

Research Article

Friends or enemies? Chemical recognition and reciprocal responses among invasive Ponto-Caspian amphipods

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Abstract

Chemical communication plays a significant role in the aquatic environment, enabling the mutual recognition of mates, predators and food items. Invasive species facing new communities are subjected to a huge variety of new scents. Their ability to recognize and interpret them adequately could be the reason of their successful establishment. We studied reciprocal chemical recognition among invasive freshwater Ponto-Caspian gammarids (*Dikerogammarus villosus*, *Dikerogammarus haemobaphes* and *Pontogammarus robustoides*) that often co-occur in native and invaded areas, to test their ability to avoid via chemical signals possible competitor and/or top intraguild-predator. We used a Y-maze with inflow of water to the Y-arms from two source tanks containing the signal (scent of living animals or the alarm cues released by crushed individuals) or a control (dechlorinated tap water), respectively. Time spent in the Y-maze zones, number of visits to the Y-arms and animal activity were analysed. Only *D. villosus* was attracted to conspecific and heterospecific alarm cues as well as to living heterospecifics. The other two species avoided the scent of *D. villosus*. We conclude that *D. villosus*, as the strongest competitor and intra-guild predator (including cannibalism) among the tested species, perceived various scents as potential food cues and posed a threat to other gammarids.

Key words: biological invasions, Ponto-Caspian gammarids, preference, avoidance, chemical communication, kairomones

Introduction

Interactions among species are often preceded by mutual recognition, based on the information sent by donors and received by recipients (Ferrari et al. 2007). Depending on the environment and the organism itself, stimuli can be chemical, visual or/and mechanical (Smith and Harper 2003). In the aquatic environment, animals rely predominantly on chemical stimuli (Ferrari et al. 2010) due to the low visibility, high density and high viscosity of the medium (Sehr and Gall 2016). Thus, the role of chemical communication cannot be underestimated and there are numerous studies confirming that different aquatic taxa respond to minute concentrations of

chemical substances released by other organisms (Brönmark and Hansson 2000). The composition of chemical signals is a highly diverse blend of compounds (Ingvarsdóttir et al. 2002; Rittschof and Cohen 2004), enabling a fine-tuned recognition of the identity of odour sources by recipient individuals. Infochemicals are used for intraspecific communication in reproductive behaviour (Krång and Baden 2004), aggregation forming (Zimmer and Butman 2000), territorialism (Jaeger and Gergits 1979), signalling the presence of a predator to conspecifics (Kobak and Ryńska 2014), and in interspecific interactions, such as identification of predators (Szokoli et al. 2015), food detection (De Lange et al. 2005) and assessment of the strength of interspecific competition (Polo-Cavia et al. 2009).

A specific situation takes place when alien species colonize a novel habitat. They form new interactions with local biota and can re-create reciprocal links among organisms originating from the same region (Strauss et al. 2006). Progressive biotic homogenization followed by subsequent invasions of alien species constitute a perfect model for studying mutual recognition among them since, as a consequence, it might facilitate the establishment of subsequent invaders. For example, this may be due to the increased possibility to detect “known” food sources, enemies or interspecific alarm signals.

Although the chemical nature of these signals perceived by peracarid crustaceans is scarcely known, their effects have been extensively studied from the aspect of sexual behaviour and food path detection (Thiel and Breithaupt 2011), but less so from the aspect of competition avoidance and antipredator responses (Baumgärtner et al. 2003; Hesselschwerdt et al. 2009). Despite the chemical communication within an invasive community in a newly invaded territory being studied by several authors (Acquistapace et al. 2004; Corkum and Belanger 2007; Jermacz et al. 2017b), knowledge about this phenomenon is still insufficient. We studied the reciprocal recognition of chemical stimuli released by three species of freshwater gammarid crustaceans (Amphipoda, Gammaroidea) originating from the Ponto-Caspian region: *Dikerogammarus villosus* (Sowinsky, 1894), *Dikerogammarus haemobaphes* (Eichwald, 1841) and *Pontogammarus robustoides* (G.O. Sars, 1894). These species have colonised all the main rivers of Europe outside their native range (Grabowski et al. 2007b) and often co-occur in their native and invaded waterbodies (Jażdżewski et al. 2004; Berezina 2007; Leuven et al. 2009). They share over one million years of co-evolution and geological history, consisting of widely varying environmental conditions that have shaped their plasticity, which has consequently contributed to their invasion success (Reid and Orlova 2002). Thus, bearing in mind their common history, the communication among these species may increase their fitness in their new range (Jermacz et al. 2015; Kobak et al. 2016; Jermacz and Kobak 2017). These Ponto-Caspian gammarids are omnivorous, occupying variable trophic positions in

various communities (Hellmann et al. 2015), but many studies have pointed out their strong inclination for being carnivorous (van Riel et al. 2006; Platvoet et al. 2009; Bacela-Spychalska and van der Velde 2013). They exhibit intra-guild predation (IGP), feeding on related species (Kinzler and Maier 2003), as well as cannibalism (Dick and Platvoet 2000). Chemical interactions among aquatic crustaceans seem to be complex and difficult to predict. They can simultaneously perceive both hetero- and con-specifics as potential predators, prey, competitors and/or sources of alarm cues in addition to detecting conspecifics as sexual receptive mates (Wudkevich et al. 1997; Chivers and Smith 1998; Dunn et al. 2008; Thiel and Breithaupt 2011). Proper identification of available information allows an individual to assess the cost/benefit trade-off between multiple chemical cues, which announce the presence of other species as either prey or a potential threat (Chivers and Smith 1998).

