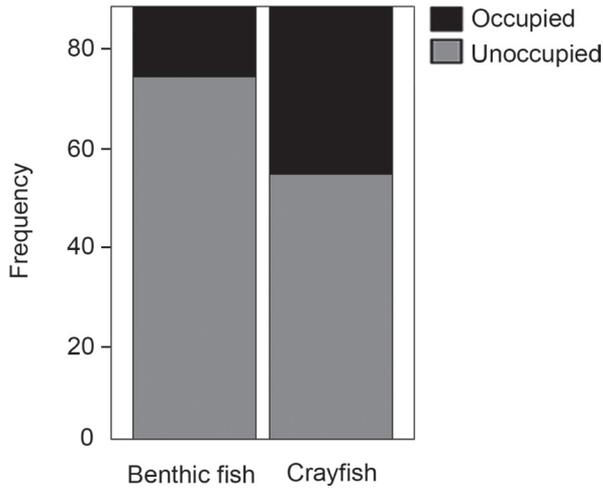
Figure 3. Status of a shelter (occupied: Black, unoccupied: Grey) before entered by crayfish or benthic fish.

Table 3. Results for the ANOVA's (generalized linear models) regarding the effect of the two crayfish species (*Faxonius limosus*: spinycheek crayfish; *Pacifastacus leniusculus*: signal crayfish) on the shelter occupation time of the benthic fish species *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead), during night and day, given as an estimate, standard error (SE), z-value, and *p*-value. Due to the different distributions, a zero-inflated distribution was used for bullhead and a Poisson distribution for a stone loach. Significant results are indicated by bold font.

Fish	Factor	Est.	SE	z	*p*
Stone loch	Intercept	5.31	0.015	338.3	< 0.001
	Signal	-0.22	0.025	-8.8	**< 0.001**
	Spinycheek	-0.37	0.030	-12.5	**< 0.001**
	Time	-1.01	0.035	-29.1	**< 0.001**
	Signal: time	-0.06	0.056	-1.1	0.261
	Spinycheek: time	0.13	0.060	2.3	**0.020**
Bullhead	Intercept	2.70	1.113	2.4	0.015
	Signal	-0.51	1.339	-0.4	0.705
	Spinycheek	-3.11	1.203	-2.6	**0.010**
	Time	-0.51	1.339	-0.4	0.705
	Signal: time	0.51	1.705	2.3	0.766
	Spinycheek: time	1.32	1.487	0.9	0.376

in a stationary position in particular spots inside the tanks for long periods (Table 4, Fig. 5a). Both benthic fish species changed their behaviour and were on average further away from the shelter when crayfish were present, which is indicated by a reduced habitat proximity index H_x (stone loach control: 0.52 ± 0.02, with spinycheck crayfish: 0.07 ± 0.003, with signal crayfish: 0.16 ± 0.01; bullheads control: 0.46 ± 0.05, with spinycheek crayfish: 0.33 ± 0.01, with signal crayfish: 0.26 ± 0.01). Similar to benthic fish, both crayfish showed stationary time inside the aquaria (Table 5, Figs 4, 5).
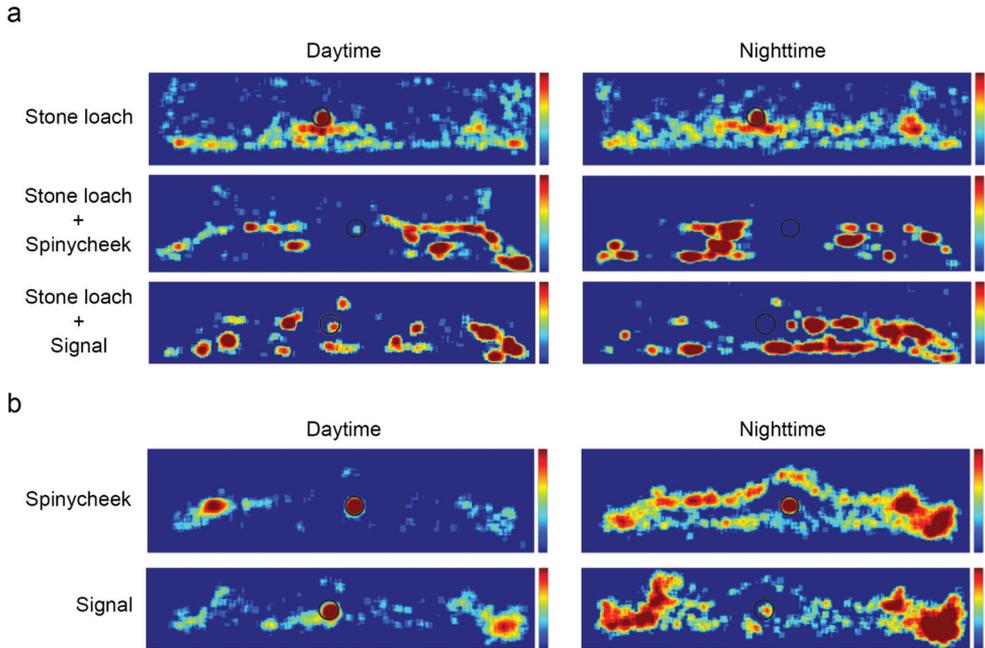
Figure 4. Heatmaps (shifting from blue over yellow to red with the increase of time spent in this respective location) is a graphical representation of the shelter occupation and animal distribution in the experimental tank **a** heatmaps showing the movement of (stone loach, *Barbatula barbatula*) in the absence or presence of invasive crayfish species (spinycheek, *Faxonius limosus* or signal crayfish, *Pacifastacus leniusculus*) during day and night **b** heatmaps illustrate the localization and dynamics of spinycheek and signal crayfish inside the aquarium during day and night.

