Behavioural differences and interactions between two sessile bivalves forming mixed-species assemblages

The invasive zebra mussel *Dreissena polymorpha* (ZM), established in Europe for a long time, has been recently joined and commonly outcompeted by a new invader, the quagga mussel *Dreissena rostriformis bugensis* (QM). To identify factors contributing to this displacement, we studied behavioural differences between the species: aggregation, movement, and responses to conspecifics, congeners, and their alarm cues. Compared to ZM, QM were more aggregated and less motile, crawling shorter distances for a shorter time at a slower speed. Conversely, QM exhibited more non-locomotor movements. Both species aggregated and burrowed less and showed more non-locomotor movements in response to conspecific and heterospecific alarm cues. They also moved shorter distances in the presence of conspecific alarm cues. ZM delayed their locomotion and non-locomotor movements, whereas QM started locomotion earlier in the presence of both alarm cues. Mussel responses to living heterospecifics resembled those to alarm cues. In mixed-species aggregations, ZM attached to conspecifics more often than to QM shells, whereas QM were non-selective. To summarize, QM are less mobile, less selective with regard to attachment site, and more aggregated than ZM. This allows QM to perform better in mixed-species assemblages by spending less energy on relocation and overgrowing ZM to a higher extent than vice-versa. Both species are capable of responding to heterospecific signals, which is helpful in mixed-species assemblages, particularly in novel areas occupied by these invasive species. Nevertheless, similar responses to alarm cues and living heterospecifics suggest a negative interaction between the congeners.

Keywords: aggregation, biological invasions, *Dreissena*, intraspecific signals, interspecific signals, movement, quagga mussel, predator cues, sessile animals, zebra mussel

Sessile animals commonly form large aggregations, structured as animal forests, reefs, or mussel beds (Rossi, Bramanti, Gori, & Orejas, 2017; Zimmer & Butman, 2000). Due to the large sizes of these aggregations (in terms of density and occupied areas), these structures can exert a strong impact on ecosystems, forming shelters for other organisms, providing rich food sources and transforming the abiotic environment (Gutiérrez, Jones, Strayer, & Iribarne, 2003; Sousa, Gutiérrez, & Aldridge, 2009). Thus, sessile animals act as ecosystem engineers with a multidimensional influence on their neighbourhood (Jones, Lawton, & Shachak, 1994) and belong to key members of aquatic communities. Due to their partial or complete immobility, these organisms exhibit a number of unique behaviours with regard to habitat selection, aggregation, reproduction, communication, and anti-predator defences (Sarà, 2009), which are remarkably different than those shown by mobile animals yet understudied so far.

In fresh waters, Ponto-Caspian dreissenid mussels (Fig. S1) provide a good example of sessile ecosystem engineers, structuring local environments (Karatayev, Burlakova, & Padilla, 2002) and affecting native biota (Sousa, Pilotto, & Aldridge, 2011). In addition, they belong to the most successful aquatic invasive species in the world, posing a threat to the economy and native communities, which further increases their importance to science and environmental protection (Gallardo, 2014). In recent years, the well-established species in Europe, the zebra mussel (ZM) *Dreissena polymorpha*, whose invasion started at the end of the 18th century (Bidwell, 2010), has been joined by its sympatric congener, the quagga mussel (QM) *D. rostriformis bugensis* (Orlova, Therriault, Antonov, & Shcherbina, 2005; Marescaux et al., 2016), which spreads rapidly and displaces the earlier invader from most co-

occupied locations (Matthews et al., 2014; Balogh, Vláčilová, G.-Tóth, & Serfőző, 2018), though a few notable exceptions from this rule have been noted (Strayer & Malcom, 2013; Zhulidov et al., 2010). In North America, where both species were introduced at shorter intervals (Ricciardi & Whoriskey, 2004), the scenario has been similar: ZM spread faster but was usually displaced in a few years after the appearance of QM (Patterson, Ciborowski, & Barton, 2005). A number of possible explanations for this displacement have been proposed, including lower energy expenditure (slower metabolism, lower investment into anti-predation defence) (Naddafi & Rudstam, 2013; Stoeckmann, 2003), faster growth (D'Hont, Gittenberger, Hendriks, & Leuven, 2018; Metz et al., 2018; Balogh, Serfőző, bij de Vaate, Noordhuis, & Kobak, 2019), earlier onset of reproduction in the season (Balogh et al., 2018), more efficient feeding (Baldwin et al., 2002), higher tolerance to cold (Orlova et al., 2005; Stoeckmann, 2003), and ability to live on soft sediments (Dermott & Munawar, 1993; Pavlova, 2012) exhibited by QM compared to ZM. Nevertheless, actual reasons for differences in the spread rate and displacement between the two invasive dreissenids remain uncertain.

Another group of traits differentiating the invasive potential of these species may be their behaviour and direct intra- and interspecific interactions taking place in mixed species assemblages, which can be complex and dependent on additional environmental factors (Babarro, Abad, Gestoso, Silva, & Olabarria, 2018). The behaviour of ZM has been relatively well studied with respect to responses to abiotic factors (e.g. temperature, light, water flow), predators, and conspecifics (Kobak, 2013). Nevertheless, comparative material concerning the behaviour of QM, as well as knowledge of reciprocal responses to each other and direct interactions between the two species has been scarce (Naddafi & Rudstam, 2013; D'Hont et al., 2018; Metz et al., 2018).

We experimentally studied mussel movement and aggregation forming in single and mixed-species assemblages and their responses to living conspecifics, congeners, and their alarm substances (predation cues) to test the following hypotheses: (1) QM are more aggregated and less selective with regard to the attachment site than ZM, which gives them an advantage in reciprocal fouling in a mixed-species assemblage; (2) QM are less mobile than ZM, losing less energy on searching for an attachment site; (3) QM respond to predation cues less strongly than ZM, saving more energy for growth and reproduction; (4) Mussels respond not only to conspecifics but also to congeneric signals, being able to identify alarm substances and the presence of living individuals interspecifically, which can be beneficial in a mixed-species assemblage. Testing these hypotheses would help determine behavioural traits of sessile organisms contributing to their competitiveness in a multi-species fouling community, and, specifically, find mechanisms contributing to the elimination of one dreissenid species by the other. Moreover, we would be able to shed more light on the interactions in a fouling community driven by intra- and interspecific communication.

METHODS

Animal collecting and housing

We collected mussels (ca. 5000 individuals of each species) in October 2019 at Keszthely station, in the nearshore zone of the western part of Lake Balaton (46°45'50.3"N 17°16'01.5"E), where both species still co-exist. We sampled mussels from the rip-rap stones (depth: 1.2–1.5 m). Directly after collection, we transported them in 50-L containers to the laboratory (1.5 h transport time), cleaned of epibionts, contaminants, and mud and identified to the species level.

