



Behavioural responses to con- and heterospecific alarm cues by an alien and a coexisting native fish

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Abstract The monkey goby *Neogobius fluviatilis* is an invasive Ponto-Caspian fish that enters habitats of the native gudgeon *Gobio gobio* in European freshwaters, likely belonging to the same prey guild. Their abilities to detect and avoid predation have been poorly understood, although these traits may contribute to the competitive advantage and drive the invasion success of the goby. We tested intra- and interspecific responses of fish from sympatric populations to damage-released alarm cues (skin extract) in laboratory. Both species of fish responded to conspecific and heterospecific cues, but their responses to conspecifics were more diverse (changed social distancing among individuals, reduced vertical and horizontal movement) than those elicited by

heterospecifics (changed social distancing only). Moreover, the fish differed in their anti-predation behaviour: the gudgeon increased whilst the monkey goby decreased inter-individual distances and only the gudgeon exhibited thigmotaxis and reduced mobility in response to the conspecific cues. Interspecific differences show that the species exhibit distant anti-predation strategies. This might be associated with their phylogenetic distance and/or higher differentiation of their ecological niches than commonly assumed. Besides, our results suggest that alien species be included in the interspecific exchange of information in local fish assemblages.

Keywords Anti-predator responses · Chemical alarm cues · *Gobio gobio* · *Neogobius fluviatilis* · Invasive species · Schreckstoff

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Introduction

For the past five decades, biological isolations have been gradually eliminated through global changes (Vilà et al., 2010; Keller et al., 2011; Early et al., 2016), resulting in coexistence of species that used to be geographically separated. This involves changes in prey guilds, i.e. groups of species that share the same predators and co-occur temporally and spatially (Mirza & Chivers, 2001; Pollock et al., 2003). The native members of the guild start to interact with

newly arrived incomers sharing no common coevolutionary history (Brown & Godin, 1997). In aquatic environments, especially in freshwater ecosystems, which are among the most invaded habitats in the world (Strayer, 2010; Ricciardi & MacIsaac 2010), interactions among formerly allopatric, now sympatric, fish species are particularly relevant because fish belong to the most commonly introduced animals into inland water systems (Keller et al., 2011). Moreover, fish, as organisms occupying higher trophic levels, can have a large cascading impact on communities and food webs (Gallardo et al., 2016).

During the past few decades, the rapid expansion of several goby fish (Gobiidae), originating from the Ponto-Caspian region, has been observed in both freshwater and marine ecosystems worldwide (Copp et al., 2005; Roche et al., 2013) with an ample potential to alter food webs, including predator–prey relationships (Barton et al., 2005). The gobies are small-sized benthic fish (mostly up to several cm in total length) commonly consumed by native predators (Reyjol et al., 2010; Płachocki et al., 2012; Mikl et al., 2017). In European inland waters, the gobies spread mainly in large rivers, including the River Danube (Jurajda et al., 2005), Rhine (Borcherding et al., 2011) and Vistula (Płachocki et al., 2020). Because there are no native Gobiidae species in these waters, the only possible option for the alien gobies is to join bottom-dwelling fish assemblages composed of species distantly related to them, belonging to other families. There is a growing number of studies comparing the alien gobies and such coexisting native fish in terms of various ecological aspects, such as competitive interactions for food/space (Van Kessel et al., 2011; Kakareko et al., 2013; Grabowska et al., 2016), habitat partitioning (Kakareko et al., 2016), feeding preferences (Błońska et al., 2016), and swimming performance (Egger et al., 2021). However, still little is known on how the gobies compare to local fish of the same prey guild in their behaviour of detection and avoidance of predation.

Chemical cues play an important role in information transfer in aquatic environments, especially in turbid and vast waters where the use of visual and auditory perception is limited (Brönmark & Hansson, 2000; Burks & Lodge, 2002; Chung-Davidson et al., 2010). In aquatic animals, scents mediate all basic life functions, such as reproduction, food location, predation avoidance and orientation in space (Liley, 1982;

Hay, 2009; Brönmark & Hansson, 2000). One of the essential cues used by animals for anti-predator defence is alarm cues released into water from damaged tissue of injured prey (Wisenden, 2015; Chivers & Smith, 1998; Wisenden & Chivers, 2006; Crane et al., 2013). They are released immediately after a predator attack, indicate high predation risk and provide reliable (context-specific) public information to guide behavioural responses of nearby animals to minimize the risk of encountering a predator (Smith, 1992; Ferrari et al., 2010; Chivers et al., 2013). Among fish, the largest and most diverse group of vertebrates (Sorensen, 2015), these unambiguous alarm cues can invoke a widespread response of potential prey individuals, including heterospecifics that are able to use such public information (Wisenden, 2003; Mathis & Smith, 1993a). Such an ability to respond to heterospecific alarm cues in mixed species groups may most likely occur when prey species are closely phylogenetically related and belong to the same prey guild (Mathis & Smith, 1993b; Mirza & Chivers, 2001; Wisenden & Chivers, 2006; Pollock et al., 2003; Dalesman et al., 2007). However, even if members of a prey guild are phylogenetically distant, the natural selection can favour cross-species communication among them, as shown for a benthic fish, the rainbow darter *Etheostoma caeruleum* Storer, 1845, and the Oklahoma salamander *Eurycea tynereensis* Moore and Hughes, 1939 (Anderson & Mathis, 2016). On the other hand, fish evolved optimal trade-offs between benefits and costs of executing adaptive behavioural anti-predator responses (Allan, 1982; Stabell & Lwin, 1997; Ferrari et al., 2010; Landeira-Dabarca et al., 2019). Thus, different species within a prey guild are likely to vary in their finely tuned mechanisms to detect the chemical alarm cues and to respond accordingly (Lima & Dill, 1990; Chivers & Smith, 1998; Mirza et al., 2003). These differences are likely to translate to inter-specific differences in competitive ability and spreading potential. In aquatic environments, invasive crustaceans are known to be able to outcompete native species due to better sensory detection of predators, manifested by the use of a broader range of chemical information and/or faster escape responses (Weis, 2016; Hazlett et al., 2003). The intriguing question is whether this phenomenon applies to various taxonomic groups of invasive species. Here, we raise the question whether the Ponto-Caspian gobiids, despite being recently

established in Polish fresh waters (about two decades ago) and phylogenetically distant from native members of their prey guild in newly invaded areas (Grabowska et al., 2010), can exchange chemical alarm cues with the native prey guild members. They may support and/or benefit from aggregate anti-predatory defence behaviours of local fish communities, depending on whether they are effective donors and/or receivers of heterospecific cues.