We conducted Y-maze experiments to determine gammarid responses to various conspecific and heterospecific scents. We applied scents of live or crushed animals to simulate the presence of an active predator that may be detected as alarm cues released from injured individuals (Sih 1986; Wudkevich et al. 1997; Chivers and Smith 1998; Abjörnsson et al. 2000). We hypothesized that *D. villosus*, regarded as the strongest competitor and very efficient intraguild predator among the studied species (Rewicz et al. 2014), would respond positively to heterospecific signals; identifying them as weaker, potential prey. Furthermore, the presence of another species may provide information about a suitable shelter since all the tested species share the same preferences towards substrata – gravel and stones (Boets et al. 2010; van Riel et al. 2009; Jermacz et al. 2015). This hypothesis is congruent with the outcome of an experimental study upon induced dispersal of Ponto-Caspian amphipods, where displacement of weaker competitors was observed (Kobak et al. 2016). Moreover, we expected a *D. villosus* scent would be avoided by the other species, which could contribute to the reduction of direct negative interactions; such as competition and predation (Jermacz et al. 2015). Furthermore, we assumed that the alarm cues released by injured conspecifics, and possibly also by related species, should elicit an avoidance reaction. However, attraction of predatory and cannibalistic species to alarm signals may be regarded as a response to potential food stimuli (Jermacz et al. 2017b).

Materials and methods

Animals

We collected *Dikerogammarus villosus* and *Pontogammarus robustoides* from the Włocławek Reservoir (lower Vistula River, central Poland) (52°37'03"N; 19°19'37"E). *Dikerogammarus haemobaphes* was obtained from the Lucieńskie Lake situated in the Vistula valley (52°29'46"N; 19°26'44"E). The sampling was conducted in May–June 2014. Animals

were transported to the laboratory in 10 L plastic containers with aerated water. In the laboratory, we placed them into stock tanks with aerated and filtered water at 18 °C (sustained by air-conditioning) and fed them daily with frozen chironomid larvae and commercial fish food pellets. We kept the animals in separate tanks before experimentation to avoid their habituation to the scents of heterospecifics. This allowed us to observe their real preference for or avoidance of the applied cues. They were used in experiments 1–4 weeks after collection. Preliminary observations confirmed that gammarids survived well during this period and did not exhibit any behavioural symptoms of exhaustion due to prolonged captivity. We assessed the size of 100 randomly selected gammarids of each species with ImageJ 1.40 g software (W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/i>), using freeze-frames from video recordings. Measurements were made from the base of the first pair of antennae to the end of the urosome in still images. We chose adult individuals within the mean body lengths of $16.2 \pm \text{S.D. } 2.4$ mm (*D. villosus*), 14.8 ± 2.8 mm (*P. robustoides*), and 13.6 ± 2.4 mm (*D. haemobaphes*), which reflected natural differences in body size between the species (Jazdzewski 1975; Grabowski et al. 2007b). We used individuals of both sexes (receptive ones and those used to prepare species odours, see below) as we aimed at studying reactions at the species level, excluding the impact of sex on the behaviour. Our field work was performed in accordance with the Polish Water Law, which considers all lotic waters in Poland as public and provides Polish citizens unhindered access to them. Our study was performed only on invasive invertebrates and did not involve any endangered or protected species. Therefore, no institutional permits were necessary.

Experimental setup

We conducted our experiments in a glass Y-maze constructed according to an established design (Baumgärtner et al. 2002) (Figure 1) used in various other behavioural studies (Lee 1992; Pearl et al. 2000; Jutfelt et al. 2017; Johannesen et al. 2017). Gravitational inflow of water (0.5 L min^{-1}) to the Y-arms was provided by plastic tubes (12 mm in diameter) from two separate 40 L source tanks containing appropriate signals or control water. The suitability of the Y-maze was preliminarily tested with dyes, which were applied to the source tanks in order to investigate the distribution of currents in the arms. This provided us with a time needed for the signal to pass through the arms. These tests determined that the water currents in both Y-arms were independent of each other and it only took several seconds for the dye to pass through the Y-maze arms. The water currents from the arms mixed only in the mixing zone. This observation has been further confirmed by the results of an earlier test, where water of various salinity was added to the source tanks of a Y-maze of the same design (Kobak et al. 2017). Kobak et al. (2017) showed that both Y-arms clearly