We kept each species separately in 300-L tanks on the stone substratum at a density of ca. 8000 individuals per square metre, which is a common density at which these species

100 occur in the wild (Karatayev, Burlakova, & Padilla, 2015). The tanks were constantly aerated
101 and connected with systems of continuous water exchange (20% of total volume per day),
102 pumping water directly from Lake Balaton. We kept the temperature in the stock tanks at 16-
103 18 °C. The photoperiod was natural (October-November), not supported by any artificial
104 lights. We fed the mussels with an algal culture (*Scenedesmus* sp.) every day. We did not
105 observe any negative effects of transport and stocking conditions on mussel survival. We
106 acclimated the mussels in the stock tanks for at least one week before the tests and used them
107 in experiments within 5–6 weeks after collection. We carried out our experiments using
108 mussels <10 mm in length (mean length ±SD of QM and ZM: 8.3 ±1.0 and 8.4 ±1.0 mm,
109 respectively). Mussels of that size are responsible for most active post-settlement relocations
110 in this species because of their lower attachment strength (implying higher detachment
111 probability) (Balogh et al., 2019; Kobak, Kakareko, & Poznańska, 2010), higher motility
112 (Toomey, McCabe, & Marsden, 2002), and due to the fact that older mussels in a colony are
113 often overgrown by conspecifics, which further impairs their ability to detach and crawl to
114 another location (Kobak, Poznańska, Kakareko, 2009). After the experiments, we humanely
115 killed the mussels by freezing.
116
117 *General experimental conditions*
118 We conducted experiments in 1-L circular opaque plastic dishes (diameter: 12 cm, height: 8
119 cm) (Fig. 1) under constant fluorescent light in conditioned tap water (settled and aerated for
120 6 days before use) to enable video recording (impossible in highly turbid Balaton water). We
121 set the water level at 5 cm above the bottom surface, which was sufficient for undisturbed
122 mussel movements but prevented excess climbing to avoid problems with focusing the
123 camera and analysing the recordings. We established the amount of space provided for
124 mussels in our experiments on the basis of earlier experiences determining appropriate initial

distances, enabling interactions among individuals (Tošenovský & Kobak, 2016). These conditions were sufficient to allow natural mussel behaviour, as they are usually crowded and generally relocate only short distances (several cm) to find a suitable attachment site (Toomey et al., 2002; Kobak & Nowacki, 2007). During the experiments, we maintained water temperature at 17°C (sustained by air conditioning), oxygen concentration at 8.5 mg/l, and conductivity at 550 μS/cm (measured with a WTW ProfiLine Multi 3320 meter). These conditions are within the range suitable for the species (Karatayev, 1995) and the test animals were acclimated to them after collection. We used aquarium aerators to aerate the dishes during the experiments and avoid oxygen limitation, except for periods of video recording in the movement tests, where air bubbles could interfere with animal behaviour and blur the picture.

We carried out all experimental procedures in our study in accordance with ethical guidelines imposed by Hungarian and Polish law. We collected macroinvertebrates and worked on invasive species under permission OKTF-KP/517-2016 issued by the Hungarian National Inspectorate of Environment and Nature Protection.

Experiment 1: Aggregation forming on hard and soft substrata

We designed this experiment to test differences in mussel aggregation behaviour. We tested mussels in experimental dishes (Fig. 1A, Fig. S2A, B) (1) on sandy substratum (2-cm layer of fine sand preventing mussels from attaching to the bottom), where other individuals were the only available hard surfaces or (2) directly on the plastic dish bottom (alternative hard substratum suitable for mussels). Moreover, we tested mussels in (1) single and (2) mixed species treatments. In each treatment, we used 12 mussels (density of ca. 1000 ind. m^{-2}, realistic for the field conditions, Karatayev, 1995; Lewandowski & Stańczykowska, 2013) arranged in a circle with their anterior parts directed inwards (to facilitate contact among

individuals moving forward). In the mixed species treatment, each individual had one conspecific and one heterospecific neighbour. To prevent dreissenids from attaching to the dish walls, we isolated them with a cylinder (8 cm in diameter) made of mosquito mesh (diameter: 1 mm, material deterring dreissenid fouling, Porter & Marsden, 2008) (Fig. 1A, Fig. S2A, B). We put the substrata (sand or dish bottom) under water 24 h before the tests to allow biofilm development, which makes submerged materials more suitable for mussels. This period is sufficient to develop biofilms affecting mussel substratum selection (Kavouras & Maki, 2003). We conducted 4 runs of the experiment on consecutive dates, deploying 30 dishes simultaneously with randomly distributed experimental treatments. Altogether, we conducted each treatment in 20 replicates (see Table S1 for details of the experimental design). We cleaned the dishes and changed the water and substratum between replicates.

After 24 h of the test, we determined the number of mussels: (1) forming monolayer aggregations, i.e. staying in physical contact with other mussels but not attached to them; (2) forming druses, i.e. attached to other mussels' shells; and (3) singletons. We calculated the following response variables: (1) percentage of all aggregated mussels (druses and monolayer aggregations pooled); (2) percentage of druse-forming mussels relative to all individuals that joined aggregations (we subtracted one individual from each group assuming that the first specimen, to which the other adhered, did not select to form an aggregation); (3) mean crowding index (according to Jarman, 1974) based on all aggregated mussels. Mean crowding is a measure of a typical aggregation size (experienced by an average individual in the treatment), calculated as:

$$(1) \quad C = \sum_{i=1}^{k} N_i^2 \Big/ \sum_{i=1}^{k} N_i$$

Where N_i – the number of individuals in aggregation i, k – the number of all aggregations (including also singletons).

We analysed the data using a Generalized Linear Mixed Model (binomial distribution, log link function) (percentage variables) or General Linear Mixed Model (mean crowding index), including (1) substratum type (categorical factor: soft or hard bottom), (2) species composition (categorical factor: QM, ZM or mixed), (3) their interaction, and (4) run date (random factor, four levels).

Experiment 2: Aggregation forming in response to alarm substances

We designed this experiment to test the effect of alarm substances produced by conspecifics and congeners on mussel aggregation behaviour. We used a similar design as in Experiment 1 (Fig. 1A, Fig. S2B), but with the addition of crushed mussels placed outside the mesh cylinder surrounding the test individuals. To produce the alarm substance, we used 3 individuals of a single species per dish, crushed manually, directly before the experiment start. Thus, we tested both mussel species in 3 treatments: (1) control, (2) with conspecific alarm, and (3) with heterospecific alarm. We decided to conduct this experiment on the sandy substratum with the expectation that the danger perceived by mussels would be higher on the substratum preventing their attachment and forcing interactions with other individuals. Mussels experience such situations in druses on the sandy bottom, where other molluscs and sparsely distributed stones are the only available substrata. We conducted 4 runs of the experiment on consecutive dates, deploying 30 dishes simultaneously with randomly distributed experimental treatments. We replicated each treatment 20 times. However, due to technical difficulties with signal application and data collection, we lost some replicates (see Table S1 for actual replicate numbers used in data analysis).

At the end of the test, we determined the number of mussels: (1) forming monolayer aggregations; (2) forming druses; (3) singletons; and (4) singletons burrowed in sand (these were always non-aggregated). We calculated the following response variables: (1) percentage

of all aggregated mussels; (2) percentage of druse-forming mussels relative to all individuals that joined aggregations; (3) percentage of burrowed mussels relative to all non-aggregated mussels; and (4) mean crowding index (based on all aggregated mussels).

We analysed the data using a Generalized Linear Mixed Model (binomial distribution, log link function) (percentage variables) or General Linear Mixed Model (mean crowding index) including (1) mussel species (categorical factor: QM or ZM), (2) alarm substance type categorical factor: (conspecific, heterospecific, or none), (3) their interaction, and (4) run date (random factor, four levels).

