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WYDZIAŁ BIOLOGII I OCHRONY ŚRODOWISKA

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Czynniki wpływające na aktywność lokomotoryczną, rozmieszczenie i ruchy muszli małża *Dreissena polymorpha* (Pallas, 1771)

Rozprawa na stopień naukowy doktora

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Spis treści

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

1.	**Dzierżyńska-Białończyk, A.**, Skrzypczak A., Kobak, J. 2018. Happy together? Avoidance of conspecifics by gregarious mussels. Current Zoology 64(1): 53-61. (współudział w zaplanowaniu pracy, wykonanie większości eksperymentów, współudział w analizie wyników i ich interpretacji, przygotowanie publikacji do druku) – **Udział 70%**	**IF – 2,070 MNiSW – 30/100 (rok 2018/2019)**
2.	**Dzierżyńska-Białończyk**, **A**., Jermacz, Ł., Maćkiewicz, T., Gajewska, J., Kobak, J. 2018. Mechanisms and impact of differential fouling of the zebra mussel *Dreissena polymorpha* on different unionid bivalves. Freshwater Biology 63: 687-699. (współudział w zaplanowaniu pracy, przeprowadzeniu eksperymentu, interpretacji wyników i przygotowaniu publikacji do druku) – **Udział 55%**	**IF – 3,404 MNiSW – 45/100 (rok 2018/2019)**
3.	**Dzierżyńska-Białończyk, A**., Jermacz, Ł., Zielska, J., Kobak, J. 2019. What scares a mussel? Changes in valve movement pattern as an immediate response of a byssate bivalve to biotic factors. Hydrobiologia 841:65-77 (współudział w zaplanowaniu pracy, wykonanie eksperymentów, współudział w analizie i interpretacji wyników, przygotowanie publikacji do druku) – **Udział 60%**	**IF – 2,325 MNiSW – 30/100 (rok 2018/2019)**

Łączny IF – 7,799
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KOMENTARZ AUTORSKI

1. WPROWADZENIE

Racicznica zmienna *Dreissena polymorpha* (Pallas, 1772) jest słodko- i słonawowodnym, częściowo osiadłym małżem, który zyskał zainteresowanie wielu badaczy przede wszystkim wykazując wysoki potencjał inwazyjny (Gallardo, 2014). Wszędzie tam, gdzie racicznica pojawia się masowo (ponad 10 tysięcy osobników na m^2) zauważalne są istotne zmiany fizykochemicznych parametrów wody (m.in. Karatayev i in. 2014; Goedkoop i in. 2011), a także zmiany w strukturze gatunkowej i liczebności zoobentosu (m.in. Naddafi, 2007). Największym problemem z perspektywy ludzkości wydaje się być zdolność racicznicy do wytwarzania nici bisiorowych i przyczepiania się nimi do powierzchni urządzeń hydrotechnicznych, co generuje ogromne straty finansowe (m.in. Gallardo, 2014; Oreska i Aldridge, 2011). Z racji tego, znaczna część badań poświęconych racicznicy koncentruje się na poznaniu jej preferencji siedliskowych i siły przyczepu na różnych materiałach (m.in. Kobak, 2013; Sanz-Ronda i in. 2013), czynników wpływających na przeżywalność larw i gamet w toni wodnej (m.in. Horvath i Crane, 2010; Ciereszko i in., 2001; Lewandowski, 1982) oraz na komercyjnym aspekcie, jakim jest testowanie podłoży, substancji, czy technik antyporostowych. Druga grupa badań nad racicznicą bierze pod szczególną uwagę wysoką wydajność filtracji tego gatunku, jego kosmopolityczny charakter występowania i szeroki zakres tolerancji na czynniki środowiskowe. Wszystkie te cechy sprawiają, że podejmowane są próby wykorzystania racicznicy w procesach rekultywacji zbiorników wodnych, a także w systemach wczesnego ostrzegania przed zanieczyszczeniami, opartych na analizie ruchów muszli (m.in. McLaughlan i Aldridge, 2013; Borcherding, 2006).