In this study, we investigated anti-predation behaviour of two fish that co-occur in European waters and fit well into the context described above: (1) the invasive monkey goby *Neogobius fluviatilis* (Pallas, 1814), and (2) the native gudgeon *Gobio gobio* (Linnaeus, 1758). The monkey goby is one of the successful invaders of Ponto-Caspian origin in European fresh waters (Čáková et al., 2008; Kakareko et al., 2009; Plachá et al., 2010), locally dominating in goby assemblages (Płachocki et al., 2020). This alien species enters habitats occupied by the gudgeon, as both species are mostly associated with sandy bottom areas (Bănărescu et al., 1999; Čáková et al., 2008; Jakubčínová et al., 2017; Płachocki et al., 2020). It has been pointed out that declines in gudgeon populations coincide with increasing monkey goby population densities (Jakovlić et al., 2015). The gudgeon and monkey goby seem to occupy similar ecological niches (Jakovlić et al., 2015; Borcharding et al., 2016). Due to the occurrence in similar habitats and similar morphology (size mostly up to several cm of standard length, streamlined shape, dappled coloration) and ecology (bottom feeders) (Kottelat & Freyhof, 2007; Plachá et al., 2010), the two species are likely to share the same predators. Therefore, the two species are a useful case model to study associations and interactions between the Ponto-Caspian invaders and their native counterparts in the light of their responses to predation pressure. The monkey goby and gudgeon are phylogenetically distant from each other, belonging to different orders: Gobiiformes and Cypriniformes (Nelson et al., 2016), respectively. They do not have a long common evolutionary history, but in the Ponto-Caspian region the goby co-exists with several species closely phylogenetically related to the gudgeon (Naseka & Bogutskaya, 2009; Mendel et al., 2008). We examined differences between the alien monkey goby and native gudgeon in their early detection and avoidance of predation through the use of alarm cues. Using a laboratory behavioural assay,

we tested behavioural responses of the fish to conspecific and heterospecific damage-released chemical alarm cues (skin extracts). We hypothesized that:

- (1) Both fish are responsive not only to conspecific but also to heterospecific alarm cues. The responses to both cues are manifested by increased aggregation and thigmotaxis, as well as reduced horizontal and vertical movements.
- (2) The fish differ from each other in the quality and strength of their responses to the damage-released alarm cues. The alien species exhibits more pronounced responses to the cues (thus maximizing its security in a mixed-species assemblage), or, alternatively, is less reactive to the cues (thus being able to partition more energy into growth and reproduction than its native counterparts).

Materials and methods

Animals

We collected fish of both species using electrofishing (EFGI 650, BSE Bretschneider Spezialelektronik, Germany) in June 2018, in the longest tributary of the River Vistula, the River Pilica near the city of Warka (51°45′49.0″N 21°08′56.7″E), east-central Poland. We caught them from the same shallow (depth: 0.2–0.5 m) near-shore area with moderate water flow and sandy bottom. The exact date of the monkey goby introduction to the River Pilica is not known, but during extensive research in 2003–2005, the monkey goby were not recorded in the area (Penczak et al., 2006). Thus, the fish have been living in sympatry in the area for no more than 14 years at the time of their collection. Immediately after capture, we transported the animals to the laboratory (ca. 3 h transport time) in plastic bags with aerated water. In the laboratory, we placed the fish in 350-L stock tanks (15–20 individuals per tank, both species together) equipped with standard aquarium filters and aerators and filled with conditioned tap water. A temperature of 20 °C, which corresponds to mean summer temperatures recorded in rivers in central Poland (Łaszewski, 2018), was maintained by air-conditioning; the photoperiod was set to 12 h day:12 h night. Light intensity at the bottom of the tanks was 5 lx (measured by a

light meter L-20A, Sonopan Ltd., Białystok, Poland). The stock tanks were equipped with ceramic shelters and had no bottom substrate. We fed the fish ad libitum daily with frozen chironomid larvae and exchanged water in the stock tanks once a week (ca. 30% of the water volume). We kept the fish in the stock tanks for at least 1 month before the start of experiments. The total length of the fish (measured using ImageJ 1.49v, freeware by W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA: <https://imagej.nih.gov/ij>, from digital photographs taken during tests) was 75 mm (min–max: 44–96 mm) and 67 mm (45–97 mm) for gudgeons and gobies, respectively. The length difference between the two fish, although relatively small, was significant (t-test: $t_{94} = 3.65$, $P < 0.001$). We took the fish for the experiment randomly, firstly from the river and then from the stock tanks, thus the interspecific length differences reflected natural differences in the size of individuals between the coexisting populations. The individuals used for the tests had virtually no external symptoms of sexual maturity and sexual dimorphism characteristics, and we did not determine their sex. We collected and used the fish under permit of the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland, statement no. 50/2017 from 28 September 2017. All procedures using fish met the European Union guidelines on the protection of animals used for scientific purposes (Directive 2010/63/UE).