Experiment 3: Selection of species as attachment sites

In the mixed species treatment of Experiment 1, the number of mussels attaching to other mussels' shells was low, which precluded more detailed analyses. Therefore, we conducted a separate experiment to check mussel selectivity for a particular species during druse formation. We put 10 QM and 10 ZM mixed randomly onto a 2-cm layer of sand in the experimental dish (Fig. 1B, Fig. S2C) and surrounded them with a mesh cylinder of 3 cm diameter, so that they were crowded inside and could form druses with other individuals of both species. We replicated this experiment 22 times.

After 24 h, we used a stereomicroscope (Olympus SZX10, magnification 10x) to determine the number of mussels of each species: (1) attached to conspecifics; (2) attached to heterospecifics; and (3) non-attached. For each species, we compared the observed percentage of mussels attached to conspecifics (relative to all individuals of this species attached to other mussels) with the percentage of available conspecifics in the dish (47%, as the number of available conspecifics was always lower by 1 from the number of heterospecifics: a mussel could not attach to itself) using a non-parametric Wilcoxon one-sample test. A significant result of this test would indicate either selectivity for or avoidance of conspecifics relative to

heterospecifics. Moreover, we used Wilcoxon paired samples tests to check for differences between percentages of conspecifics and heterospecifics attached to shells of each species.

Experiment 4: Movement activity in response to living mussels and alarm substances

We designed this experiment to check how chemical cues released by mussels (alarm substances or signals released by live individuals) affect movement activity of dreissenids. We used the same experimental dishes as in Experiment 1 (Fig. 1C, Fig. S2D). To exclude the possibility of mussel attachment to the bottom and increase their activity, we tested mussels on a 2-cm layer of sandy substratum, but did not surround them by a mesh cylinder, so they could find a suitable attachment site after reaching the dish wall or move further, depending on their preference. We placed a single mussel in the centre of the dish and tested it for 24 h in: (1) control water (conditioned tap water), (2) presence of a conspecific alarm substance, (3) presence of a heterospecific alarm substance, (4) presence of living conspecifics, (5) presence of living heterospecifics. We placed the signal source (3 crushed or living mussels) inside a mosquito mesh enclosure (diameter 4 cm) located at one of the walls of the experimental arena (Fig. 1C, Fig. S2D, Fig. S3). We recorded dreissenid behaviour under constant fluorescent light by an IP video camera (SNB-6004, Samsung, South Korea) placed vertically above the tanks. We replicated each treatment 27 times, 9 replicates per each of the three video cameras located in different parts of the laboratory room. We randomly assigned replicates of various treatments under each camera to 10 trial dates (see Table S1 for details of the experimental design).

We used Noldus Ethovision 10.1 video analysis software to determine the following behavioural variables: (1) distance moved, (2) percentage of time spent in locomotion, (3) percentage of time spent in non-locomotor movement (wriggling around or moving there and back without relocation >0.01 cm/min), (4) locomotion speed (only for relocation periods),

(5) turning angle (mean angle between directions moved in neighbouring 1-minute intervals of relocation periods), (6) timing of locomotion movements from the start of the experiment, and (7) timing of non-locomotor movements from the start of the experiment.

We calculated variables 6 and 7 according to formula:

$$(2) \quad D = \sum_{i=1}^{t} M_i \Big/ t$$

Where M_i – time (in min) from the beginning of the test for each minute i with mussel movement noted, t – total movement time (in min). High or low values of this index indicated that most of the movement took place late or early during the test duration, respectively.

We analysed the data using General Linear Mixed Models including (1) mussel species (categorical factor: QM or ZM), (2) treatment (categorical factor: single, with living conspecifics, living heterospecifics, conspecific alarm, or heterospecific alarm), (3) their interaction, and (4) run (random factor: 3 video camera locations in the lab).

General remarks on data analysis

We checked the General Linear Mixed Model assumptions using Shapiro-Wilk (normality) and Levene (homoscedasticity) tests. We log-transformed the movement data from Experiment 4 to meet these assumptions. We further examined the significant effects of General and Generalized Linear Mixed Models with sequential-Bonferroni corrected Fisher LSD tests and pairwise contrasts, respectively, as post-hoc procedures. We completed all analyses using SPSS 25.0 statistical package (IBM Inc.).

RESULTS

Experiment 1: Aggregation forming on hard and soft substrata

The percentage of aggregated mussels depended on the species composition of the group and substratum type, as shown by a significant interaction between these predictors in the Generalized Linear Mixed Model (Table 1A). QM aggregated more on the hard substratum than on sand, whereas the ZM aggregation level was independent of substratum type (Fig. 2A). Accordingly, on the hard substratum, QM aggregated more than ZM and the species did not differ from each other in their aggregation level on sand. Mussels in the mixed-species treatment aggregated similarly to those in both single-species treatments on sand and similarly to those in the ZM treatment on the hard substratum. However, mixed-species mussels were more aggregated on the hard substratum than on sand, similar to the QM individuals (Fig. 2A).

Mussels formed druses (Fig. 2B) more often on sand than on the hard substratum, as shown by a significant main effect of substratum in the Generalized Linear Mixed Model (Table 1B). Moreover, QM formed druses more often than ZM and mixed species groups, as indicated by a significant main effect of species composition (Table 1B).

Mean crowding (aggregation size) of mussels (Fig. 2C) was higher in QM on the hard substratum than in the other species compositions and on sand, as shown by a significant substratum x species composition interaction in the General Linear Mixed Model (Table 1C).

Experiment 2: Aggregation forming in response to alarm substances

Irrespective of their species, mussels aggregated less in the presence of alarm substances, both conspecific and heterospecific, than under control conditions (Fig. 3A), as shown by a significant effect of alarm source in the Generalized Linear Mixed Model (Table 2A). Moreover, QM formed druses more often than ZM (Fig. 3B), as indicated by a significant main effect of species in the Generalized Linear Mixed Model (Table 2B). The presence of alarm substances did not affect druse formation by mussels. In contrast, mean crowding was

higher in ZM than in QM (Fig. 3C), without any effects of alarm substances, which resulted in a significant main effect of species in the General Linear Mixed Model (Table 2C).

In the absence of alarm substances, non-aggregated QM more often burrowed in sand than ZM (Fig. 3D). The presence of alarm substances of both types decreased QM burrowing and the difference between the species disappeared, resulting in a significant species x alarm source interaction in the Generalized Linear Mixed Model (Table 2D). Nevertheless, the inhibiting effect of the conspecific alarm on QM burrowing was stronger than that of the heterospecific alarm (Fig. 3D).

Experiment 3: Selection of species as attachment sites

Significantly more ZM attached to conspecifics than to heterospecifics (medians: 29 vs. 10% of all individuals, 1st-3rd quartile ranges: 20-38 vs. 0-20, respectively, Wilcoxon one sample test: $Z = -3.72$, $P < 0.001$). In contrast, QM did not differentiate between species (medians: 29 vs. 20%, 1st-3rd quartile ranges: 13-39 vs. 10-28 attached to conspecifics and heterospecifics, respectively, Wilcoxon one sample test: $Z = -0.70$, $P = 0.485$). Moreover, more QM than ZM attached to QM shells (Wilcoxon paired samples test: $Z = -2.50$, $P = 0.012$), whereas the percentages of both species attached to ZM shells were the same (Wilcoxon paired samples test: $Z = -72$, $P = 0.472$).

Experiment 4: Movement activity in response to living mussels and alarm substances

ZM moved longer distances than QM (mean: 8.5 vs. 3.5 cm, maximum: 54 vs. 52 cm) and both species reduced their distances moved in the presence of a conspecific alarm substance (Fig. 4A), as shown by significant main effects of species and treatment, respectively, in the General Linear Mixed Model (Table 3A). Furthermore, mussels also showed a non-significant

tendency to reduce locomotion in the presence of living conspecifics (Fig. 4A). In 65% of cases, mussels exhibited non-locomotor movements before starting locomotion.