W wodach Polski gatunek ten zyskał miano poinwazyjnego (Kołodziejczyk, 2009), a jego liczne kolonie (podobnie, jak w innych krajach) pełnią przede wszystkim funkcję siedliskotwórczą i stanowią bazę pokarmową dla wielu gatunków zwierząt związanych ze środowiskiem wodnym (m.in. Kobak i in. 2014; DeVanna i in. 2011; Gergs i Rothhaupt, 2008b; Botts i in. 1996). Ponadto, problem obrastania przedmiotów zanurzonych przez racicznicę nie jest tak dotkliwy, jak w innych krajach. Niemniej jednak jest to w dalszym ciągu gatunek, którego behawior (szczególnie związany z rozmieszczeniem, tworzeniem i kształtowaniem kolonii) i oddziaływanie na rodzimą faunę powinny zostać dogłębnie przebadane.

Mimo wielu lat badań, wciąż słabo poznane są pewne aspekty biologii racicznicy, zwłaszcza związane z interakcjami między osobnikami tego gatunku i formowaniem przez nie agregacji. Jest to zaskakujące, ponieważ właśnie ta cecha małża warunkuje jego znaczącą rolę siedliskotwórczą i wpływ na gospodarkę. Ważne są także relacje między innymi gatunkami małży (zwłaszcza z rodziny Unionidae) a racicznicą, która obrastając je, może mieć negatywny wpływ na ich kondycję (m. in. Sousa i in. 2011; Schloesser i in. 1996). Dostępne w literaturze dane dotyczące znaczenia tego zjawiska nie są jednoznaczne i różnią się w zależności od regionu geograficznego, badanych gatunków małży i zastosowanej metodyki. Na szczególną uwagę zasługują również badania dotyczące wpływu czynników środowiskowych na ruchy muszli *D. polymorpha*. Ten najmniej poznany aspekt behawioru racicznicy może dostarczyć ważnych informacji na temat wczesnych reakcji małży na czynniki o charakterze stresogennym. Dogłębne poznanie cyklu otwierania i zamykania muszli jest także warunkiem koniecznym w kwestii usprawnienia istniejących już systemów wczesnego ostrzegania opartych na aktywności racicznicy.

2. OPIS MERYTORYCZNY

Nie ulega wątpliwości, że racicznica zmienna jest jednym z najlepiej poznanych gatunków wodnych pod względem biologii, ekologii i behawioru. Jednakże, w dalszym ciągu brakuje kompleksowych badań, które pozwoliłby na poznanie: 1) czynników i procesów mających wpływ na rozmieszczenie małży, 2) charakteru relacji racicznicy z małżami z rodziny skójkowatych (Unionidae), 3) zmian aktywności racicznicy (otwieranie i zamykanie muszli) w odpowiedzi na czynniki środowiskowe. W ramach niniejszej rozprawy doktorskiej zostały przedstawione wyniki badań eksperymentalnych, które przyczyniają się do poszerzenia wiedzy z zakresu trzech wyżej wymienionych zagadnień. Ponadto, autorka rozprawy wyraża głęboką nadzieję, że będą one stanowić swego rodzaju naukową inspirację do dalszych badań nad behawiorem tego ważnego z punktu widzenia ekologii i gospodarki wodnej gatunku małża.