Preparation of alarm cues

We prepared two consecutive samples of the damage-released chemical alarm cue during the study. Each time, we took 3–7 donor individuals of each species from the stock tank, stunned them by a blow to the head and then severed their spinal cord. To obtain the chemical cue, whose sources are located in the epidermal layer (for review, see Ferrari et al., 2010), we gently removed skin patches from the dorsal and lateral parts of the body using a scalpel and tweezers (1 g total skin weight), homogenized them in 100 ml of chilled (4 °C) distilled water (all the donors of the same species pooled) and filtered through qualitative cellulose filters Whatman no. 1 (11 µm) to remove any particles, yielding the skin homogenate (Pollock et al., 2003; Souza-Bastos et al., 2014). We collected the filtrate into 2-ml plastic Eppendorf tubes, and stored

at –80 °C until use (no longer than for a month). This ultra-low temperature is recommended for long-term storage of biological samples like sex hormones (TwoRoger & Hankinson, 2006). Such pooled signal samples allowed us to reduce potential intraspecific differences in signal strength and focus on fish responses to typical cues representative of particular species. We also froze distilled water for use in control conditions in the same way.

Experimental setup

We conducted experiments in 84-L glass tanks (bottom: 60 × 40 cm, height: 35 cm, water level: 28 cm, water volume 67.2 L) filled with settled, aerated tap water, without any bottom substrate. To reduce the impact of external disturbances on the fish, we isolated the tanks on all sides with Styrofoam screens. Each tank was equipped with a standard aquarium filter (in the corner) and aerator. A single dose of the cue was taken from pooled contents of nine Eppendorf tubes with the stored extract (alarm substance) or distilled water (control cue). We thawed the cue at 20 °C and immediately added it to the experimental tank by use of a peristaltic pump (Watson-Marlow 323U, Falmouth, United Kingdom) at a rate of 19.8 ml min⁻¹ (accuracy to the nearest 0.1 ml min⁻¹). The cue fully reached the water column of the tank in 50 s. Thus, a single dose of the cue consisted of 16.5 ml of the alarm substance (i.e. skin extract obtained from 0.165 g of the tissue). Immediately before the injection of the test substance, we filled the outlet tube of the peristaltic pump with fresh water from the tank by use of reverse operation of the pump to avoid pumping air bubbles to the tank. The outputs of the peristaltic pump (vinyl tubes with an inner diameter of 1.59 mm) were attached near the output nozzle of the aquarium filter, which provided water movement distributing the cue in the water column and creating conditions similar to natural riverine habitats where both species occur. We recorded the experiment using an IP video camera (SNB–6004P, Samsung, South Korea) placed 1 m above the water level in the tank. The camera was facing down to catch the view of the entire tank from above.

Experimental procedure

We tested responses of triplets (i.e. 3 conspecifics placed together in the same tank) of the two fish species to conspecific and heterospecific alarm cues. The triplets consisted of individuals collected randomly from the same stock tank, and thus were familiar to one another from the beginning of the test. Each individual was used only once in the experiment. We selected this number of individuals during preliminary observations, which indicated that fish in such groups behave naturally in contrast to pairs or singletons. Each of the two treatments, i.e. fish exposed to conspecific or heterospecific alarm cues, was replicated 8 times ($n = 8$). In total, 96 individuals were tested (2 species \times 2 treatments \times 3 individuals \times 8 replicates).

Acclimation of fish to experimental conditions after transferring them to the experimental tank lasted for 72 h. During the acclimation period, we fed the fish daily with frozen chironomid larvae ad libitum. The last feeding took place 12 h before the beginning of the experiment. An extended acclimation period ensured that the experiment would not be disturbed by the presence of the camera. At the end of the acclimation period, individuals of both species behaved naturally, i.e. explored the environment, did not cling to the wall of the tank, did not hide behind the filter and did not exhibit any rapid erratic swimming. We carried out the experiments during the daytime, between 7:00 and 12:00, when the activity of the fish, based on our preliminary observations, was highest. We established the duration of the experiment and dose of the alarm cue on the basis of preliminary research and literature data (Mathuru, 2016; Pollock et al., 2003; Souza-Bastos et al., 2014). One replicate lasted for 18 min and consisted of two consecutive 9-min periods (Fig. 1). We injected distilled water (control cue) into the experimental tank at the beginning of the first period to make it a control period. Then, at the beginning of the second period, we injected the alarm substance to the tank (to observe fish responses to the alarm cue, compared to their regular behaviour during the control period). We counted the exposure time from the moment when the first drop of the dosed substance reached the experimental tank. It is worth noting that the duration of the test itself (18 min) was much shorter than the entire stay and pre-experimental acclimation of the fish in the

experimental tank (72 h) and the period of their maximum activity (5 h) (during which the tests were conducted exclusively). Therefore, it is highly unlikely that any time-related changes in fish behaviour obscured or masked the potential effect of the stimulus during the tests. We conducted all tests at the same temperature, photoperiod and light intensity as those set in the stock tanks.