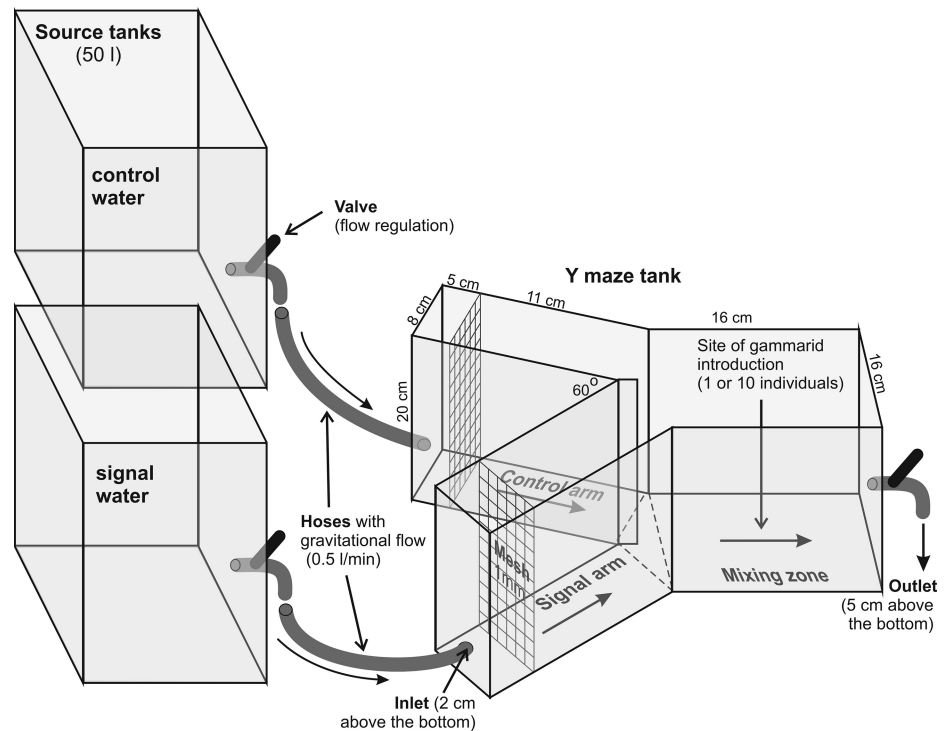


Figure 1. A diagrammatic representation of the Y-maze tank, including any accessories, used in the experiments. The arrows with full tips indicate the flow direction of the liquid containing the scent or control.

differed in salinity level, with the mixing zone having an intermediate concentration of ions. In order to keep the same flow rate and concentration of infochemicals, water in the source tanks was replaced and replenished to the initial level. Fresh signal was added to the signal tank before each replicate. After each trial, the source tanks were dried and washed with 96% ethanol before proceeding. The signal and control arms were switched between each replicate. The outlet from the mixing zone was covered by 1 mm mesh preventing tested animals from leaving the tank. Both inlets were separated from the part available for gammarids by mesh barriers which prevented the gammarids from moving upstream and also provided them with shelters. Homogenous indirect light conditions (520 lx) were established and controlled with a light meter (L-20A, Sonopan Ltd., Białystok, Poland). Water quality parameters (mean \pm SD), monitored with a multimeter Multi340i (WTW GmbH, Weilheim, Germany) were as follows: temperature: 17.7 ± 0.7 °C, pH: 7.69 ± 0.3 , conductivity: 473 ± 8.64 μ S/cm, oxygen saturation: $80.7 \pm 2.5\%$. Similar values were measured in the stock tanks.

Water containing living gammarid signals was obtained by incubation of ca. 400 individuals of a given species in a filtered and aerated 200 L tank filled with dechlorinated tap water (added to the tank 24 h before the start of the incubation) for 24 h at 18 °C. Gammarids in the incubation tank were not fed to avoid food cues in the signal water. The entire source tank was filled with water from the incubation tank. The control water was dechlorinated tap water prepared according to the same procedure as described above, but without any gammarids. The alarm cues of injured

gammarids were obtained by manual crushing of 20 individuals with 10 ml of control water. Such a mixture was filtered (40 μm gauze) and immediately added to the source tank filled earlier with control water. To adjust the experimental setup, preliminary tests were performed using established numbers of incubated and crushed individuals (Jermacz et al. 2017b), as well as an incubation time sufficient to obtain active infochemicals.

The signal water or alarm substance was added to the source tank immediately before each trial, ensuring the activity of the chemical signal. We tested each species in the presence of one of the above-mentioned cues (2 types of cues: 3 alarm signals and 3 scents of living animals, obtained from individuals of *D. villosus*, *D. haemobaphes*, *P. robustoides*) in one of the Y-arms, with control water in the other. We also conducted a control treatment with control water in both arms of the Y-maze to check for equality. Such a design allowed us to test whether gammarids responded to the detection of a signal positively, negatively or not at all. We intended to detect the presence of gammarid responses to particular signal sources, rather than their relative strength and/or interactions, thus we did not conduct treatments with two different gammarid signals in the Y-arms.

After stabilizing the flow, we introduced 10 gammarids into the mixing zone of the Y-maze. Each experimental trial lasted for 35 min, including 5 min for adaptation and 30 min of behavioural observations. Our experimental protocol was adjusted to the results of a study that estimated the activity time of gammarid chemical cue as approximately 3 h (Wisenden et al. 2009). Nevertheless, we applied the signal water immediately after it was prepared to reduce the risk of degradation. The experiment was recorded using an IP video camera (SNB-6004, Samsung, South Korea) placed above the tank. The experiment was replicated 10 times for each signal type.