2.1. Reakcje *Dreissena polymorpha* na osobniki własnego gatunku (publikacja nr 1)

Racicznica zmienna tworzy duże kolonie, co w literaturze jest przede wszystkim tłumaczone preferencją względem osobników własnego gatunku (m. in. Burks i in. 2002;

Wainman i in. 1996). Paradoksalnie, do tej pory nie przeprowadzono badań, które potwierdziłyby to dawno przyjęte za pewnik stwierdzenie, choć charakter relacji wewnątrzgatunkowych może być kwestią kluczową w procesach tworzenia i kształtowania skupisk racicznicy. Biorąc pod uwagę wszystkie zalety i trudności życia w kolonii, formowanie agregacji (druz) przez racicznice wydaje się być kompromisem pomiędzy obroną przed drapieżnikami, prądami wody i dogodnymi warunkami rozrodu, a rosnącym stężeniem metabolitów wtórnych i konkurencją wewnątrzgatunkową o zasoby środowiskowe.

W odpowiedzi na powyższy problem, przeprowadzone zostały badania (dwa eksperymenty laboratoryjne), których **główna hipoteza zakładała, że agregacje racicznicy powstają na skutek preferowania osobników własnego gatunku (pozostawanie w druzie bez względu na jej zagęszczenie, ruch w kierunku innych małży), bądź braku alternatywnego podłoża stanowiącego dogodne miejsce przyczepu.**

Za pomocą eksperymentu nr 1 sprawdzono wpływ podłoża (twarde – szkło lub miękkie – piasek) i wielkości druzy (2, 4 ,9, 12, 25 osobników) na „rozchodzenie się" agregacji (utworzonych przez małże w warunkach laboratoryjnych). Zjawisko to zostało opisane parametrami takimi jak procent osobników odczepionych od druzy, osobników, które oddaliły się od druzy oraz wskaźnikiem agregacji (ang. mean crowding).

Wyniki pierwszego eksperymentu nie potwierdziły istnienia wyraźnej preferencji racicznicy względem osobników własnego gatunku. Małże częściej odczepiały się i oddalały od dużych druz w porównaniu z mniejszymi, szczególnie mając do dyspozycji korzystne, twarde podłoże. Niekorzystny z powodu braku możliwości przyczepienia się piasek sprawiał, że małże oderwane od druzy zostawały w jej pobliżu. Taki stosunek racicznicy do osobników własnego gatunku może być dowodem na unikanie przez racicznicę zbyt zagęszczonych kolonii. Opisany behawior może tłumaczyć zjawisko jednowarstwowych, rozległych powierzchniowo kolonii w zbiornikach, gdzie elementów stałego podłoża (np. kamieni, skał lub obiektów pochodzenia antropogenicznego) jest pod dostatkiem (np. litolitoral Jeziora Hańcza, obserwacje własne). Warto również zauważyć, że w szczególnych sytuacjach aktywne odczepianie się małży od kolonii (np. od obficie obrośniętych przez racicznicę burt statków) może mieć pośredni wpływ na kolonizowanie przez racicznicę odległych terenów.

W eksperymencie nr 2 obserwacji poddano reakcje lokomotoryczne pojedynczych osobników racicznicy w obecności małży własnego gatunku. W tym celu osobniki badane były znakowane i umieszczane na piaszczystym podłożu w arenie eksperymentalnej z wydzieloną strefą, w której znajdowały się małże stanowiące źródło sygnału (strefa sygnału). W eksperymencie wzięto pod uwagę odległość osobnika badanego od strefy sygnału oraz moc sygnału (liczbę małży w strefie) jako czynniki mogące mieć wpływ na wynik obserwacji. Każde powtórzenie było nagrywane, a następnie analizowane pod kątem procentowego udziału osobników przemieszczających się, pozostających w bezruchu oraz wykazujących jedynie ruchy niezwiązane z lokomocją (obracanie się wokół własnej osi oraz otwieranie i zamykanie muszli). Aktywność osobników została opisana parametrami takimi jak: pokonany dystans, kierunek ruchu, czas spędzony na lokomocji oraz czas spędzony na wykonywaniu ruchów nielokomocyjnych.