ZM spent more time in locomotion than QM (Fig. 4B; mean: 5.5 vs. 3.0% of the 24-h test duration, maximum: 35 vs. 41%, respectively) but less time in non-locomotor movements (Fig. 4C; mean: 2 vs. 7.5%, maximum: 26 and 60%, respectively), as shown by significant main effects of species in the General Linear Mixed Models (Table 3B and C, respectively). Mussels of both species spent more time in non-locomotor movements in the presence of heterospecifics (both living mussels and their alarm substances) and the conspecific alarm substance than single mussels and those accompanied by living conspecifics (Fig. 4C), as indicated by a significant main effect of treatment in the General Linear Mixed Model (Table 3C).

ZM exhibited higher locomotion speed (Fig. 4D) than QM (mean: 10.5 vs. 6.7 cm/h, maximum: 28 vs. 17 cm/h, respectively), as shown by a significant main effect of species in the General Linear Mixed Model (Table 3D). The presence of living mussels and alarm substances did not affect locomotion speed. The mean turning angle of relocating mussels did not depend on species or treatment (Table 3E) and was quite high (mean: 57 degrees/min, 95% confidence intervals: 55-60 degrees/min), indicating that mussels moved in circles, commonly changing the direction of their relocation.

Mussels initiated their non-locomotor movements on average 1 h (ZM) or 3 h (QM) after the start of the test. Locomotion started after 1.5 and 5.5 h, respectively. The fastest individuals of both species initiated their movements after a few min of the test, except locomotion of QM, which never started earlier than 14 min after the beginning of the test. The timing of movement events during the test depended on an interaction between species and treatment in the General Linear Mixed Models (Table 3F and G for locomotion and non-locomotor movements, respectively). ZM exhibited their movements earlier than QM in all

344 treatments (Fig. 4D). Moreover, ZM delayed their locomotion in the presence of living and
345 crushed QM (compared to their behaviour in the presence of conspecifics), and postponed
346 their non-locomotor movements in the presence of living QM and both alarm substances (Fig.
347 4D). In contrast, QM did not change timing of their non-locomotor movements in response to
348 any mussel cues, whereas their locomotion took place earlier during the exposure to ZM and
349 the conspecific alarm substance than in the presence of living conspecifics.
350
351 **DISCUSSION**
352 *Behavioural differences between quagga and zebra mussels*
353 In our study, QM and ZM clearly differed from each other in their behaviour (see Table S2
354 for a summary). QM were more crowded on the hard than on soft substratum and tended to be
355 more crowded than ZM. The former result contrasted our hypothesis, as we expected higher
356 mussel aggregation on sand, where no hard substratum alternative to mussel shells was
357 available. However, unlike ZM, QM can thrive on soft sediments (Dermott & Munawar,
358 1993; Pavlova, 2012). Moreover, due to their rounded ventral side (Beggel, Cerwenka,
359 Brandner, & Geist, 2015), a single QM may experience difficulties in keeping the upright
360 position on a flat hard surface without any support. Perhaps that is why they more often
361 selected contacts with other mussels on hard materials.
362 Compared to ZM, QM seem more adapted to life in large aggregations due to their
363 lower metabolic rate (and thus lower oxygen demands) (Stoeckmann, 2003) and higher
364 starvation tolerance (Baldwin et al., 2002). Accordingly, in our study, their crowding, in
365 particular the affinity to attach to other mussels' shells, was greater than that of ZM. The
366 higher crowding of QM vs. ZM was also observed by D'Hont, Gittenberger, Hendriks, &
367 Leuven (2018). Nevertheless, it should be noted that in our study both species generally
368 avoided forming druses. When during their movement over an experimental arena they

contacted another mussel, they could attach to its shell, stay in its vicinity, or continue relocation. The percentage of mussels attaching to other mussels' shells on the hard substratum (relative to all mussels that joined aggregations) was well below 50% (Fig. 2B), which shows that most of the individuals staying in the vicinity of another mussel did not attach directly to its shell. Similar results were previously obtained for ZM (Dzierżyńska-Białończyk, Jermacz, Maćkiewicz, Gajewska, & Kobak, 2018; Dzierżyńska-Białończyk, Skrzypczak, & Kobak, 2018), suggesting their avoidance of conspecific shell substratum as much as possible. In the current study, QM exhibited a similar, though somewhat weaker tendency. In a mussel bed, a strategy of attaching in the vicinity of other mussels, but not directly to them, may be an optimal utilization of crowding benefits (anti-predator protection, availability of partners for reproduction), while avoiding costs of life in a colony (increased competition, possibility of unwanted relocation with a mobile substratum, exposure of topmost individuals to hydrodynamical forces) (Burks, Tuchman, Call, & Marsden, 2002; Tuchman, Burks, Call, & Smarrelli, 2004). Therefore, if conditions permit, mussels are often observed to form wide monolayer aggregations with individuals densely packed next to one another but attached to the non-shell substratum (Dzierżyńska-Białończyk, Jermacz, et al., 2018), whereas druses are formed only when an alternative hard substratum is missing (Dzierżyńska-Białończyk, Skrzypczak, et al., 2018), which was also shown in the present study. In fact, a higher affinity for conspecific aggregations was exhibited by marine mussels, such as *Mytilus edulis* (Commito et al., 2014; Commito, Gownaris, Haulsee, Coleman, & Beal, 2016) and the salt-water dreissenid *Mytilopsis sallei* (He et al., 2019), which is likely due to the more demanding sea environment (more numerous and more diverse predators, stronger hydrodynamics), increasing benefits of contagious distribution. Indeed, Tošenovský & Kobak (2016) observed that zebra mussels aggregated more in flowing water conditions, compared to stagnant, but they still avoided druse formation when alternative hard substratum

394 was available. Nevertheless, dreissenids are common in lakes, and in rivers dominate at
395 locations with reduced flow (e.g. dam reservoirs or transition lake-river zones) (Lewandowski
396 & Stańczykowska, 2013), thus our results obtained in stagnant conditions explain their
397 behaviour in a large part of their field range.

398 In our study, ZM did not show any differences in their crowding level between the soft
399 and hard substratum. This is in contrast with the results by Kobak & Ryńska (2014) but in
400 accordance with those by Tošenovský & Kobak (2016). These contrasting outcomes may
401 result from different densities used in various studies; higher aggregation on sand than on the
402 hard material was observed in mussels tested at lower density (Kobak & Ryńska, 2014),
403 whereas no difference between substrata was found at higher experimental densities
404 (Tošenovský & Kobak, 2016, this study). The disadvantages of aggregated life (competition,
405 waste accumulation, shortage of food and oxygen) are manifested more drastically at higher
406 densities. Therefore, at higher densities, mussels less often group with other individuals even
407 on sand, which leads to the disappearance of the difference between the substrata.
408 Nevertheless, it should also be noted that profound inter-population differences might exist
409 within dreissenid species, as postulated by Marsden & Lansky (2000), which may be another
410 explanation of differences between our current results and some earlier studies.