Table 4. Mean (±SE) times of different behaviours (minutes) for the benthic fish species *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead) alone (control), or in presence of crayfish (*Faxonius limosus*: spinycheek crayfish; *Pacifastacus leniusculus*: signal crayfish) inside and outside (moving or stationary) the shelter during day and night in the tank.

Fish situation		Stone loach			Bullhead		
		Control	Spinycheek	Signal	Control	Spinycheek	Signal
Inside shelter	day	154.7±55	161.0±33	137.4±19	0.0±0.0	218.9±24	69.3±31
	night	41.3±25	67.0±13	56.3±17	0.0±0.0	110.3±23	23.5±13
Outside shelter	moving, day	98.8±32	85.8±19	43.2±18	143.2±30	42.9±13	62.2±21
	moving, night	405.8±18	203.8±20	207.4±45	234.2±108	178.7±25	134.3±34
	stationary, day	105.5±47	113.1±32	179.4±24	216.8±30	98.2±26	228.9±30
	stationary, night	33.0±19	229.2±29	216.2±49	245.8±108	191.0±25	322.2±31

As a result of their inability to occupy the shelter in the presence of crayfish species, the stone loach expressed hiding behaviour (i.e., reduced general activity, lying low). In general, the stone loach was more active at night than the daytime. Such activity was reduced in the presence of both crayfish species, which was indicated by a significantly lower activity index ($F_{2,22}$ = 7.7, P = 0.002, Fig. 6a, Table 4). During the day, the
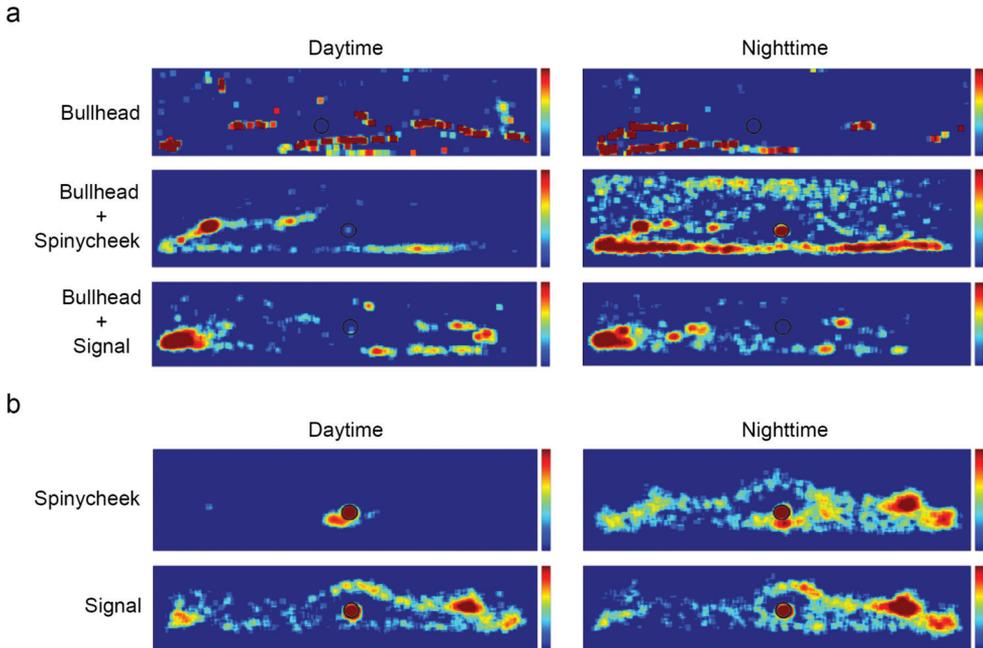
Figure 5. Heatmaps (shifting from blue over yellow to red with the increase of time spent in this respective location) is a graphical representation of the shelter occupation and animal distribution in the experimental tank **a** heatmaps showing the movement of bullhead, (*Cottus gobio*) in the absence or presence of invasive crayfish species (spinycheek, *Faxonius limosus* or signal crayfish, *Pacifastacus leniusculus*) during day and night **b** heatmaps illustrate the localization and dynamics of spinycheek and signal crayfish inside the aquarium during day and night.

Table 5. Mean (±SE) times of different behaviours (minutes) for the crayfish species *Faxonius limosus* (spinycheek crayfish) and *Pacifastacus leniusculus* (signal crayfish) tracking inside and outside the shelter (moving or stationary) in the presence of *Barbatula barbatula* (stone loach) and *Cottus gobio* (bullhead) during day and night in the tank.

Fish situation		Stone loach		Bullhead	
		Spinycheek	Signal	Spinycheek	Signal
Inside shelter	day	156.1±21.8	67.2±26.5	62.0±33.4	33.5±21.7
	night	95.2±30.3	26.2±16.1	19.7±18.8	59.4±42.8
Outside shelter	moving, day	109.6±19.7	170.0±33.7	127.2±30.7	174.1±29.6
	moving, night	330.3±35.7	217.0±50.8	389.5±28.3	359.2±34.9
	stationary, day	82.6±14.2	110.0±30.5	139.8±14.9	86.5±30.4
	stationary, night	78.2±15.3	255.7±49.3	102.2±17.5	129.8±36.9