Wyniki eksperymentu nr 2 pokazują, że małże nie wykazują ruchu kierunkowego (od/do źródła sygnału). Wraz z rosnącą liczbą małży w strefie sygnału malał odsetek przemieszczających się osobników. Ograniczenie aktywności i pozostawanie w miejscu niezapewniającym możliwości przyczepu (piasek) może mieć charakter podobny do reakcji małży na czynniki stresowe, takie jak intensywne światło czy obecność drapieżnika (m.in. Naddafi i Rudstam, 2013; Kobak i Nowacki, 2007). Ograniczenie lokomocji może być konsekwencją niezdolności do ruchu kierunkowego – w ten sposób minimalizowane jest ryzyko przypadkowego dołączenia do przegęszczonej kolonii. Ponadto, w wariantach z największą liczbą małży w strefie sygnału odnotowano wydłużenie czasu przeznaczonego na ruchy niezwiązane z lokomocją. Obracanie się wokół własnej osi oraz otwieranie i zamykanie muszli przez małże powodowało ich częściowe zakopywanie się w podłożu, co mogło być związane z poszukiwaniem najbliższego dogodnego miejsca przyczepu. Obie wyżej opisane reakcje behawioralne mogą wskazywać na wykształcenie przez racicznicę specyficznego mechanizmu obronnego, który w pracy nr 1 został opisany jako „bierne unikanie” osobników własnego gatunku.

2.2. Preferencje racicznicy względem różnych gatunków małży z rodziny Unionidae (publikacja nr 2)

Intensywne obrastanie małży z rodziny skójkowatych (Unionidae) przez racicznicę zmienną jest powszechnie znanym zjawiskiem (m. in. Burlakova i in. 2000;

Lewandowski, 1976), które może mieć negatywny wpływ na najważniejsze procesy życiowe i behawior Unionidae (m.in. filtrację, lokomocję i zakopywanie się w osadach). Wiele badań ukazuje zależność między słabnącą kondycją skójkowatych (w tym wysoką śmiertelność) a rosnącym stopniem ich obrośnięcia przez racicznicę (m. in. Bódis i in. 2014; Ricciardi i in. 1996). Do tej pory w wodach Polski nie odnotowano jednak spadku liczebności skójkowatych, który można byłoby bezpośrednio powiązać jedynie z ich obrośnięciem przez racicznicę. Ponadto, w dalszym ciągu nie są poznane mechanizmy kierujące wyborem poszczególnych gatunków Unionidae jako podłoża przez *D. polymorpha*.

W ramach niniejszej pracy zebrano próby terenowe, a także przeprowadzono eksperyment terenowy i laboratoryjny, których **celem było zweryfikowanie następujących hipotez: 1) stopień obrośnięcia małża z rodziny Unionidae przez racicznicę zależy od jego gatunku, 2) racicznica częściej wybiera muszle Unionidae niż inne stałe podłoża (kamienie), 3) obrośnięcie przez racicznicę wpływa negatywnie na biomasę Unionidae oraz 4) racicznice porastające małże skójkowate tworzą na nich wielopoziomowe struktury (druzy), co świadczy o wysokiej preferencji względem osobników własnego gatunku.**

Badania terenowe przeprowadzono na Zbiorniku Włocławskim (dolna Wisła). Obrośnięte osobniki Unionidae były pobierane z dwóch stanowisk o podłożu piaszczystym i mulistym. Stwierdzono obecność pięciu gatunków skójkowatych (szczeżuja pospolita *Anodonta anatina,* szczeżuja wielka *Anodonta cygnea,* szczeżuja chińska *Sinanodonta woodiana,* skójka malarska *Unio pictorum* i skójka zaostrzona *Unio tumidus*), z czego osobniki *A. cygnea* jedynie na stanowisku mulistym. Po identyfikacji, osobniki Unionidae zostały zważone i zmierzone, a zebrane z nich racicznice podzielone na dwie grupy: osobniki bezpośrednio przyczepione do muszli Unioniade i przyczepione do innych osobników własnego gatunku. Obie grupy zostały zważone, a każdy z osobników zmierzony.