411 In Experiment 4 (mussel motility), ZM were more mobile than QM; they moved longer
412 distances at a higher speed, started their relocation earlier, and spent more time in locomotion.
413 This would help them find a more suitable attachment site faster but also requires higher
414 energetic investment in locomotion, which may result in a shift in the trade-off between
415 locomotion and other life activities, such as growth and reproduction. Perhaps, lower habitat
416 selectivity, shown by QM in our study, reduces their needs to relocate in search of an
417 appropriate attachment site, allowing them to partition more energy into growth and

418 reproduction, which has been confirmed by field evidence (Balogh et al., 2018; D'Hont et al.,
419 2018; Metz et al., 2018).

420 Theoretically, differences in movement activity might have been accounted for by a
421 difference in physical condition between the compared mussel species, with weaker condition
422 associated with lower movement. However, QM were found to have higher glycogen (storage
423 material suitable as a condition indicator) contents than ZM at the same location as that used
424 for collecting specimens for our experiments (Balogh et al., 2019). Thus, this explanation of
425 our results can be excluded and we can confirm that we observed the actual interspecific
426 differences.

427 One type of activity that was exhibited more by QM than by ZM was non-locomotor
428 movements. In most cases, they consisted in turning around the central point without
429 relocation. Dreissenids seem unable to move directionally towards a chemical signal source
430 (Dzierżyńska-Białończyk, Skrzypczak, et al., 2018), which was also suggested by our current
431 Experiment 4, as mussels tended to move along a highly curved path, in circles, with many
432 turns indicating a random search for a suitable site around them. Thus, an attempt to find an
433 appropriate direction for subsequent locomotion may be rejected as an explanation for these
434 non-locomotor movements. Conversely, they may indicate attempts to burrow in sand instead
435 of attachment or find a suitable attachment site on the spot, without relocation. The former
436 solution is only available for QM, which is capable of surviving in soft sediments (Dermott &
437 Munawar, 1993; Pavlova, 2012). However, it should be noted that the intensity of non-
438 locomotor movements of mussels exposed to predation cues increased (Experiment 4),
439 whereas burrowing activity decreased in response to the same stimuli (Experiment 2). This
440 supports the third hypothesis, of non-locomotor movements being attempts to re-attach
441 without relocation as the first option tried by a mussel on unsuitable substratum. In natural
442 conditions, potential hidden attachment sites available to mussels on the soft substratum could

443 be some hard materials, e.g. gravel pellets buried in sand. It is only if this option fails that
444 mussels start locomotion, with ZM selecting this alternative more often than QM.
445 We found no clear differences in the intensity of responses of both species to predation
446 cues. This is in contrast to findings by Naddafi & Rudstam (2013), who observed weaker anti-
447 predation defences in QM compared to ZM and attributed this to the higher energetic
448 investments of the former species in growth and reproduction. This strategy seems beneficial
449 when predators exert relatively low consumptive effects on well armoured alien prey, to
450 which they are not well adapted after its recent invasion. This would be a likely contribution
451 to the higher competitive ability of QM over ZM. However, we have to discriminate between
452 two types of danger cues: indirect cues that indicate the occurrence of a predator somewhere
453 in the neighbourhood (e.g. predator kairomones, prey exudates in predator faeces) and direct
454 cues that indicate the presence of a foraging predator in the direct vicinity (alarm substances
455 released by crushed prey). Whereas the reduction in responses to indirect cues may be
456 beneficial under some circumstances (like those described above for QM), direct cues cannot
457 be neglected by a recipient. ZM exhibit clear qualitative differences in their responses to these
458 two cue types: in the presence of fish kairomones they are known to increase attachment
459 strength and aggregation (Kobak et al., 2010; Naddafi & Rudstam, 2013), whereas when
460 exposed to conspecific alarm cues, they cease all activity, including adhesion and metabolic
461 rate (Czarnołęski, Müller, Adamus, Ogorzelska, & Sog, 2010; Czarnołęski, Müller, Kierat,
462 Gryczkowski, & Chybowski, 2011; Antoł, Kierat, & Czarnołęski, 2018). Accordingly, in our
463 study, both species responded to alarm substances with similar strength, by reducing their
464 overall activity (aggregation, burrowing, locomotion). Such a behavioural change may reduce
465 the probability of detection of prey by a predator responding to movement (visual cues, water
466 currents generated by active mussels, chemicals released from the exposed mantle surface)
467 (Antoł, Kierat, & Czarnołęski, 2018). The observed activity reduction supports the above

cited studies but contradicts that by Kobak & Ryńska (2014), who found increased ZM locomotion in response to conspecific alarm cues in light. This may be accounted for by the presence of a mesh cylinder with the signal source in the experimental arena in our current study (Fig. 1C, Fig. S2D). As mussels were previously found unable to move directionally (Dzierżyńska-Białończyk, Skrzypczak, et al., 2018), they responded to the presence of a signal, rather than to its location in the arena. Therefore, they could use the cylinder as a shelter and cease their activity after reaching its wall, which accounts for shorter distances covered by threatened individuals. This setup seems more realistic than that used by Kobak & Ryńska (2014), where mussels had no shelter in the arena and moved endlessly in a circular dish. The results of these two studies together indicate that mussels move in response to danger cues in search for an appropriate shelter.

Interspecific interactions between quagga and zebra mussels

We found a profound difference in reciprocal interactions between both dreissenid species (see Table S3 for a summary). In Experiment 3 (mussel attachment to conspecific and heterospecific shells), QM attached equally to the shells of both species, whereas ZM more often attached to conspecifics. This is unlikely to result from an unequal locomotion rate of QM and ZM (see Experiment 4 on mussel motility) and the following difference in availability of both species as a substratum. In such cases, both species would be unequally distributed and QM, as the less mobile species, would be a more available substratum. Thus, ZM exhibited either avoidance of QM or preference for conspecifics. Other studies showed that ZM rather reluctantly attached to conspecific shells, selecting other substrata (Dzierżyńska-Białończyk, Skrzypczak, et al., 2018; Kavouras & Maki, 2003), including other bivalve shells if available (Dzierżyńska-Białończyk, Jermacz, et al., 2018). Moreover, in our Experiment 3, ZM generally attached to other mussels' shells less often than QM. These

493 results suggest that the hypothesis of QM avoidance by ZM is more likely. Antifouling
494 properties in chemical structure and texture of the shell have been found in marine bivalves,
495 helping them defend themselves against excessive fouling by sessile biota, impairing the
496 functioning of a fouled individual (Bers et al., 2006, 2010). Such relations between both
497 mussel species are likely to favour QM in mixed druses, as they would attach willingly to
498 other mussels' shells irrespective of their species identity. In contrast, ZM might waste more
499 energy for site selection and finally be forced to attach to undesired substratum, particularly
500 when QM start to prevail in the assemblage. The lower habitat selectivity of QM vs. ZM (with
501 regards to exposure to light) was also observed by D'Hont et al. (2018). Such a trait benefits
502 QM in a variable environment, where optimum substratum is limited, allowing it to take up
503 available sites earlier and thrive on a wider range of materials.
504 This difference in attachment site selection preferences between the species may also
505 account for the intermediate aggregation levels obtained in the mixed species treatment in
506 Experiment 1 (mussel aggregation in various species compositions). It is likely that QM
507 aggregated irrespective of their neighbour species identity, whereas ZM had less possibilities
508 than in the single species treatment, which resulted in the higher aggregation level on the hard
509 substratum than on sand (due to QM responses) but also in the overall lower aggregation than
510 in the single species QM treatment on the hard substratum (due to the avoidance of QM by
511 ZM).
512 Both dreissenid species were able to detect signals not only from conspecifics but also
513 from congenerics. This is highly beneficial in a mixed-species assemblage of organisms
514 occupying a similar ecological niche, as they can use such information to find a suitable site
515 (Vaughn, Nichols, & Spooner, 2008) or prepare for predator attacks (Chivers & Smith, 1994;
516 Rachalewski, Jermacz, Bącela-Spychalska, Podgórska, & Kobak, 2019). Interestingly, in
517 Experiment 4 (motility in response to mussel cues), mussel responses to living congenerics