Processing video data

We split each 9-min test period (with control water or the alarm substance) into three 3-min sub-periods (Fig. 1). This allowed us to test the effect of time after the cue application on fish responses and to detect immediate behavioural changes occurring only at the first contact with the cue. We used VirtualDub 1.10.4 (freeware by Avery Lee, www.virtualdub.org/index) to extract video frames from the recorded videos, which facilitated determination of behavioural variables and ensured better precision. Because the individuals were visually very similar to one another, it was not possible to track them without mistaking particular individuals on video frames. Instead, we tracked the entire group of three individuals; thus a replication of the experiment was the responses of all individuals in the group, which were summed up and averaged (see below for the details). We measured all necessary distances in these frames, using ImageJ 1.49v, with the centre of the fish head (established as an equidistant point between the eyes) as a reference point (Online Resource 1A, C). We manually noted four anti-predation behavioural responses (Chivers & Smith, 1998) of the tested fish for each experimental sub-period:

1. Thigmotaxis (T). This was the average distance of individuals to the nearest tank wall measured in 6-s intervals (Online Resource 1A) and calculated according to the following formula:

$$T = \frac{DW}{(3 \times 30)}$$

where: DW – the total sum of measured distances of individuals to the nearest tank wall, 3 – the number of individuals in the tested group, 30 – the number of frames (measurements) per sub-period. The filter in the tank was treated as part of the wall, i.e. if a fish was closer to the filter than to the glass

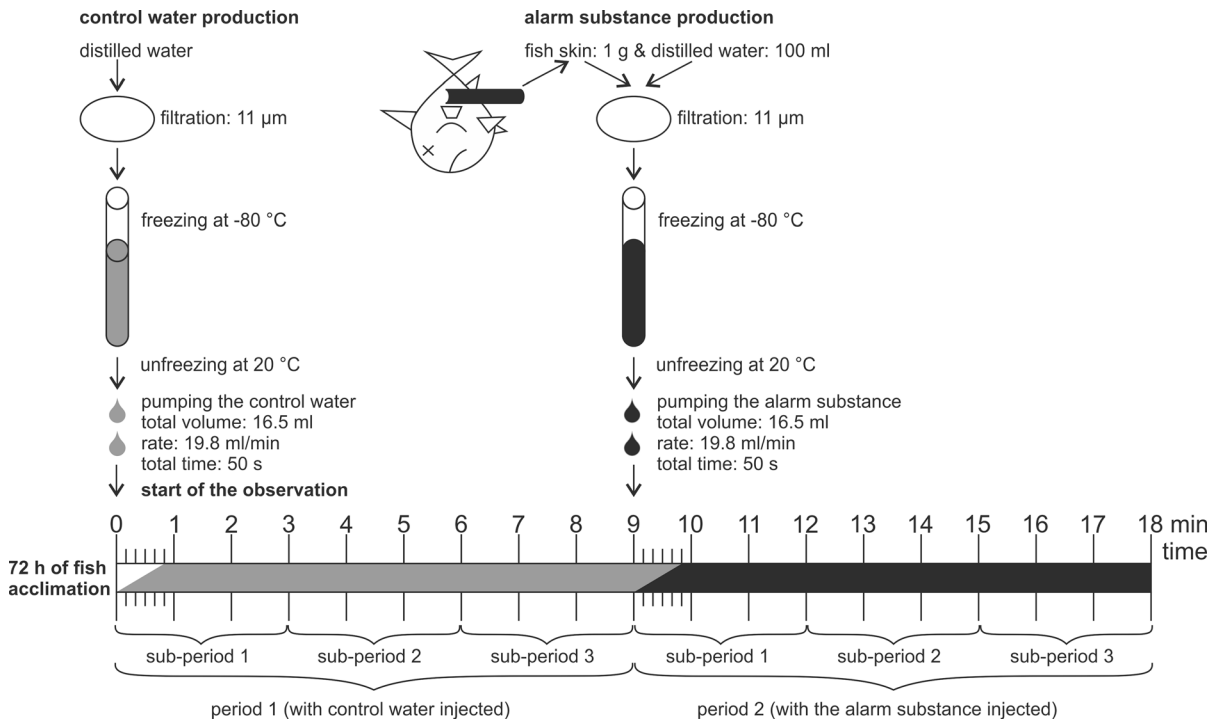


Fig. 1 Experimental setup

wall, the distance to the filter was taken into account). We used only cases where a fish had contact with the bottom, i.e. did not move up to the water column (Online Resource 1B), assuming that the corner between the vertical wall and bottom of the tank provides a fish with shelter.

2. Relations with conspecifics (R). This was the average horizontal distance of individuals to their nearest neighbour, measured in 6-s intervals (Online Resource 1A) and calculated according to the following formula:

$$R = \frac{DN}{(3 \times 30)}$$

where: DN – the total sum of measured distances of individuals to their nearest neighbours, 3 – the number of individuals in the tested group, 30 – the number of frames (measurements) per sub-period.

3. Immobility (I). This was the average percentage of time spent by individuals without movement, calculated according to the following formula:

$$I = \frac{SP}{(3 \times 29)} \times 100$$

where: SP – the total number of cases when an individual stayed in the same position in neighbouring video frames 6 s apart, 3 – the number of individuals in the tested group, 29 – the number of frame transitions (measurements) per sub-period. We assumed a fish to move when its horizontal displacement between the frames was equal to or longer than its body length (Online Resource 1C).

4. Vertical movement frequency: determined as binary events (present/not present) when at least one individual was observed to swim up to the water column (Online Resource 1B) and then sank to the bottom. To notice such events and collect quantitative data for analysis, each experimental sub-period was divided into nine 20-s intervals (more useful than frames for seeing these events from above, as established in preliminary trials). If at least one vertical movement occurred during a given interval, it was counted as one event. Thus, the number of vertical movement events that could be observed during each 3-min sub-period ranged from 0 to 9.

Statistical analysis

We were interested mainly in qualitative or quantitative differences in species behavioural responses to the alarm cues, rather than in general interspecific differences in activity or distances kept by the fish. Therefore, we conducted analyses separately for each species. Thus, to control for the inflated type I error rate, we considered the main effects and interactions as statistically significant at $P < 0.025$ (Bonferroni correction for 2 analyses). We log-transformed the thigmotaxis, distance among conspecifics and immobility variables to achieve normality and analysed them using a General Linear Model. We tested the frequency of vertical movements using a Generalized Linear Model with binomial distribution (vertical movement observed or not during each of 9 intervals) and log link function. Each model included alarm source (conspecific or heterospecific) as a between-subject factor, as well as cue type (consecutive 9-min periods with distilled water or alarm substance as a cue) and exposure time (consecutive 0–3, 3–6 or 6–9 min sub-periods within each 9-min period, see *Processing video data* for description) as within-subject factors. Initially, we included all main effects and interactions and then applied backward simplification of the models by removing non-significant higher-order interactions.