Eksperyment terenowy zaprojektowano tak, aby był kompatybilny z badaniami terenowymi, ale wykluczał wpływ podłoża (a więc potencjalne różnice między gatunkami Unionidae w zdolności do zakopywania się w osadach) na obrośnięcie przez racicznicę. W ramach eksperymentu odłowione i pozbawione racicznic Unionidae zostały umieszczone w ażurowych koszach i zawieszone w toni wodnej. Po 73 dniach (lipiec-

-październik) kosze zostały wyłowione, a małże poddano procedurze podobnej jak w badaniach terenowych.

Zadaniem eksperymentu laboratoryjnego było sprawdzenie preferencji racicznicy względem wyżej wymienionych gatunków Unionidae (prócz *A. cygnea*), przy jednoczesnym zapewnieniu alternatywnego podłoża w postaci kamieni. Obiekty eksponowano w dwóch wariantach – z jedno i pięciocentymetrową warstwą piasku, którego zadaniem było odpowiednio ograniczenie lub umożliwienie zakopania się skójkowatych. Dzięki temu zabiegowi, możliwe było sprawdzenie wpływu zdolności do zakopywania się w osadach poszczególnych gatunków na stopień ich obrośnięcia przez racicznicę. W tym przypadku do testów użyto juwenilnych (<10 mm) osobników racicznicy, które wybierały między różnymi rodzajami podłoży (żywe Unionidae i kamienie) dostępnymi na eksperymentalnej arenie.

Z badań terenowych wynika, że najczęściej wybieranymi przez *D. polymorpha* małżami były obcy w wodach Polski gatunek *S. woodiana* oraz rodzimy *A. anatina*, zaś najrzadziej *U. tumidus*, *U. pictorum* i *A. cygnea* (gatunki rodzime). Wyniki eksperymentów w dużej mierze potwierdzają wyżej opisane obserwacje. Ponadto, wskazują one, że różnice między gatunkami małży w zdolności do zakopywania się nie są jedynym czynnikiem wpływającym na stopień ich obrośnięcia przez racicznicę. Jedynie w przypadku *S. woodiana* (w warunkach laboratoryjnych) odnotowano negatywny wpływ obecności podłoża/ możliwości zakopania się małża na liczbę przyczepionych do niego racicznic. Niemniej jednak, zakopywanie się *S. woodiana* nie wydaje się być wystarczająco skutecznym mechanizmem obronnym przed racicznicą, czego dowodem są powyższe wyniki badań terenowych.

Negatywny wpływ racicznicy na biomasę obrośniętych małży odnotowano jedynie dla osobników *S. woodiana* odłowionych ze stanowiska o podłożu mulistym. Wrażliwość *S. woodiana* na obrośnięcie przez *D. polymorpha* można częściowo tłumaczyć tym, że są to gatunki allopatryczne, które prawdopodobnie do lat 60. XX wieku (w Polsce do lat 80.) nie miały ze sobą styczności. Brak znajomości zagrożenia, niedostatecznie wykształcone mechanizmy obronne oraz muliste podłoże niesprzyjające filtracji i utrzymaniu się na powierzchni dna są prawdopodobną przyczyną odnotowanego spadku biomasy *S. woodiana*.

Eksperyment laboratoryjny nie wykazał preferencji racicznicy wobec muszli Unionidae w porównaniu z podłożem alternatywnym (kamieniami). Ponadto, badania terenowe wykazały, że wraz ze wzrostem biomasy racicznic zebranych z przedstawicieli Unionidae maleje procentowy udział osobników bezpośrednio przyczepionych do muszli Unionidae. Natomiast w przypadku osobników słabo obrośniętych, racicznice zebrane bezpośrednio z muszli skójkowatych stanowiły 100% wszystkich osobników tego gatunku. Wynik ten (podobnie jak wyniki z publikacji nr 1) podważa hipotezę o wysokiej preferencji racicznicy względem osobników własnego gatunku. Tak więc, w warunkach naturalnych, wybór muszli innych małży, a zwłaszcza osobników własnego gatunku, wydaje się być w dużym stopniu podyktowany brakiem alternatywnego podłoża.