In each treatment, we tested 10 groups of 10 gammarids according to the protocols of similar experiments already performed by other authors (Jermacz et al. 2017b; Wisenden et al. 2009). We were not able to follow individuals separately due to technical difficulties, but our experimental protocol allowed us to obtain the mean activity of the 10 gammarids. Group testing allowed us to avoid the influence of abnormal behaviour of particular individuals and the effect of their “personality” on the results (Kaldonski et al. 2007). Furthermore, amphipods always occur in high densities reaching up to a few thousand ind/m² (Dedju 1967). Thus, the experiment was set with respect to their natural conditions, since being alone would have been a deviation for those animals. It should be noted that in our experiment, when a group of gammarids were exposed to the conspecific cue, it was possible that the scent from the source tank (and that of the other individuals being tested) may have interfered with each other, weakening gammarid responses to the cue from the Y-arm. Nevertheless, tests with healthy conspecific signals were necessary to check whether gammarids reacted differently to conspecific and heterospecific

scents of particular taxa, rather than responding similarly to a general “gammarid cue” released by any species. Every individual in the experiment was used only once to avoid pseudoreplication.

Data analysis

We analysed the video recordings using Noldus Ethovision® XT 10 software. The individuals present in a single replicate were not independent of one another and the software sometimes switched the identities of the individuals that had touched during the experiment. To avoid pseudoreplications, all 10 individuals used in a single replicate were treated as a unit. Cumulative values (summed up for all group members) for each replicate were divided by 10 to obtain the average behaviour of gammarids in the group and were used as data points in any subsequent analyses (Jermacz et al. 2017b).

We assumed that gammarids sensing a cue from the signal zone would change: (1) the amount of time spent in this zone (relative to the control zone) and (2) the number of visits to the zone. We calculated an electivity index based on the Ivlev’s index (Ivlev 1961):

$$E = (R_s - R_c) / (R_s + R_c)$$

where R_s and R_c are the values of a response variable measured in the signal and control Y-arms from a given replicate, respectively. This approach considers the difference relative to the overall magnitude of gammarid behaviour (occupation time or number of visits to both Y-arms). Positive and negative difference values would indicate a preference for, or avoidance of, the signal. For the control treatment, we randomly assigned the Y-arms as signal or control. We assumed that the reduction in the time spent in the signal arm (compared to the control arm) and/or the reduction in the number of entrances to the signal arm (negative values of electivity indices) would indicate avoidance of a particular signal. The increase in one or both of these parameters (positive values of electivity indices) would indicate the preference. Moreover, an increase in the time spent in the signal zone accompanied by the reduction in the number of entrances to this zone could indicate a freezing response: the reduction in activity after signal detection.

We also expected that the signals might affect gammarid behaviour throughout the entire experimental tank, regardless of their zone selection. Such responses could not be detected by comparisons between the maze arms. Therefore, we also analysed (3) gammarid activity in the signal zone (where we expected the strongest response), expressed as the percentage of time spent while moving, relative to the total time spent in the zone. Moreover, it was possible that gammarids decreased their time spent in both Y-arms, staying in the mixing zone throughout particular treatments. To check this, we compared (4) the time spent in the mixing zone during each treatment.

Table 1. Statistical analysis of the behavioural response data from the groups of gammarids to conspecific and heterospecific signals (one-way ANOVA). Values in bold indicate statistically significant differences.

	Variable	Species	df ¹	MS ¹	F	P
A	Electivity based on the occupancy time of the signal and control y-arm	<i>D. villosus</i>	6, 63	0.53, 0.07	7.27	< 0.001
		<i>D. haemobaphes</i>	6, 63	0.59, 0.17	3.55	0.004
		<i>P. robustoides</i>	6, 63	0.28, 0.08	3.71	0.003
B	Occupancy time of the mixing zone	<i>D. villosus</i>	6, 63	0.42, 0.05	8.44	< 0.001
		<i>D. haemobaphes</i>	6, 63	0.61, 0.13	4.67	0.001
		<i>P. robustoides</i>	6, 63	0.10, 0.06	1.81	0.111
C	Electivity based on the number of visits to the signal and control y-arm	<i>D. villosus</i>	6, 63	0.15, 0.04	3.34	0.006
		<i>D. haemobaphes</i>	6, 63	5.49, 2.59	2.12	0.064
		<i>P. robustoides</i>	6, 63	1.42, 0.68	2.09	0.067
D	Activity in the signal zone	All species, only control treatments	2, 27	21.72, 0.49	44.29	< 0.001
E	Activity in the signal zone	<i>D. villosus</i>	6, 63	1.49, 0.26	5.81	< 0.001
		<i>D. haemobaphes</i>	6, 63	0.40, 0.25	1.63	0.155
		<i>P. robustoides</i>	6, 63	4.99, 0.20	25.46	< 0.001

¹ values for effect and error terms, respectively.

We used one-way ANOVAs to compare the following response variables among treatments (including the control treatment without signal in both arms): (1) electivity index based on the time spent in each of the Y-arms, (2) electivity index based on the numbers of visits to both Y-arms, (3) activity (log-transformed to stabilize variance and normality) and (4) the time spent in the mixing zone (log-transformed). We checked the normality and homoscedasticity of the data with Shapiro-Wilk and Levene tests, respectively. We analysed the behaviour of each species separately, as particular cues were not equivalent for them in terms of perceiving conspecific and heterospecific signals (i.e. for each species, different signals are either conspecific or heterospecific signals). For significant ANOVA effects, we compared gammarid behaviour in the presence of particular signals with those observed in the control treatment (in which we assumed no zone selectivity and basic activity unaffected by signals) using sequential Bonferroni corrected Fisher LSD tests.