When both species displayed a qualitatively similar response to the alarm cue (which happened only in the case of vertical movements, see the Results), we checked whether the strength of their responses differed between the species. For each replicate, we calculated a relative change in fish behaviour (Ch) after the application of the alarm cue compared to the corresponding sub-period (0–3, 3–6 and 6–9 min) after the application of the control cue:

$$Ch = \frac{B_{Alarm} - B_{Control}}{B_{Alarm} + B_{Control}}$$

where B_{Alarm} , $B_{Control}$ are the measurements of the behaviour of fish exposed to the alarm cue and control cue, respectively. Higher values of this index indicate a greater change in fish behaviour relative to its absolute magnitude.

We analysed these indices using General Linear Models including species as a between-subject factor and exposure time (the three sub-periods after the cue application) as a within-subject factor. We only used

responses to conspecific alarm cues in these analyses, as fish changed significantly the analysed behaviours only in response to that cue (see the Results).

We further examined significant model effects using sequential-Bonferroni-corrected pairwise Fisher LSD tests (for the General Linear Models) and pairwise contrasts (for the Generalized Linear Model).

We used IBM SPSS Statistics 25.0 for the statistical analysis (IBM Inc., USA).

Results

Compared to the control period, gudgeon stayed closer to the tank walls when exposed to conspecific alarm cues, but not in the presence of heterospecific alarm cues (Fig. 2), as shown by a significant alarm source \times cue type interaction (Table 1A, Online Resource 2A). Monkey goby did not change their position relative to the tank walls in response to any stimuli applied in the experiment (Table 1A).

Distances among gudgeon individuals increased in response to both conspecific and heterospecific alarm sources, though only during the first 3-min period after

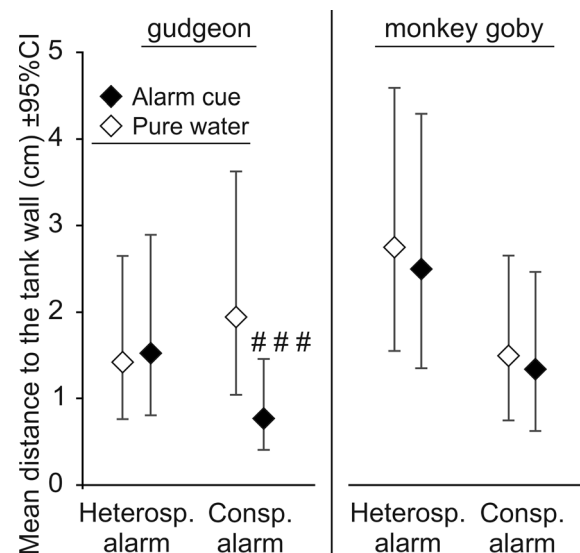


Fig. 2 Thigmotaxis responses of monkey gobies and gudgeons to conspecific and heterospecific alarm cues. Presented values (back-transformed after the analysis of log-transformed data) are means predicted by the General Linear Models (Table 1A). Time of exposure to the cue is not included (pooled) as non-significant in the models. Hashtags (#) indicate significant differences in the behaviour of animals exposed to the control water and alarm cue: ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$

Table 1 Analysis of the impact of alarm cue source (con-specific or heterospecific), cue type (pure water or alarm substance applied to the tank in two consecutive 9-min

intervals) and exposure time (0–3, 3–6 or 6–9 min after the application of the cue) on the behaviour of gudgeon and monkey goby

Response variable	Species	Effect	df	F	P
A. Thigmotaxis (distance to the tank wall) (General linear model)	Gudgeon	Alarm source	1, 14	0.21	0.656
		Cue type	1, 59	16.66	< 0.001 [#]
		Exposure time	2, 41	0.43	0.652
		Source*Cue type	1, 58	22.84	< 0.001 [#]
	Goby	Alarm source	1, 14	2.65	0.126
		Cue type	1, 46	0.57	0.454
		Exposure time	2, 23	0.49	0.618
B. Distance among fish (General linear model)	Gudgeon	Alarm source	1, 14	0.37	0.551
		Cue type	1, 57	0.58	0.450
		Exposure time	2, 38	0.88	0.425
		Cue type*Time	2, 38	5.44	0.008 [#]
	Goby	Alarm source	1, 13	4.20	0.061
		Cue type	1, 53	9.62	0.003 [#]
		Exposure time	2, 46	1.39	0.259
C. Immobility time (General linear model)	Gudgeon	Alarm source	1, 29	0.21	0.650
		Cue type	1, 43	11.97	0.001 [#]
		Exposure time	2, 20	0.13	0.877
		Source*Cue type	1, 42	12.86	0.001 [#]
	Goby	Alarm source	1, 14	0.47	0.502
		Cue type	1, 58	0.27	0.603
		Exposure time	2, 39	1.61	0.212
D. Vertical movements (Generalized linear model, binomial distribution, log link function)	Gudgeon	Alarm source	1, 90	0.54	0.464
		Cue type	1, 90	24.74	< 0.001 [#]
		Exposure time	2, 90	0.88	0.419
		Source*Cue type	1, 90	27.26	< 0.001 [#]
	Goby	Alarm source	1, 90	1.21	0.275
		Cue type	1, 90	0.45	0.504
		Exposure time	2, 90	6.35	0.003 [#]
		Source*Cue type	1, 90	8.32	0.005 [#]

Cue type and exposure time were included in the models as within-subject factors. Non-significant higher order interactions were dropped to simplify the models

Hashtags (#) indicate significant effects with a Bonferroni correction for two separately analysed species ($P < 0.05/2 = 0.025$)

cue application (Fig. 3), as shown by a significant cue type x exposure time interaction, as well as non-significant model terms involving the alarm source (Table 1B, Online Resource 2B). Monkey goby stayed closer to one another when exposed to conspecific and heterospecific alarm sources compared to control conditions, irrespective of the exposure time (Fig. 3), as shown by a significant main effect of cue type (Table 1B).