2.3. Wpływ czynników biotycznych na ruchy muszli racicznicy zmiennej (publikacja nr 3)

Otwieranie i zamykanie muszli u małży jest związane z najważniejszymi procesami życiowym takimi jak filtracja, wydalanie, rozmnażanie, czy kontaktowanie się ze środowiskiem zewnętrznym (m.in. Gabriel-March i in. 2008; Gosling, 2003). Równocześnie, jest to najmniej poznany aspekt behawioru racicznicy zmiennej. Z drugiej strony, monitoring ruchu muszli wykorzystywany jest w projektowaniu komercyjnych systemów wczesnego ostrzegania opartych na rejestracji aktywności małży. Co więcej, może być on cennym źródłem informacji o pierwszych zauważalnych reakcjach na czynniki stresowe, co jest istotne z punktu badań dotyczących mechanizmów obronnych tego gatunku.

Badania prowadzone w ramach niniejszej pracy miały na celu ukazanie charakterystycznych reakcji dotyczących otwierania i zamykania muszli u racicznicy zmiennej w obecności wybranych biotycznych czynników środowiskowych o potencjalnie stresogennym charakterze. W trakcie eksperymentów laboratoryjnych sprawdzono wpływ: 1) substancji alarmowej wydzielanej przez uszkodzone osobniki *D. polymorpha*, 2) zapachu płoci *Rutilus rutilus*, której głównym pokarmem jest racicznica zmienna oraz 3) obecności kiełża *Dikerogammarus villosus*, którego masowa obecność w koloniach racicznicy może powodować mechaniczne drażnienie syfonów małży. **Na potrzeby eksperymentu sformułowano trzy hipotezy badawcze: 1) *Dreissena polymorpha* ogranicza aktywność (czas spędzony w szerokim rozwarciu oraz liczbę ruchów muszli) w obecności wyżej wymienionych czynników, 2) dieta**

drapieżnika ma wpływ na reakcje racicznicy oraz 3) różne czynniki stresogenne działające równocześnie wywołują silniejszą odpowiedź małży niż każdy z nich z osobna (działanie synergistyczne).

Przed eksperymentem małże były przyklejane do szkiełek podstawowych, a ruchomą połówkę muszli znakowano kolorowym znacznikiem. Aktywność małży (w obecności wybranych czynników) była nagrywana przez 2 godziny, a następnie analizowana pod kątem: a) średniego rozwarcia muszli w trakcie całego eksperymentu, b) czasu spędzonego z zamkniętą muszlą oraz w małym i dużym rozwarciu muszli (rozwarcie poniżej 20% i powyżej 80% całkowitego rozwarcia), a także c) liczby poszczególnych otwarć muszli.

Czynnikami najbardziej modyfikującymi ruchy muszli racicznicy w porównaniu z wariantem kontrolnym były obecność *D. villosus* oraz substancji alarmowej. W obecności kiełży małże spędzały więcej czasu z muszlą zamkniętą, bądź rozwartą jedynie do 20% całkowitego rozwarcia (rozwarcie takie nie pozwala na wysunięcie syfonów). *D. villosus* jest opisywany jako gatunek wybitnie drapieżny i agresywny (Kley i Maier, 2005), ale do tej pory nie odnotowano jego jednoznacznie negatywnego wpływu na procesy życiowe oraz kondycję racicznicy zmiennej. Wcześniejsze badania pokazują, że w obecności *D. villosus* racicznica zmienia siłę przyczepu i ogranicza lokomocję, co notowano również w obecności drapieżnika i substancji alarmowej (Kobak i in. 2012; Czarnołęski i in. 2010; Kobak i in. 2010). Wszystkie opisane powyżej reakcje behawioralne mają charakter obronny, jednakże, aby potwierdzić negatywny wpływ kiełży na kondycję małży, należy przeprowadzić badania o charakterze behawioralnym i fizjologicznym z wydłużonym czasem ekspozycji małży na działanie *D. villosus*.