behavioural reaction was less clear, and the activity index was not significantly different ($F_{2,22}$ = 1.9, P = 0.171, Fig. 6b, Table 4). Bullheads spent more time moving inside the aquaria all day (Table 4). This time decreased upon exposure to both crayfish species, especially during the daytime as evidenced by the significantly reduced activity index ($F_{2,21}$ = 4.3., P = 0.02, Fig. 6c). During the night, bullhead's behaviour slightly changed, and the activity index was not significantly different ($F_{2,21}$ = 1. 6, P = 0.216, Fig. 6d).
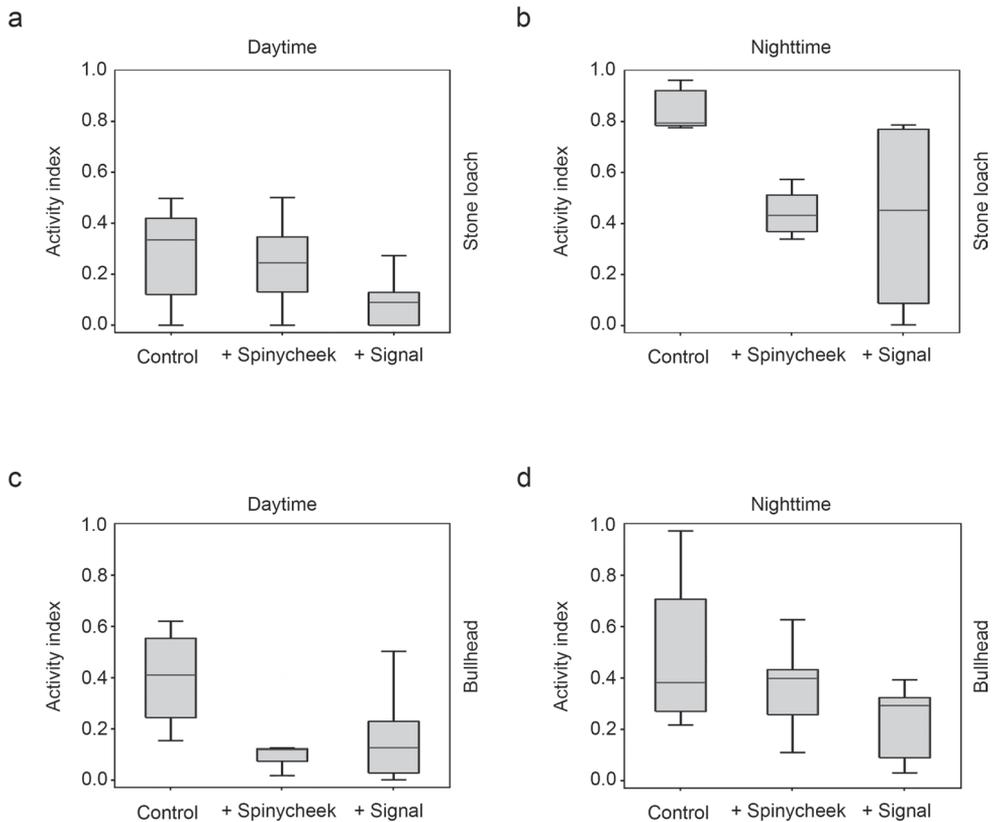
Figure 6. Box plots showing the activity index (*AI*) for the benthic fish species stone loach (*Barbatula barbatula*) (**a, b**) and bullhead (*Cottus gobio*) (**c, d**) in the trials with benthic fish only (Control), in the presence of spinycheek crayfish (*Faxonius limosus*), and in the presence of signal crayfish (*Pacifastacus leniusculus*) during day and night times.

Furthermore, the activity levels of both crayfish species varied, with differences in activity levels observed between the two species as well as the time of the day (Suppl. material 1).

Behavioural interaction

Crayfish species have different levels of agonistic behaviour, with the lowest being an exploratory movement of the antennae and accidentally getting into physical contact with the other animal (non-aggressive interactions) and the highest level being fighting (aggressive interactions). Although there was no statistically significant difference between the two crayfish species in terms of aggressive movement scores towards benthic fish, both species demonstrated aggressive interactions with benthic fish ($F_{1,76} = 0.07$, $p = 0.792$). Stone loaches were significantly more exposed to aggressive interactions from both crayfish species than bullheads, as evidenced by the fact that the score of aggressive behaviour was significantly higher ($F_{1,76} = 20.5$, $p < 0.001$, Fig. 7a) and the long-lasting interactions ($F_{1,76} = 18.8$, $p < 0.001$, Fig. 7b). In addition, crayfish sexes probably affected