518 resembled those exhibited in the presence of alarm cues. This suggests negative interactions
519 between the species, which seem to exhibit behavioural symptoms of stress in a mixed-
520 species group. Actually, life in a mixed-species aggregation may be associated with several
521 costs. First of all, ZM may suffer from the presence of a superior competitor, which feeds
522 more effectively (Baldwin et al., 2002) and fouls congeneric shells more efficiently (this
523 study). Moreover, both species may suffer during spawning, when some gametes would be
524 wasted for failed fertilization or hybrid forming during random interspecific encounters in the
525 water column (Babcock, 1995), given that gamete recognition mechanisms between
526 dreissenids are not tight and the formation of hybrids has been documented experimentally
527 (Nichols & Black, 1994).
528
529 *Summary and conclusions*
530 We have shown that both dreissenid species clearly differ in behaviour with QM being less
531 mobile, less selective for attachment site, and more aggregative than ZM. Moreover,
532 dreissenids reciprocally perceived other species signals, responding negatively to
533 heterospecifics. These behavioural differences are likely to contribute to the competitive
534 superiority of QM, but also suggest a suite of traits likely to be beneficial in sessile mixed-
535 species assemblages in general. These traits include lower selectivity for attachment site,
536 which decreases the need for relocation in search for a suitable location (thus saving energetic
537 resources). This may be made possible by the higher tolerance to crowding, e.g. due to more
538 efficient feeding and/or lower metabolic rate, as shown for QM vs. ZM (Baldwin et al., 2002;
539 Stoeckmann, 2003). Another advantage of a sessile organism in a mixed-species aggregation
540 is the superiority in settling on and overgrowing other members of the assemblage. This may
541 help it find better environmental conditions (on the top of a colony) and limit negative
542 impacts of other colony members (Burks et al., 2002; Tuchman et al., 2004). Furthermore,

543 organisms living in multi-species assemblages may benefit from detecting heterospecific
544 signals, as we showed for both dreissenid species in our study. This is particularly important
545 for individuals occurring outside their native range, exposed to unknown stimuli produced by
546 their new environment. The presence of familiar signals released by co-occurring species and
547 informing of the presence of shelter, food or, as in our case, danger, may help them survive
548 the initial post-introduction period (Rachalewski et al., 2019). Finally, we demonstrated that
549 the mechanisms of mixed-species aggregation forming may include situations where animals
550 group together despite their preferences, with the lack of alternative substratum as the main
551 driver, or because the avoidance of one species (ZM) is not enough to overrule the preference
552 or non-selectivity of the other fouler (QM).

553 The lower locomotion activity of QM may limit its long-distance dispersal by reducing
554 the probability of attachment to mobile objects, such as boat hulls. Moreover, higher short-
555 term attachment rates (Balogh et al., 2019; Peyer, McCarthy, & Lee, 2009) and shell strength
556 (Balogh et al., 2019; Casper & Johnson, 2010), as well as better survival in air (Collas,
557 Karatayev, Burlakova, & Leuven, 2018) exhibited by ZM contribute to their better ability to
558 use human vectors to spread (Collas et al., 2018). This may account for the overall lower
559 dispersal rate of QM noted in most of the habitats invaded by dreissenids in Europe and North
560 America (van der Velde, Rajagopal, & bij de Vaate, 2010). Conversely, QM, as less selective
561 with regard to microhabitat (this study, D'Hont et al., 2018) and capable of living on soft
562 substratum (Dermott & Munawar, 1993), may be more likely to find a suitable site and
563 survive when accidentally dropped in a new area.

564 Differences between the dreissenid species may also affect their environmental and
565 economic impact, which seems especially important given the replacement of ZM by QM
566 taking place across Europe and North America (Ricciardi & Whoriskey, 2004; Patterson et
567 al., 2005; Matthews et al., 2014). QM, as more tolerant to crowding, and also to soft

substratum (Dermott & Munawar, 1993), may be able to reach higher densities when the availability of hard surfaces is limited (e.g. in areas with lower human impact). However, the lower attachment strength observed in QM (Peyer et al., 2009; Grutters, Verhofstad, van der Velde, Rajagopal, & Leuven, 2012) may facilitate mechanical eradication of dreissenid assemblages dominated by this species. Nevertheless, some studies show that this picture may be more complex, as QM seems to make up for its initial weaker adhesion after longer exposure (Peyer et al., 2009) and/or at larger size (Balogh et al., 2019). More crowded QM colonies will probably provide aquatic invertebrates with better anti-predator protection (Karatayev et al., 2002) by forming more complex 3-D structures on the bottom. Furthermore, the environmental impact of dreissenids, which is strongly related to their clumping and activity, can be reduced by non-consumptive effects of high predation pressure, inhibiting their locomotion, aggregation (this study), valve movements (Dzierżyńska-Białończyk, Jermacz, Zielska, & Kobak, 2019), and attachment (Czarnołęski et al., 2010).

Our study contributes to the growing body of evidence demonstrating profound behavioural, physiological and life history-based differences between both dreissenid species. The question remains open whether these differences will translate into changes in the impact and functioning of freshwater mussel beds in invaded ecosystems in the light of the ongoing replacement of ZM by QM. Our study suggests such possibilities, but this environmental change deserves further research explaining its mechanisms and consequences.

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Table 1. Analysis of the effects of substratum type and species composition of the group on mussel aggregation (Experiment 1) with Generalized Linear Mixed Model (binomial distribution, log link) (a-b) and General Linear Mixed Model (c). The models include a random run date factor (not shown, non-significant in all cases). Asterisks indicate significant effects.

	Response	Predictor	*df*	*F*	*P*	
(a)	% aggregated mussels	Substratum	1	7.42	0.007	*
		Species composition	2	7.97	0.001	*
		Interaction	2	4.87	0.009	*
		Error	113			
(b)	% druse-forming mussels	Substratum	1	8.67	0.004	*
		Species composition	2	5.27	0.007	*
		Interaction	2	2.94	0.057	
		Error	106			
(c)	Mean crowding	Substratum	1	20.10	0.002	*
		Species composition	2	1.20	0.306	
		Interaction	2	5.93	0.004	*
		Error	111			

Table 2. Analysis of effects of conspecific and heterospecific alarm substances on mussel aggregation (Experiment 2) with Generalized Linear Mixed Model (binomial distribution, log link) (a, b, d) and General Linear Mixed Model (c). The models include a random run date factor (not shown, non-significant in all cases). Asterisks indicate significant effects.