To check interspecies differences in activity, independent of the applied signals, we applied a one-way ANOVA on the data using the control treatments only.

Results

Time spent in the zones

The analysis of the Y-arm electivity revealed that all the species responded to gammarid cues (Table 1A). *Dikerogammarus villosus* stayed longer in the signal zone in the presence of the cues of living *P. robustoides* and alarm substances released by crushed conspecifics and crushed *D. haemobaphes* (Figure 2A). *Dikerogammarus haemobaphes* avoided the signals of living *D. villosus* and *P. robustoides* (Figure 2B). *Pontogammarus robustoides* was repelled from the signal zone by the cues of living *D. villosus* and crushed conspecifics (Figure 2C). The time spent by gammarids in the mixing zone

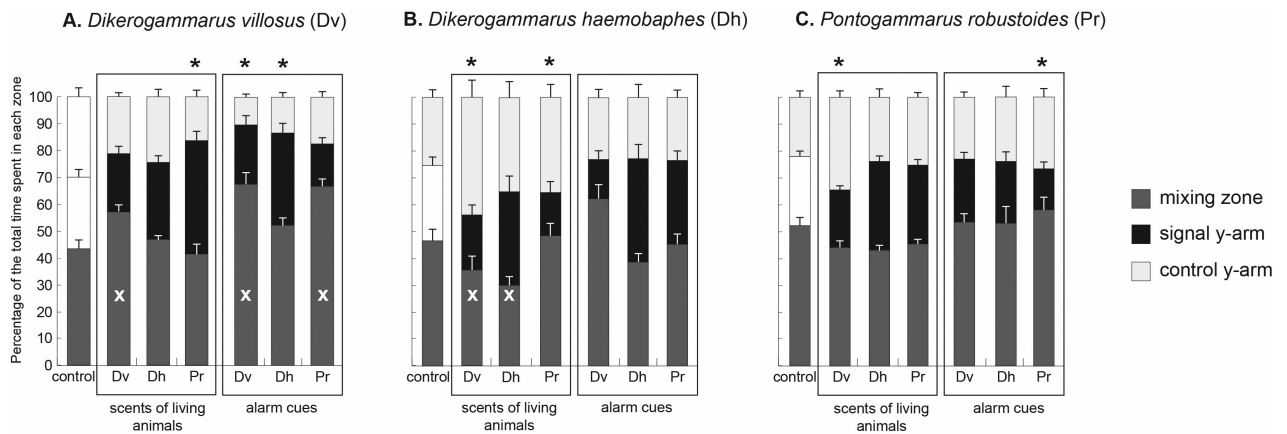


Figure 2. Percentage of time spent by gammarids in the mixing zone, as well as in the signal and control Y-arms. Asterisks indicate which treatments significantly different from the control via the electivity index and based on the time spent by gammarids in each Y-arm. “X” symbols indicate which treatments significantly different from the control, and are expressed as the time spent by gammarids in the mixing zone. Dv, Dh and Pr are abbreviations of *Dikerogammarus villosus*, *Dikerogammarus haemobaphes* and *Pontogammarus robustoides*, respectively. The error bars reflect the standard deviation of the mean.

differed among treatments for *D. villosus* and *D. haemobaphes*, but not for *P. robustoides* (Table 1B). Compared to the control treatment, *D. villosus* stayed in the mixing zone more often in the presence of crushed conspecifics and crushed *P. robustoides*, as well as in response to living conspecifics. *Dikerogammarus haemobaphes* exposed to the cue of living conspecifics and live *D. villosus* stayed in the mixing zone for a shorter time period than the control individuals (Figure 2B).

Number of visits to the zones

The gammarids moved from the mixing zone to one of the Y-arms 13.8 ± 10.6 times (mean per one individual \pm SD) during the analysed period. The notable exception was *D. haemobaphes*, which was much less active and switched between the mixing zone and Y-arms 1.1 ± 0.4 times on average. We observed significant differences in the electivity index based on the numbers of visits between the zones only for *D. villosus* (Table 1C), which entered the zone containing the living conspecific cues less frequently than the control Y-arm (Figure 3A). It was noted that the results for the other species were only marginally non-significant (Table 1C).

Activity

Activity of the species in the control treatments differed from one another (Table 1D). *Dikerogammarus villosus* was the most active and *D. haemobaphes* was the most immobile (Figure 4). *Dikerogammarus villosus* and *P. robustoides* modified their activity in response to gammarid cues, whereas the activity of *D. haemobaphes* did not vary significantly among treatments (Table 1E). *Dikerogammarus villosus* and *P. robustoides* reduced their activity in the presence of *D. haemobaphes* (both living and crushed). Additionally, *D. villosus* spent less time moving in response to living *P. robustoides* (Figure 4).