the mean score of aggressive movements towards bullheads with the male individuals being more aggressive than females (7.4, 5.3, $F_{1,36}$ = 3.68, p = 0.054, Fig. 7c). For the stone loach, there was no significant difference between crayfish males and females in the mean score of aggressive movements (10.2, 9.85, $F_{1,36}$ = 0.136, p = 0.714, Fig. 7c). The two crayfish species differed markedly with respect to the frequency of the different aggressive acts. Stone loaches were exposed to several aggressive behaviours from both crayfish species, but antennal approaches (score 1) occurred significantly more often than other aggressive acts (Table 6). Half-raised (score 3) or fully raised cheliped (score 4) were the most used aggressive acts by signal crayfish species toward bullheads (Table 6). The frequency of the crayfish aggressive movements increased with the crayfish body mass. In the case of the stone loach, the frequency of aggressive movements correlated positively with carapace length, chela length, and crayfish body mass (r = 0.465, n = 40, p = 0.003; r = 0.560, n = 40, p < 0.001; r = 0.520, n = 40, p < 0.001), respectively (Suppl. material 2). There was no statistically significant relationship between the frequency of aggressive movements and standard length and body mass of stone loach (r = 0.183, n = 40, p = 0.252; r = 0.256, n = 40, p = 0.111), respectively (Suppl. material 2). Accordingly, there were non-significant relationships between the frequency of aggressive movements of crayfish towards bullhead and the carapace length, chela length, crayfish body mass, standard length, and body mass of bullheads (r = 0.119, n = 40, p = 0.464; r = 0.200, n = 40, p = 0.217; r = 0.262, n = 40, p = 0.103; r = 0.027, n = 40, p = 0.869; r = 0.007, n = 40, p = 0.966), respectively (Suppl. material 3). It is worth mentioning that three benthic fish mortalities were recorded due to crayfish aggression within the time frame of the experiment. During the 40 experiments, signal crayfish killed and ate one bullhead and one stone loach, while spinycheek crayfish preyed only on one stone loach.