Immobility time of gudgeon depended on a significant alarm source x cue type interaction (Table 1C, Online Resource 2C), whereas monkey goby did not change their horizontal mobility in response to any alarm cues. Gudgeon moved less in the presence of conspecific alarm cues, but not in response to heterospecific alarm cues (Fig. 4).

Vertical movements of both species depended on a significant alarm source x cue type interaction

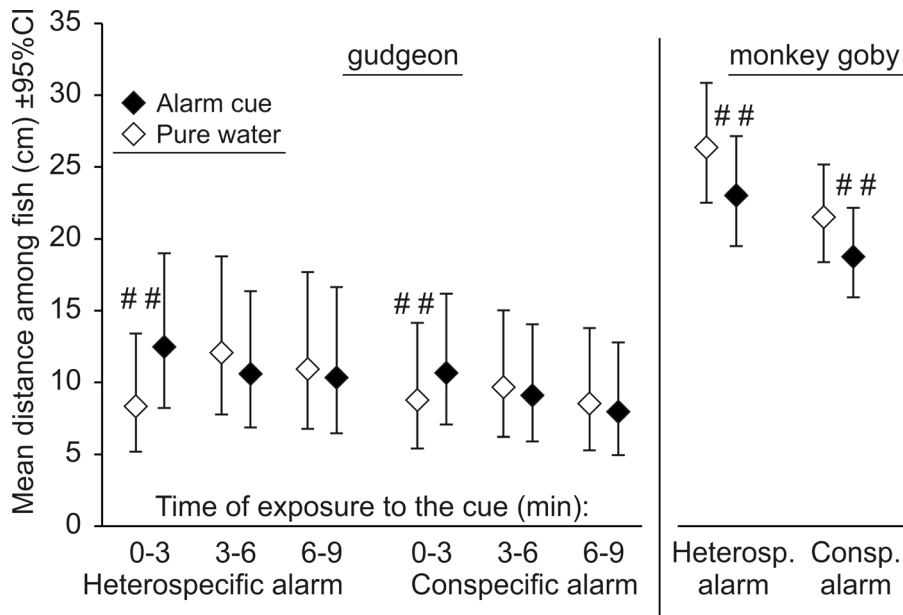


Fig. 3 Social distancing responses of monkey gobies and gudgeons to conspecific and heterospecific alarm cues depending on exposure time after receiving the cues. Presented values (back-transformed after the analysis of log-transformed data) are means predicted by the General Linear Models (Table 1B).

Time of exposure to the cue is not included (pooled) for the monkey goby as non-significant in the model. Hashtags (#) indicate significant differences in the behaviour of animals exposed to the control water and alarm cue: ###*P* < 0.001, ##*P* < 0.01, #*P* < 0.05

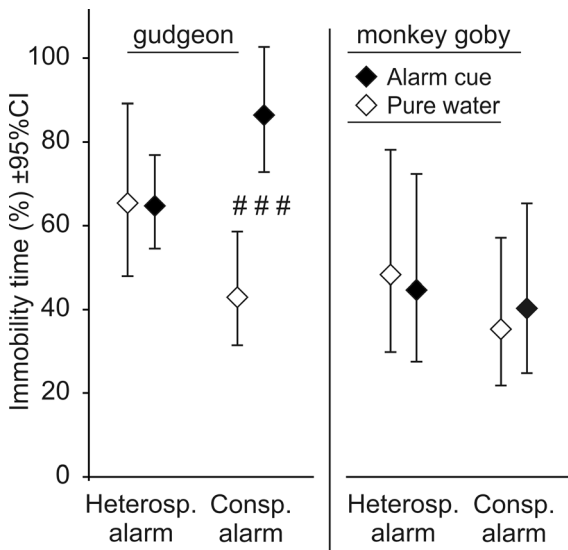


Fig. 4 Immobility responses of monkey gobies and gudgeons to conspecific and heterospecific alarm cues. Presented values (back-transformed after the analysis of log-transformed data) are means predicted by the General Linear Models (Table 1C). Time of exposure to the cue is not included (pooled) as non-significant in the models. Hashtags (#) indicate significant differences in the behaviour of animals exposed to the control water and alarm cue: ###*P* < 0.001, ##*P* < 0.01, #*P* < 0.05

(Table 1D, Online Resource 2D). In the presence of conspecific alarm cues, the fish more often stayed on the tank bottom compared to the control period (Fig. 5). Heterospecific alarm cues did not cause any changes in vertical movement of fish. The responses of gudgeon were generally stronger than those of monkey goby (Fig. 5), which was confirmed by a significant effect of species in the analysis of relative changes in fish vertical movements (mean relative change ± SE: 71 ± 28 and 15 ± 28% for the gudgeon and monkey goby, respectively) (Table 2).