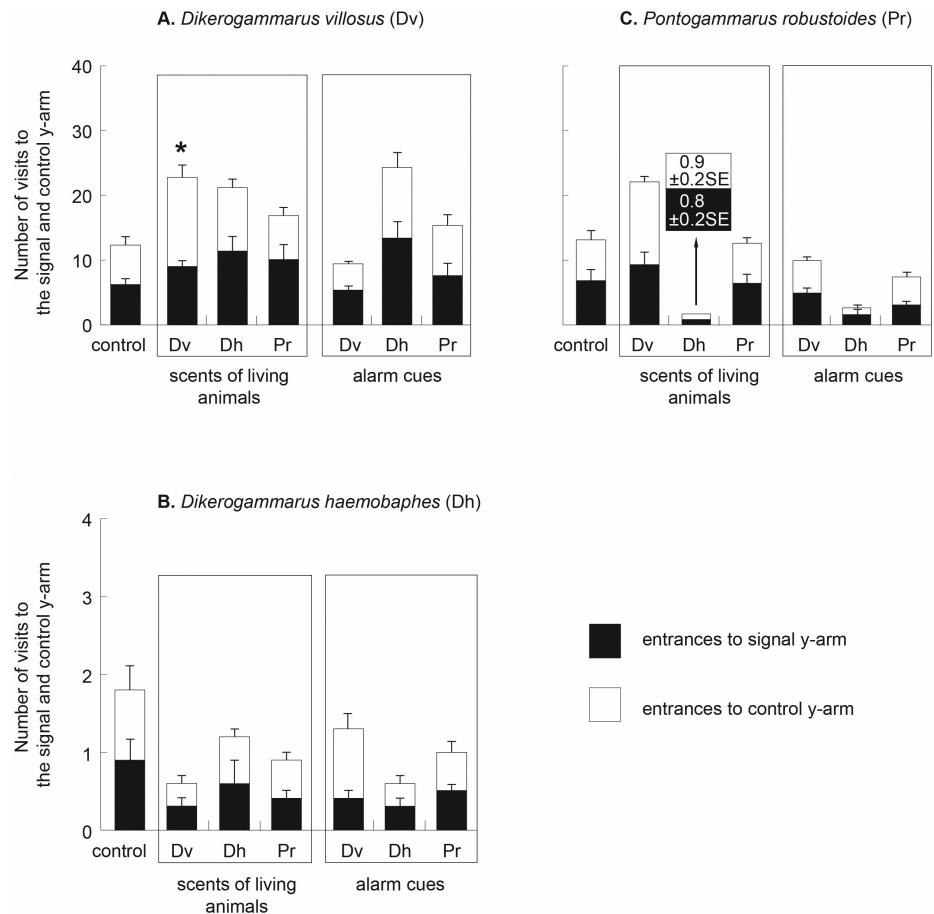


Figure 3. The number of entrances by amphipods to the signal and control Y-arms. Asterisks indicate which treatments significantly different from the control using the electivity index and based on gammarid visits to the Y-arms. Dv, Dh and Pr are abbreviations of *Dikerogammarus villosus*, *Dikerogammarus haemobaphes* and *Pontogammarus robustoides*, respectively. The error bars reflect the standard deviation of the mean.

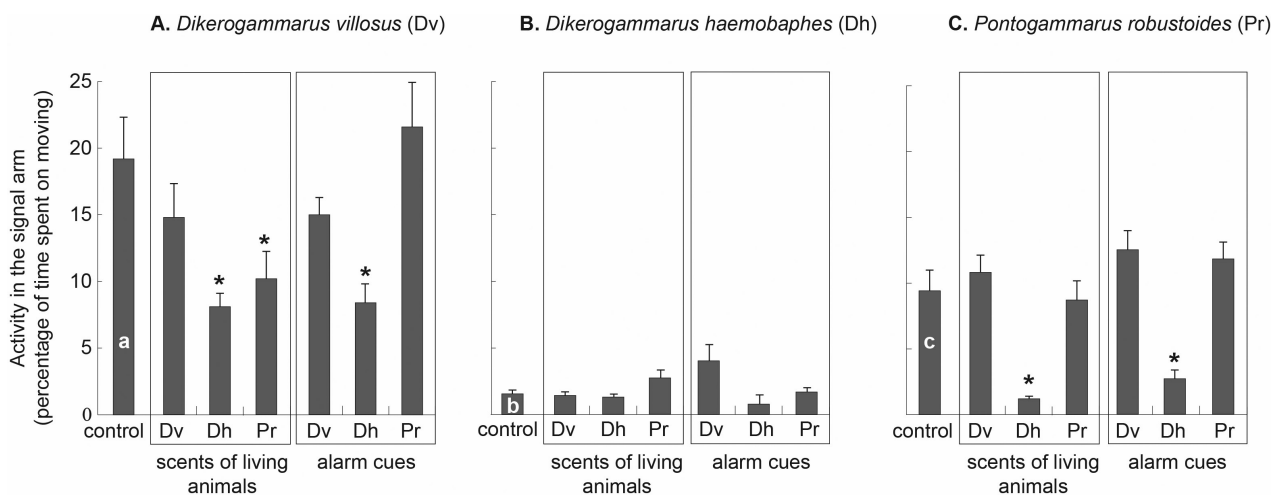


Figure 4. The activity of the gammarids (percentage of time spent on moving) in the signal Y-arm. Asterisks' indicate which treatments differ significantly from the control. Different letters on the control bars indicate which species significantly differing from another regarding their activity, under control conditions. Dv, Dh and Pr are abbreviations of *Dikerogammarus villosus*, *Dikerogammarus haemobaphes* and *Pontogammarus robustoides*, respectively. The error bars reflect the standard deviation of the mean.