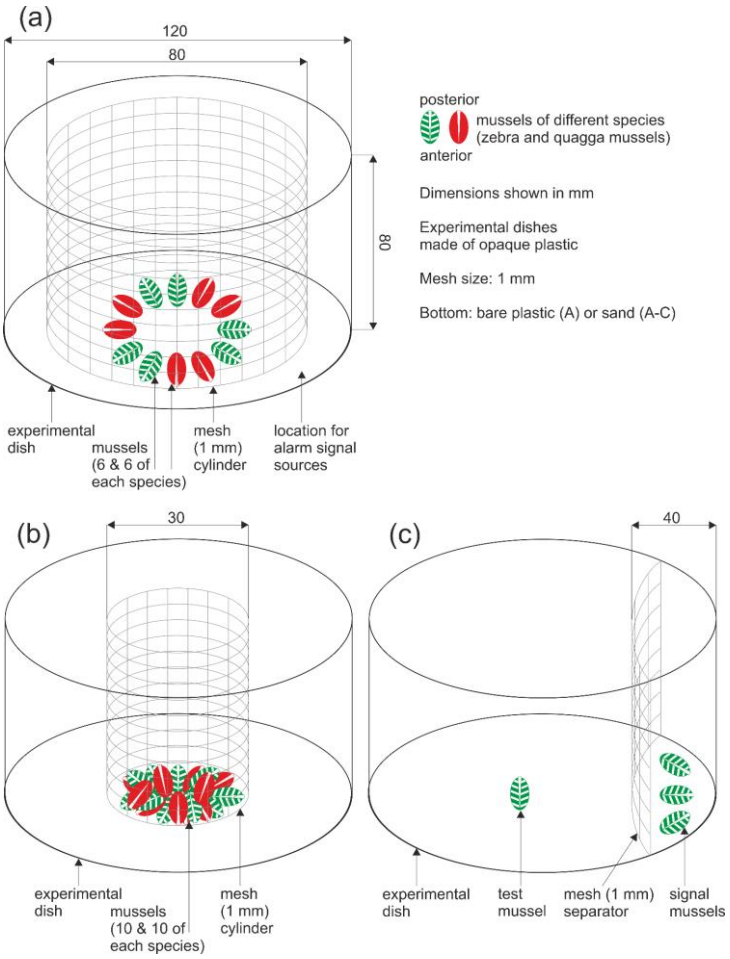
	Response	Predictor	*df*	*F*	*P*	
(a)	% aggregated mussels	Species	1	1.93	0.169	
		Alarm source	2	3.51	0.032	*
		Interaction	2	0.24	0.787	
		Error	92			
(b)	% druse-forming mussels	Species	1	9.92	0.002	*
		Alarm source	2	0.91	0.406	
		Interaction	2	1.08	0.345	
		Error	87			
(c)	Mean crowding	Species	1	5.31	0.024	*
		Alarm source	2	2.42	0.095	
		Interaction	2	1.12	0.330	
		Error	87			
(d)	% of burrowed mussels	Species	1	10.02	0.002	*
		Alarm source	2	3.92	0.024	*
		Interaction	2	7.35	0.001	*
		Error	72			

Table 3. Analysis of effects of species and presence of living conspecifics, heterospecifics, or their alarm substances on mussel movement activity (Experiment 4) with General Linear Mixed Model. The models include a random run (video camera location) factor (not shown, non-significant in all cases). Asterisks indicate significant effects.

	Response	Predictor	*df*	*F*	*P*	
(a)	Distance moved	Species	1	26.59	<0.001	*
		Treatment	4	2.49	0.044	*
		Interaction	4	0.98	0.422	
		Error	258			
(b)	% time in locomotion	Species	1	15.78	<0.001	*
		Treatment	4	1.77	0.136	
		Interaction	4	0.94	0.440	
		Error	258			
(c)	% time in non-locomotor movement	Species	1	81.89	<0.001	*
		Treatment	4	2.93	0.021	*
		Interaction	4	1.10	0.355	
		Error	258			
(d)	Locomotion speed (relocating mussels only)	Species	1	39.28	<0.001	*
		Treatment	4	1.30	0.272	
		Interaction	4	1.87	0.117	
		Error	199			
(e)		Species	1	0.79	0.374	
		Treatment	4	1.07	0.372	

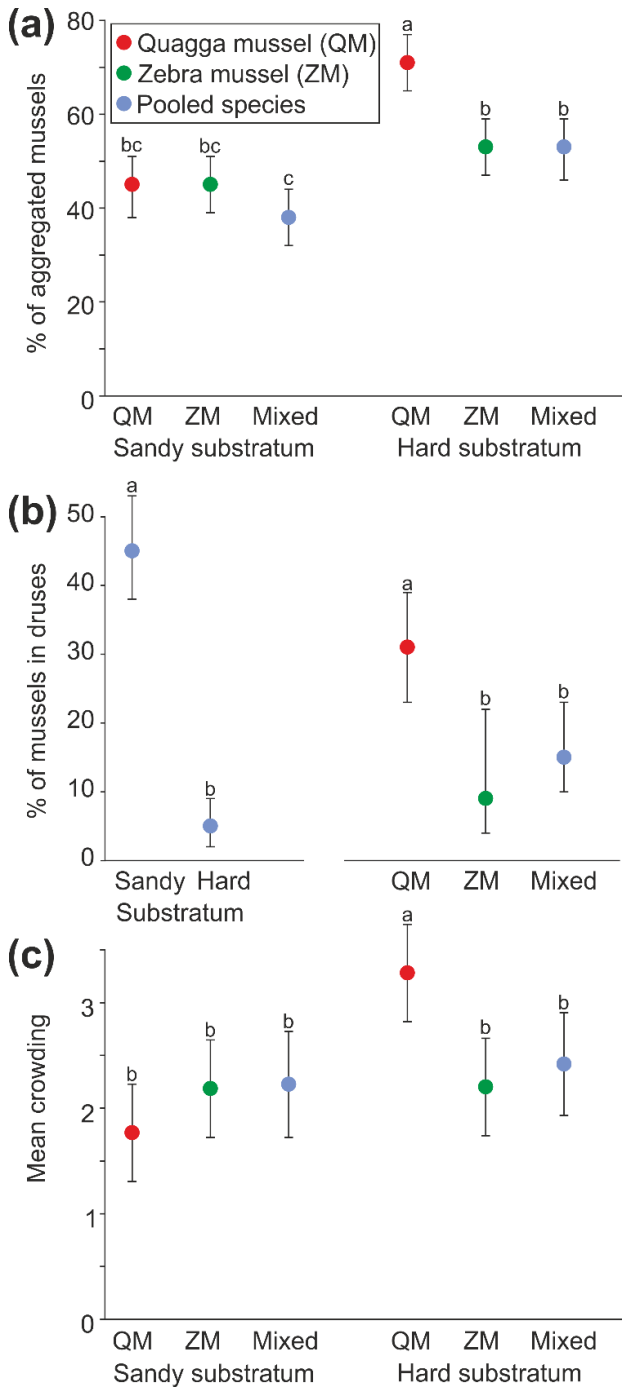
	Turning angle (relocating mussels only)	Interaction	4	0.38	0.821	
		Error	199			
(f)	Timing of locomotion from the trial start (relocating mussels only)	Species	1	73.20	<0.001	*
		Treatment	4	1.95	0.104	
		Interaction	4	3.69	0.006	*
		Error	199			
(g)	Timing of non-locomotor movements from the trial start	Species	1	83.88	<0.001	*
		Treatment	4	3.17	0.015	*
		Interaction	4	4.21	0.003	*
		Error	225			

831 **FIGURES**



832

833 Fig. 1. Experimental design: (a) Experiment 1 and 2, (b) Experiment 3, and (c) Experiment 4.



834

835 Fig. 2. Aggregation of quagga mussels (QM), zebra mussels (ZM) and mixed species groups

836 (Mixed) on hard and soft (sandy) substrata (Experiment 2). (a) Percentage of all aggregated

837 mussels (druses and monolayers pooled); (b) Percentage of druse-forming mussels (attached

838 to other mussel shells) relative to all mussels that joined aggregations; (c) Mean crowding

839 index (aggregation size experienced by an average individual). Presented values are

840 estimates predicted for significant terms of General and Generalized Linear Mixed Models

(Table 1). Treatments labelled with the same letters do not differ significantly from one another (post-hoc comparisons).