Kolejnym czynnikiem, który znacząco zmienił ruchy muszli racicznicy w trakcie eksperymentu była substancja alarmowa. Małże w obecności substancji alarmowej wykazywały mniejsze średnie rozwarcie muszli oraz skrócony czas spędzony w największym rozwarciu (powyżej 80% całkowitego rozwarcia). W warunkach naturalnych substancja alarmowa sygnalizuje obecność aktualnie żerującego drapieżnika. Zmniejszenie rozwarcia muszli może mieć na celu ograniczenie emisji sygnałów chemicznych lub fizycznych (zapach, prąd wody z syfonu), które pomagają drapieżnikowi w lokalizacji ofiary. Zaskakującym wynikiem był brak wyraźnego wpływu zapachu samego drapieżnika (bez względu na jego dietę) na ruchy muszli testowanych

małży. Można więc wysunąć wniosek, że racicznica zmienna zmienia zakres ruchów muszli tylko w obecności bezpośredniego zagrożenia (w tym przypadku pod wpływem substancji alarmowej). Kolejnym zupełnie niespodziewanym zjawiskiem był brak istotnej reakcji racicznicy na substancję alarmową w połączeniu z zapachem drapieżnika. Początkowo założono, że połączenie wspomnianych czynników będzie miało działanie synergistyczne, czego efektem byłaby nasilona reakcja racicznicy na potencjalnie stresogenne bodźce. Hipoteza ta została odrzucona, a zapach drapieżnika prawdopodobnie „maskował" obecność substancji alarmowej, czego przejawem były reakcje małży zbliżone do reakcji odnotowanych w wariancie kontrolnym (bez żadnego czynnika). Podobny „efekt maskowania" został już wcześniej opisany, aczkolwiek tylko w odniesieniu do maskowania tzw. „dietary alarm cues", a więc substancji alarmowej zawartej w odchodach drapieżnika odżywiającego się określonym gatunkiem ofiar (Wisenden, 2015). Jest to pierwsze takie doniesienie w przypadku relacji racicznica – - drapieżnik. Niewątpliwie zjawisko „maskowania" sygnałów alarmowych wymaga szerszej analizy eksperymentalnej.

2.4. Najważniejsze wyniki i wnioski

1) *Dreissena polymorpha* nie przejawia reakcji jednoznacznie wskazujących na silne powinowactwo w stosunku do osobników własnego gatunku. Jest to wynik, który podważa hipotezę o aktywnym preferowaniu przez racicznicę innych małży, czego jedynym dowodem są zaobserwowane w środowisku naturalnym kolonie składające się z licznych osobników. Racicznica nie wykazuje zdolności do ruchu kierunkowego, co uniemożliwia skuteczne zbliżanie się, a także oddalanie od innych osobników. Ponadto przejawia tendencje do odrywania się od dużych druz i podejmowania wędrówki w obecności alternatywnego stałego podłoża. Zjawisko ograniczenia lokomocji małży w obecności osobników własnego gatunku może wskazywać na specyficzną formę reakcji o charakterze obronnym (opisanej w rozprawie jako „bierne unikanie"), mającej na celu ograniczenie ryzyka dołączenia do zbyt zagęszczonej kolonii. Niewątpliwie relacje międzyosobnicze mają wpływ na rozmieszczenie racicznicy zmiennej w skali mikrosiedliska i prawdopodobnie mogą być jednym z pośrednich powodów wysokiego potencjału inwazyjnego tego gatunku (aktywne odczepianie się i dryfowanie wraz z prądami wody powinno ułatwiać kolonizowanie nowych terenów).