Table 6. Mann-Whitney test table to estimate the difference between the frequency of different aggressive interactions of invasive crayfish species (*Faxonius limosus*: spinycheek crayfish; *Pacifastacus leniusculus*: signal crayfish). Significant results are indicated by bold font.

Aggressive react	Stone loach		Bullhead		Mean Rank			
	U	P	U	P	Stone loach		Bullhead	
					spinycheek	signal	spinycheek	signal
Antenna wave	52.0	**<0.001**	147.5	0.173	27.90	13.10	17.76	22.13
Cheliped touching	150.0	0.176	167.0	0.369	23.00	18.00	22.15	18.85
Cheliped half raise	164.5	0.337	118.0	**0.043**	18.73	22.28	16.21	23.60
Cheliped full raise	189.5	0.775	134.5	**0.055**	21.00	19.98	17.23	23.78
Grappling and pull	187.0	0.541	184.0	0.513	19.85	21.15	19.70	21.30

Discussion

Crayfish and benthic fish often overlap in their niches, showing a high temporal overlap in their needs for shelter (Cooper et al. 2009; Kubec et al. 2019). For crayfish, shelter use can even be of a higher value than access to food (Bergman and Moore 2003) as it lowers the risk of predation and cannibalism (Garvey et al. 1994; Söderbäck 1994; Hill and Lodge 1999; Fero et al. 2007).
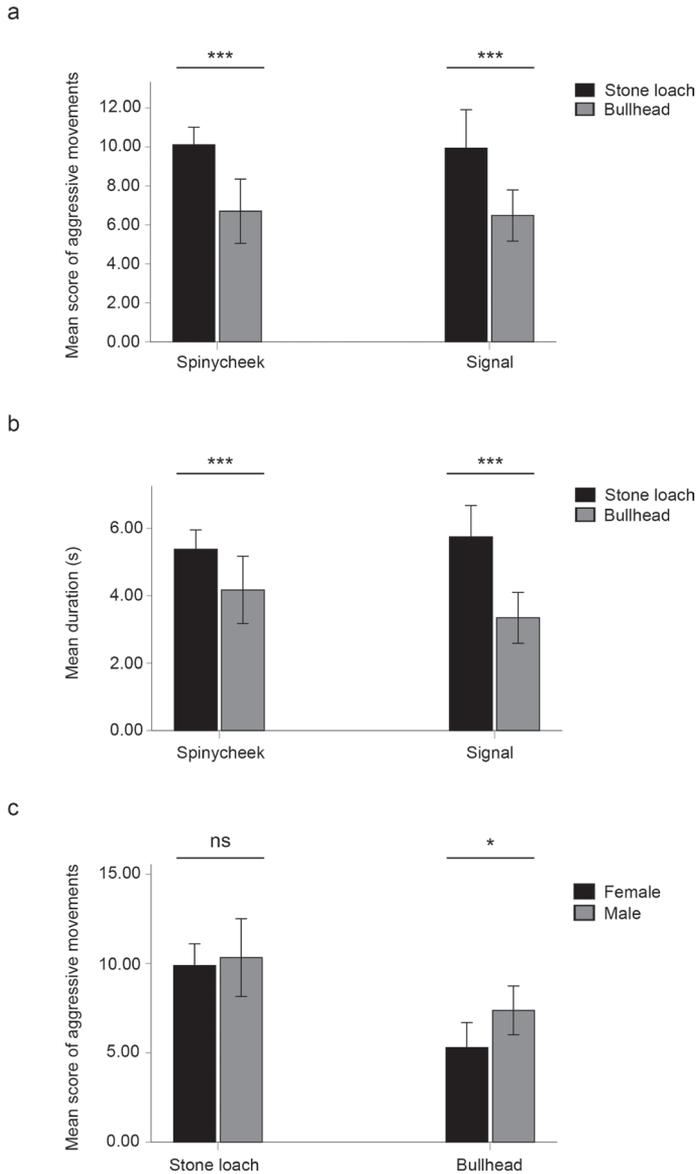
Figure 7. Comparison of aggressive interactions of the two crayfish species (spinycheek crayfish, *Faxonius limosus* or signal crayfish, *Pacifastacus leniusculus*) towards the benthic fish species bullhead (*Cottus gobio*) and stone loach (*Barbatula barbatula*). Error bars indicate the standard errors of the mean. (ANOVA; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Our results demonstrate that both stone loach and bullhead, displayed increased hiding behaviour, changing shelter use as well as their activities and movements in the presence of crayfish. Being superior competitors, spinycheek crayfish and signal crayfish succeeded in gaining control of the shelter by displacing stone loaches from the