Discussion

The first noteworthy finding of our study is the indication that, in accordance with our first hypothesis, the two phylogenetically distant species with a short history of co-existence are able to respond to heterospecific alarm cues from each other. This finding opens the possibility that *N. fluviatilis*, which is a successful invasive fish species in European freshwaters (Płachocki et al., 2020), has a potential to affect native fish communities not only negatively, but

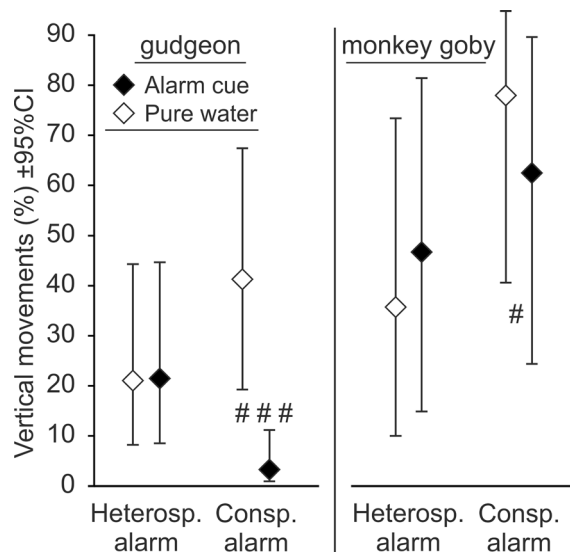


Fig. 5 Vertical movement responses (percentages of 20-s intervals with vertical movements) of monkey gobies and gudgeons to conspecific and heterospecific alarm cues. Presented values are means predicted by the Generalized Linear Model (binomial distribution, log-link function) (Table 1D). Time of exposure to the cue is not included (pooled) as non-significant in the models. Hashtags (#) indicate significant differences in the behaviour of animals exposed to the control water and alarm cue: ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$

Table 2 Analysis of the change in vertical movements of the studied species (gudgeon vs. monkey goby) exposed to the conspecific alarm cue relative to their behaviour in the presence of the control cue (pure water) after various exposure times (0–3, 3–6 or 6–9 min after the application of the cue)

Response variable	Effect	df	F	P
Vertical movements	Species	1, 12	9.52	0.009 [#]
	Exposure time	2, 19	2.01	0.161

The analysis was conducted for this response variable, for which both fish species displayed a similar response type. Exposure time was included in the model as a within-subject factor. Non-significant interactions were dropped to simplify the models

Hashtags (#) indicate significant effects ($P < 0.05$)

also positively. They can act as eavesdropping competitors (Anderson & Mathis, 2016), but also as supportive companions for native prey species in early detection of predator presence, being both receivers and donors (senders) of the public alarm cues. The use of heterospecific alarm cues likely provides a greater amount and broader range of information about the

environmental predation risk compared to what can be obtained from conspecifics (Seppänen et al., 2007). Thus, our results put it forward for consideration that a native species might sometimes benefit from the presence of alien species (Pollock & Chivers, 2004; Pollock et al., 2003). How much this matters for the fitness of the studied species is an open question, given that we showed much more pronounced behavioural responses to conspecific alarm cues compared to those to heterospecific cues. Notwithstanding these differences, it should be emphasized that invasive species affect simultaneously multiple aspects of their environment and invaded communities, thus it is likely that the net effect of the monkey goby on the local benthic fish assemblage would be negative, even if any benefits from interspecific enhancement of anti-predator defences do exist. It is worth considering that the result may also apply to other, closely phylogenetically related Ponto-Caspian goby species as members of a single clade within the family (Thacker & Roje, 2011).

Here, we tested specifically *N. fluviatilis* living in sympatry with *G. gobio* for up to 14 years and lacking a long coevolutionary history (see Materials and methods). Thus, we observed interspecific interactions probably mainly developed over such a period. However, it cannot be ruled out that the two species have shared common alarm substances, as these chemicals are conserved across fish taxa and neither sympatry nor phylogenetic relatedness are necessary for recognition of heterospecific alarm cues in this taxonomic group (Magellan et al., 2020). It is worth emphasizing that *G. gobio* is absent from the Ponto-Caspian region, while *N. fluviatilis* co-occurs in its native Ponto-Caspian area with other species of the genus *Gobio* (Naseka & Bogutskaya, 2009; Copp et al., 2005; Mendel et al., 2008; Roche et al., 2013), closely related to *G. gobio*. Thus, in contrast to the gudgeon, the monkey goby had a chance to evolve recognition mechanisms towards gudgeon alarm cues (Pollock & Chivers, 2004). Nonetheless, we found that both of the two co-existing species responded to heterospecific alarm cues, though, as we mentioned earlier, the heterospecific responses were less pronounced than those to the cues released by conspecifics. In our study, the two fish rely primarily on cues from individuals of their own species, possibly because they are the ones they encounter significantly more often than heterospecifics in the environment.

On the other hand, behavioural responses of fish to alarm cues are highly plastic and reflect the overlap or differentiation between their ecological niches, as was demonstrated in juveniles and adults of sympatric sunfish: *Lepomis macrochirus* Rafinesque, 1819 and *Lepomis gibbosus* (Linnaeus, 1758) (Xia et al., 2018). We suppose that the recognition of heterospecific cues is suppressed by the fact that the prey guild and habitat overlap between the two species is not as strong as commonly assumed. It is worth noting that the tested specimens of both species differed in size, which reflects natural interspecific differences in the field. Nevertheless, it cannot be excluded that, if any of the studied behaviours are size-dependent, this may contribute to different responses of the species observed in our study.