Table 2. A summary of gammarid behavioural responses to conspecific and heterospecific signals (compared to the control treatment).

Species tested	Variable	Signal source					
		Living individuals			Alarm substances		
		Dv	Dh	Pr	Dv	Dh	Pr
<i>Dikerogammarus villosus</i> (Dv)	Time spent in the mixing zone	+	0	0	+	0	+
	Electivity (time in the y-arms)	0	0	+	+	+	0
	Electivity (visits to the y-arms)	–	0	0	0	0	0
	Activity in the signal y-arm	0	–	–	0	–	0
<i>Dikerogammarus haemobaphes</i> (Dh)	Time spent in the mixing zone	–	–	0	0	0	0
	Electivity (time in the y-arms)	–	0	–	0	0	0
	Electivity (visits to the y-arms)	0	0	0	0	0	0
	Activity in the signal y-arm	0	0	0	0	0	0
<i>Pontogammarus robustoides</i> (Pr)	Time spent in the mixing zone	0	0	0	0	0	0
	Electivity (time in the y-arms)	–	0	0	0	0	–
	Electivity (visits to the y-arms)	0	0	0	0	0	0
	Activity in the signal y-arm	0	–	0	0	–	0

+ stands for a preference or value increase, – stands for an avoidance or value decrease, 0 stands for no significant response.

Discussion

The study species differed from one another with respect to their responses to gammarid scents. They responded differently to the cues of various species (Table 2). *Dikerogammarus villosus* was the only species attracted to alarm cues and the scents of living heterospecifics. The other species either avoided or did not respond directionally to the infochemicals.

Responses of Dikerogammarus villosus

Numerous examples of avoidance behaviour induced by alarm signals have been reported so far (Ferrari et al. 2010; Verheggen et al. 2010; Richter et al. 2018; Ferzoco et al. 2019). Nevertheless, *D. villosus* were not only unrepelled, but were attracted by such stimuli released by conspecifics and the congener species, *D. haemobaphes*. Theoretically, a similar outcome might have been obtained as an effect of a freezing response to the alarm cue. For example, an animal entering the zone with the predation signal would have stopped its movement, which may eventually have resulted in an aggregation in this zone, giving a false impression of a positive response. However, we can exclude this explanation because the number of visits of *D. villosus* to the signal Y-arm was never lower than the number of visits to the control Y-arm in all the preference cases. This was not expected for a freezing response, which would have been associated with a lower number of visits to the zone.

Conversely, we found that *D. villosus* stayed in the mixing zone in the presence of a crushed gammarid signal. This could happen because *D. villosus* exhibits “sit and wait behaviour” with low locomotor activity (Becker et al. 2016), a high affinity for inhabited territory, and its tendency for spontaneous migrations is reduced in the presence of other species (Kobak et al. 2016). Nevertheless, when moved, *D. villosus* still did not avoid the signal Y-arm containing the alarm cues of various gammarid species.

Actually, it even selected the alarm cues released by conspecifics and *D. haemobaphes* (though, interestingly, it did not respond to those released by *P. robustoides*). Regardless of the time spent by this species in the mixing zone, we concluded that positive responses of *D. villosus* to conspecific and congener alarm cues were viable. It is likely that *D. villosus*, which is an omnivore, a scavenger and a cannibal, followed all these scents, perhaps by interpreting them as suitable food items (van Riel et al. 2006; Jermacz et al. 2017b), such as fresh carrion and/or wounded individuals that are easy to prey on.

Conspecific alarm odour emerges when a predator captures its prey, so should be recognized by conspecifics as potential danger. Błońska et al. (2015) found that *D. villosus* was not frequently consumed by goby fish when alternative gammarid prey was available. This is because this species has been found to have harder exoskeleton (Błońska et al. 2015), avoid fish predation more efficiently (Kobak et al. 2014; Beggel et al. 2016), be less mobile (Becker et al. 2016; Beggel et al. 2016) and to form protective aggregations more effectively (Jermacz et al. 2017 a) than other gammarids. Accounting for this, we suggest that *D. villosus* individuals in our experimental system might have not been under any eminent threat, probably due to the relatively low predatory pressure at the sampling site. The sampling site had an abundant bottom community including chironomid larvae, oligochaetes and other potential alternative prey for fish (Poznańska et al. 2009). A similar positive response detected in the isopod *Caecidotea intermedius* (Forbes, 1876) was observed to wounded conspecific cues. This has been attributed to a low predatory pressure in the wild (Spivey et al. 2015). Therefore, the scent of a wounded conspecific might indicate a potential food source for this gammarid, which has been frequently observed to feed on members of its own species (summarised in Rewicz et al. 2014). This might be particularly important at high densities, where the probability of encountering a wounded conspecific is relatively high and the availability of other food sources may be reduced due to high intraspecific competition, promoting cannibalism. Moreover, it has been observed that *D. villosus* is also attracted to the scent of fish predators that feed on gammarids, including *D. villosus* itself (Jermacz et al. 2017b). In our case, the crushed conspecific scent may be recognized by *D. villosus* as a food-related signal rather than a typical alarm cue, triggering avoidance responses.