shelter, thereby decreasing the time of shelter occupation by stone loaches. Bullheads, on the other hand, showed increased shelter use when crayfish were present. This suggests that unlike stone loaches, which avoided the shelter to evade crayfish chasing, bullheads hid inside the shelter. Eviction and displacement of fish species from their own shelter by invasive crayfish under laboratory conditions have been shown not only for small benthic fish like bullheads (Bubb et al. 2009), Paiute sculpin *Cottus beldingi* (Light 2005), cobitid fish *Lefua echigonia* (Matsuzaki et al. 2012), and Johnny darter *Etheostoma nigrum* (Rahel and Stein 1988), but also for bigger and more mobile fish species such as Atlantic salmon *Salmo salar* (Griffiths et al. 2004) and Japanese crucian carp *Carassius auratus* (Matsuzaki et al. 2012). This eviction of fish from the shelters seems to be of ecological relevance as it can markedly increase the risk of direct predation by crayfish or other predators (Rahel and Stein 1988; Matsuzaki et al. 2012).

In addition to the change in shelter use, both benthic fish species showed an increased hiding behaviour in response to the presence of crayfish and because crayfish monopolized the shelter. Fish reduced their general activity and spent long periods stationary at certain places outside the shelter. As a consequence of those behavioural changes, they maintained a greater average distance from the shelter than when crayfish were not present. Perales et al. (2021) showed that virile crayfish (*Faxonius virilis*) shifted away from their habitats as the rusty crayfish (*Faxonius rusticus*) population increased, and paiute sculpin (*Cottus beldingi*) changed their habitat to high-velocity areas in the presence of signal crayfish (Light 2005). These observations of behavioural changes support our interpretation that crayfish are the superior competitors, displacing the benthic fish toward less preferred habitats with fewer competitors.

Our findings demonstrate that both benthic fish species are negatively affected by crayfish invasion, regardless of whether they increased or decreased their shelter use. On the other hand, stone loach is perceived to be more endangered than bullheads. Our data shows that crayfish caused the stone loach to stay away from the shelter to a greater extent than the bullhead. This could imply a more drastic reduction in the stone loach population due to increased susceptibility to predation compared to bullheads. This observation aligns with (MacKenzie and Greenberg 1998), who demonstrated that stone loaches utilized shelters as a means to evade predators.