In accordance with our second hypothesis, both species differed from each other in their anti-predation behaviour. Thigmotaxis was exhibited only by the gudgeon as a tendency to avoid the central part of the experimental tank and stay at its walls (wall-hugging). Thigmotaxis is a well-validated index of anxiety in animals (Schnörr et al., 2012). It can be assumed that this reaction is a passive and defensive attitude, reducing the risk of death (Węsierska & Turlejski, 2000). In our study, the proximity of the walls/filter was the only shelter and probably increased the sense of safety in fish under predation risk. This clear preference for the peripheral part of the tank can be considered as increasing caution in exploring the environment by tested fish. Interestingly, instead of schooling, the gudgeon exhibited brief (during the initial 3-min sub-period) dispersion. It is generally accepted that schooling is a behavioural mechanism which decreases the probability of predation (Jarman, 1974; Magurran, 1990; Jachner, 1995). A predator facing aggregated prey may experience the confusion effect, i.e. difficulty in focusing on and picking a single individual from a group and therefore chances of escape of prey individuals increase (Heczko & Seghers, 1981; Speedie & Gerlai, 2008). Additionally, the likelihood of detection of predation risk by a shoal is greater than by a single individual (Jachner, 1995; Lima, 1995). We posit that the thigmotaxis and dispersion revealed by the gudgeon should be considered together. These two behaviours probably act together in the same direction to enhance the probability of success in seeking shelters. The monkey goby, in contrast to the gudgeon, gathered together

when exposed to the alarm stimuli, though this reaction was the weakest among all those observed in our study and should be interpreted with caution. Both fish moved less in the presence of the alarm cue, but the response of the monkey goby here was weaker than that of the gudgeon. Only gudgeon reduced their horizontal movements and their reduction in vertical movements was greater than that of the monkey goby. Reduction in locomotor activity is a common anti-predator defence induced by alarm cues (Mathis & Smith, 1993a; Kopack et al., 2015). Potential prey individuals, while on the move, send clear visual and mechanical stimuli attracting the attention of predators (Sih, 1986; Johansson, 1992). Immobility helps avoid predators due to the reduction in water movements informing the predator of the presence of the prey individuals (Klemm, 2001; Barbosa-Júnior et al., 2010).

Thus, the monkey goby exhibited less pronounced anti-predator responses than the gudgeon (the lower number of different reaction types and lower intensity of those shown by both species). The lower responsiveness of the invasive monkey goby to the alarm cues could be attributed to their phylogenetical and ecological distance from the gudgeon. In nature, even closely related species within the same prey guild may differ in their responses to alarm cues, as shown for lobsters (Briones-Fourzán et al., 2006), flatfish (Borsma et al., 2008) and hemipteran bugs (Ferzoco et al., 2019). Thus, not only phylogenetic but also ecological aspects, such as habitat use, should be considered, including a combination of these factors (Sullivan et al., 2003), to understand the nature of the behavioural differences between the two species. Unfortunately, the in-depth research elucidating fine-scale differences in habitat use between the species with concomitant discrepancies in their behaviour is missing.

In connection with the weak anti-predator responses of the monkey goby and its high invasive potential in contrast to the gudgeon, a question arises whether these traits might be associated with the invasiveness. We tested two co-occurring species, one invasive, the other with no invasive history in the world. In some locations relative changes in their abundances were seen to coincide with the appearance of the invader (Jakovlić et al., 2015). It is worth noting that when an alien species enters a local assemblage and starts to compete with its members, the outcome of

this competition depends on differences between them, rather than on its general comparisons with other biota all over the world. Therefore, it is not important whether its responses to predators are generally strong or weak, but how they compare to those of local, co-occurring competitors. Assuming that the monkey goby in new areas “escape” from their natural competitors and predators, the need for defence against the enemies may be reduced in contrast to the gudgeon, facing its natural enemies. The energy saved this way can be allocated, for example, into faster growth or more effective reproduction (Keane & Crawley, 2002; Heger & Jeschke, 2014). However, it cannot be excluded that the weaker responses of the monkey goby to the alarm cues result from its ability to assess the predation risk more efficiently compared to the gudgeon because alarm substances are not the only cues indicating predation risk (Wisenden, 2015) and they alone may be too little of a stimulus to encourage greater fear responses in the fish. In both cases, the observed differences may contribute to the advantage of the invasive species over the native one. Nevertheless, we admit this is just a correlational reasoning based on two species. Therefore, further studies on a higher number of species and cues associated with predation are needed to confirm whether the interspecific differences in behavioural responses to alarm cues observed in our study are related to the invasive potential.

We have shown that two co-existing bottom-dwelling fish differ in their behavioural responses to predation cues, with the invasive species being generally less responsive than the non-invasive one. Moreover, both species turned out to be capable of detecting heterospecific alarm cues, which may be beneficial in a multi-species bottom fish assemblage. Nevertheless, their responses to conspecific alarms were clearly stronger than those to the other species’ cues.

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Authors’ contributions PK, TK, MPK, JK, RP and EJ collected and maintained fish. PK, JK, TK, EJ and RP conceived the ideas and designed methods. PK and MA conducted experiments under TK supervision. PK analysed

video data. PK, JK and TK analysed data. PK, TK, JK and MPK wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data Availability The datasets generated and analysed during the current study are available from the corresponding author on request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval We collected and used the fish under permit of the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland, statement no. 50/2017 from 28 September 2017. All procedures using fish met the European Union guidelines on the protection of animals used for scientific purposes (Directive 2010/63/UE). We took care to ensure compliance with animal welfare guidelines to minimize the welfare impact on subjects. We carried out the fishing and transportation with great care to minimise the duration of these operations as we did the direct handling of the fish to avoid any harmful effects (e.g. injuries, scale loss) that might cause undue stress and mortality. During the acclimation period in the stock tanks, we did not observe any negative consequences of transport or stock conditions (the fish were active, foraged and occupied shelters and their mortality was sporadic). During the tests, we noticed neither injuries nor mortality. Each individual was tested only once. After the tests, we released the native gudgeon into the wild, whereas the monkey goby, because of their invasive status, had to be killed (with an overdose of MS-222) and disposed of according to the Regulation of the Polish Minister of the Environment from 9 September 2011 (Journal of Laws No. 210, item 1260). We kept the number of individuals killed in order to obtain the alarm substances to a minimum. Killing was carried out by a qualified person holding an appropriate certificate (No. 2355/2015) issued by the Polish Laboratory Animal Science Association.

Consent to participate Not applicable.

Consent for publication Not applicable.

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