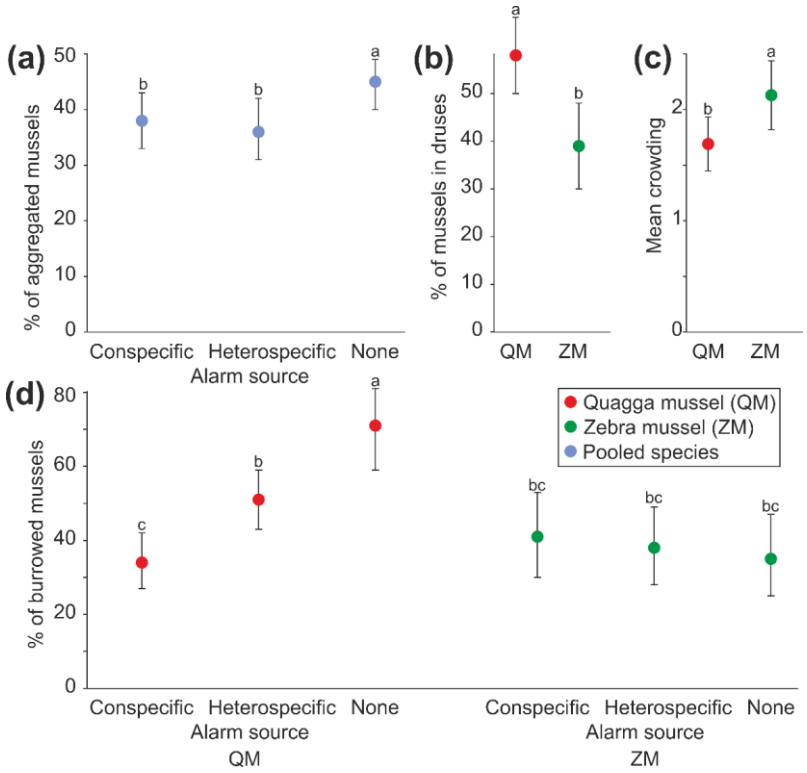
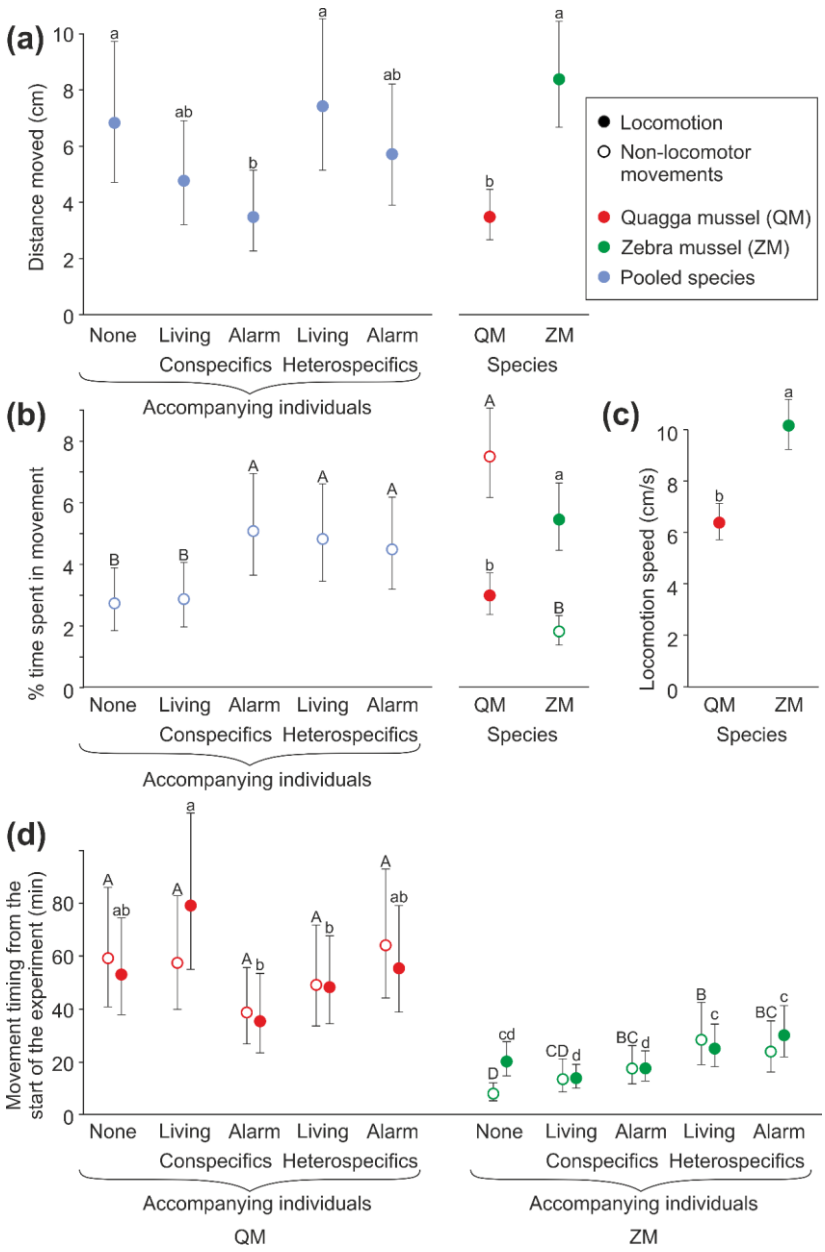


Fig. 3. Aggregation of quagga (QM) and zebra mussels (ZM) in response to conspecific and heterospecific alarm substances (Experiment 2). (a) Percentage of all aggregated mussels (druses and monolayers pooled); (b) Percentage of druse-forming mussels (attached to other mussel shells) relative to all mussels that joined aggregations; (c) Mean crowding index (aggregation size experienced by an average individual); (d) Percentage of mussels burrowed in sand (relative to all non-aggregated individuals). Presented values are estimates predicted for significant terms of General and Generalized Linear Mixed Models (Table 2). Treatments labelled with the same letters do not differ significantly from one another (post-hoc comparisons).



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858

859 Fig. 4. Movement activity of quagga (QM) and zebra mussels (ZM) in response to living

860 conspecifics, heterospecifics, and their alarm substances (Experiment 4). (a) Distance

861 moved by mussels; (b) Percentage of time spent on locomotion and on non-locomotor

862 movements; (c) Locomotion speed; (d) Timing of movement events from the start of the

863 experiment (lower and higher values indicate that most of the movement took place early or

864 late during the test duration, respectively). Solid and open symbols refer to locomotion and

865 non-locomotor movements, respectively. Presented values are estimates predicted for

866 significant terms of General Linear Mixed Models (Table 3). Treatments labelled with the
867 same lowercase and capital letters do not differ significantly from one another (post-hoc
868 comparisons) in locomotion and non-locomotor movements, respectively.
869
870