In addition to shelter and microhabitat use, the aggressive behaviour of invasive crayfish itself can also impact native benthic fish. Highly aggressive behaviour is known to be a key feature of successful invaders (Weis 2010). Furthermore, aggressive dominance is considered an advantage in the competition for shelter use, implying the importance of aggressiveness in replacements among crayfish species (Usio et al. 2001). We observed that both crayfish species displayed several aggressive acts against both species of benthic fish, indicated by the high scores of aggressive interactions. Such antagonistic actions can cause the weaker competitor to change habitat use, and a displacement to suboptimal feeding habitats may impair their population development. This was previously shown for juvenile burbot (*Lota lota*) in Lake Constance exposed to spinycheek crayfish in experimental mesocosms, where burbot was evicted from their preferred shelter (Hirsch and Fischer 2008). Consistently, another

study showed that the native benthic fish Paiute sculpin (*Cottus beldingi*) declined in growth rate due to a reduction of feeding as they spent more time fleeing when signal crayfish was present (Light 2005). Similar effects might be expected for stone loach and bullhead although our experimental arena was not designed to test that kind of behavioural response.

The level of aggression and resource-holding potential of animals can be influenced by many factors such as species, size, age, sex, or energy levels and can therefore serve as the key indicator of crayfish dominance (Marden and Waage 1990; Nijman and Heuts 2000). In this study, we observed that factors such as body mass, carapace length, and chela length enhanced the aggressive behaviour of crayfish against stone loach, while crayfish sex had a more significant impact in case of bullheads. These findings are consistent with observations made for other fish species (Figler et al. 1995; Huber and Schroeder 2001; Daws et al. 2002; Klocker and Strayer 2004; Fero et al. 2007; Moore 2007; Patullo et al. 2009). This was also consistent with the reported influence of these factors on the outcome and aggression level in animal fights in general (Marden and Waage 1990; Edsman and Jonsson 1996; Nijman and Heuts 2000).

Previously reported gut content analyses showed that crayfish can feed on bullheads (Guan and Wiles 1998). In line, another study showed that two bullhead mortalities were observed upon exposure to signal crayfish under lab conditions (Bubb et al. 2009). We noted three benthic fish mortalities due to the aggressive behaviour of crayfish within the time frame of our study. Our data suggest that fish mortalities due to the direct predation by crayfish or because of the constitutive stress caused by the presence of crayfish can serve as a relevant factor for fish density.

We conclude that the remarkable dominance of the invasive crayfish species over benthic fish, which we observed under laboratory conditions, such as displacing them from the shelter and changing their behaviour, can render them susceptible to predation. Furthermore, both invasive crayfish species exhibit aggression towards benthic fish.

Acknowledgements

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Supplementary material 1

Activity index (*AI*) box plots
Authors: Essmat Mohammed, Rahma Amen, Hoda M. Abdelwahab, Carola Winkelmann
Data type: image (.png file)
Explanation note: Box plots showing the activity index (*AI*) for the crayfish species signal (*Pacifastacus leniusculus*) (a, b) and spinycheek (*Faxonius limosus*) (c, d) in the trials with benthic fish bullhead (*Cottus gobio*) and stone loach (*Barbatula barbatula*) during the day and night times.
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Link: https://doi.org/10.3897/neobiota.83.102975.suppl1

Supplementary material 2

Scatterplot of the frequency of crayfish aggressive movements vs. carapace length, chela length, body mass, stone loach standard length, and stone loach body mass

Authors: Essmat Mohammed, Rahma Amen, Hoda M. Abdelwahab, Carola Winkelmann

Data type: image (.png file)

Explanation note: Data for both crayfish species were pooled. a-c) Scatterplots show aggression towards stone loaches which was positively correlated with carapace length (r=0.465, p=0.003), chela length (r=0.560, p<0.001), and crayfish body mass (r=0.520, p=0.001).

Link: https://doi.org/10.3897/neobiota.83.102975.suppl2

Supplementary material 3

Correlation scatterplot of the crayfish frequency of aggressive movements, carapace length, chela length, body mass, bullhead standard length, and bullhead body mass

Authors: Essmat Mohammed, Rahma Amen, Hoda M. Abdelwahab, Carola Winkelmann

Data type: image (.png file)

Explanation note: Data for both crayfish species were pooled. a-e) Scatterplots display aggression towards bullhead.

Link: https://doi.org/10.3897/neobiota.83.102975.suppl3