Cannibalism seems to be the likely reason for the positive response of *D. villosus* to the crushed conspecific cue. This is further evidenced by *D. villosus* lacking a positive response to living conspecifics in our study. The latter signal was the only one to trigger the avoidance behaviour of *D. villosus*, indicated by the lower number of visits to the Y-arm with the scent of living conspecifics. This could be explained by its tendency to be cannibalistic (Kinzler et al. 2009), or possibly reveals an attempt to

decrease intraspecific competition, which is likely to be stronger than interspecific interactions. *Dikerogammarus villosus* tended to follow the scent of living *P. robustoides*. It is likely that *D. villosus* could select areas or shelters with existing gammarid communities as suitable habitat for its development, possibly due to their similar habitat demands but also because inhabited shelters are preferable by gammarids due to the benefits from aggregation forming (Abjörnsson et al. 2000; Musko 1994). Alternatively, this could have resulted from the antagonistic relationship between those species, particularly those with similar predatory habits. *Dikerogammarus villosus* has been commonly reported to exhibit intra-guild predation (Kinzler and Maier 2003).

All things considered, *D. villosus* is presently the most widespread invasive Ponto-Caspian crustacean and has recently dominated European freshwaters. Its apparent boldness, expressed by the ability to recognize and follow various amphipod scents, including those of crushed conspecifics, clearly differentiates it from other related species. Such behaviour is likely to help it find suitable habitats and food sources and suggests that it seems relatively safe from predation pressures. These traits may contribute to its invasive potential and increased success in newly invaded areas.

Responses of the other Ponto-Caspian species

Both *D. haemobaphes* and *P. robustoides* expressed avoidance behaviour in response to the scent of living *D. villosus*, suggesting that such infochemicals were interpreted as a potential threat. This also corroborates the positive response of *D. villosus* to the scents of heterospecific gammarids observed in our study. It has been shown that both *P. robustoides* and *D. villosus* share similar microhabitat preferences, but their interactions result in the displacement of *P. robustoides* (Jermacz et al. 2015). Moreover, these results, combined with the aforementioned preference of *D. villosus*, suggest that the tendency of these species to form single-species aggregations can be explained by the efficient avoidance of mixed groups by the weaker *P. robustoides*, rather than by the behaviour of *D. villosus* (Jermacz et al. 2017a). Both *Dikerogammarus* spp. exhibit similar habitat demands (Musko 1994) and the weaker species (*D. haemobaphes*) has been shown experimentally to be displaced by stronger *D. villosus* (Kobak et al. 2016). *Dikerogammarus haemobaphes* in our study also avoided the scent of living *P. robustoides*, which is another highly aggressive species (Bacela-Spychalska and van der Velde 2013) that is capable of displacing *D. haemobaphes* from its shelters (Kobak et al. 2016). We have shown that weaker gammarid species are able to recognize and avoid the scents of their stronger competitors, reducing the probability of direct encounters.

Pontogammarus robustoides was the only species in our experiments that avoided crushed conspecific alarm cues. Other species in our experiments

did not express such behaviour, despite the fact that this may indicate the presence of a foraging predator in the direct vicinity (Sehr and Gall 2016; Wudkevich et al. 1997).

Dikerogammarus haemobaphes appeared to be less active than the other species and did not switch the Y-maze arms often. The reason for this could be due to its tendency to remain within an established habitat. This was found to be higher in *D. haemobaphes* than in the other species (Kobak et al. 2016). A physical contact with a stronger competitor (*D. villosus*) is the primary reason for it to leave its site and migrate (Kobak et al. 2016).

Summary

Our study has demonstrated the importance of intra- and inter-specific chemical recognition with respect to the behaviour of freshwater invasive amphipods. It has shed new light on the nature of their reciprocal interactions from multiple perspectives. This is the first study researching the ability of gammarids to recognize chemical stimuli from other gammarid individuals and was tested on the most ubiquitous invasive species of the Ponto-Caspian gammarid community. We have shown that *D. villosus* is perceived as a threat by other amphipod species and that, even without a direct physical contact, other Ponto-Caspian species (*D. haemobaphes* and *P. robustoides*) avoid *D. villosus*. This may be one of the mechanisms of their displacement from occupied habitats (Kobak et al. 2016). Subsequently, *D. villosus* might follow displaced species, which could contribute to the large-scale spread of the Ponto-Caspian gammarids. Understanding the outcomes of the chemical perception in these species may contribute to the knowledge of factors that influence amphipod community composition in European fresh waters.

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Authors' Contribution

ŁJ, KBS and JK designed the study. ŁJ and JK designed the experiments. MR, ŁJ, MP and JK collected animals and carried out the experiment. MR and JK analysed and interpreted data. KBS and JK secured the funding. MR and JK wrote the manuscript with contributions from all the authors.

Declarations of interest

None

Ethics and Permits

All the authors acknowledge that they have complied with the institutional and/or national policies governing the humane and ethical treatment of the experimental subjects, and that they are willing to share the original data and materials if so requested. All research pertaining to this article did not require any research permit.

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