2) Ważnym czynnikiem wpływającym na rozmieszczenie racicznicy zmiennej jest obecność różnych małży z rodziny skójkowatych Unionidae, które nierzadko są jedynym stałym podłożem dostępnym na dużej powierzchni miękkich osadów dennych w zbiornikach wodnych (obserwacje własne). Wybór skójkowatych przez racicznicę jest specyficzny. Najbardziej preferowanymi przez *D. polymorpha* gatunkami są szczeżuja pospolita *A. anatina* oraz szczeżuja chińska *S. woodiana* (gatunek obcy w wodach Polski). Pomimo tego, że w obecnych badaniach nie wykazano negatywnego wpływu racicznicy na biomasę rodzimego gatunku *A. anatina*, należy mieć szczególne baczenie na jego stan w miejscach naturalnego współwystępowania z racicznicą.

3) Racicznica reaguje istotną zmianą w cyklu otwierania i zamykania muszli na czynniki takie, jak substancja alarmowa (wydzielana przez uszkodzone osobniki tego samego gatunku) oraz obecność kiełża *D. villosus* (licznie zasiedlającego kolonie racicznicy). Wydłużenie czasu spędzonego w zamknięciu, czy ograniczenie średniego rozwarcia muszli może mieć korzystny wpływ na obronę przed mechanicznym drażnieniem miękkich części ciała małża (powodowane przez kiełże) oraz na ograniczenie emisji sygnałów pomagających drapieżnikowi w lokalizowaniu ofiary. Ponadto, małże tylko w niewielkim stopniu modyfikują ruchy muszli w odpowiedzi na zapach samego drapieżnika. Prawdopodobnie reakcje racicznicy są indukowane tylko bezpośrednim zagrożeniem (np. obecnością substancji alarmowej świadczącej o bliskości żerującego drapieżnika). Niezwykle ciekawym wynikiem, aczkolwiek niepozwalającym jeszcze na wyciąganie ostatecznych wniosków, jest brak znaczącej reakcji małży na działanie dwóch potencjalnie stresogennych czynników jednocześnie, tj. substancji alarmowej i zapachu drapieżnika. W rozprawie wysunięto przypuszczenie, iż może to być przypadek zjawiska „maskowania" przez drapieżnika sygnału chemicznego ostrzegającego małże o bezpośrednim zagrożeniu.

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4. STRESZCZENIE

Racicznica zmienna *Dreissena polymorpha* jest zaliczana do najbardziej inwazyjnych gatunków w Europie i Ameryce Północnej, co czyni ją najlepiej poznanym słodkowodnym małżem na świecie. W wodach Polski stała się stałym elementem fauny dennej, w związku z czym uważana jest za gatunek obcy poinwazyjny. Małże te tworzą liczne kolonie, dostarczając schronienia i pokarmu gatunkom związanym ze środowiskiem wodnym, a także, dzięki wydajnej filtracji, znacząco wpływają na przejrzystość wody. Co więcej, podejmowane są próby wykorzystania racicznicy w rekultywacji zbiorników wodnych i w systemach wczesnego ostrzegania przed zanieczyszczeniami. Niemniej jednak, wiele pytań dotyczących relacji racicznicy z osobnikami własnego gatunku oraz innymi organizmami, a także jej behawioru związanego z otwieraniem i zamykaniem muszli, pozostaje w dalszym ciągu bez odpowiedzi. Zagadnienia te są ściśle związane z mechanizmami tworzenia i kształtowania kolonii racicznicy, a także z jej krótkoterminowymi reakcjami na czynniki środowiskowe.

Celem niniejszych badań było eksperymentalne sprawdzenie: 1) charakteru relacji racicznicy z osobnikami własnego gatunku (publikacja nr 1), 2) preferencji racicznicy względem różnych gatunków małży z rodziny skójkowatych (Unionidae) i wpływu obrośnięcia Unionidae przez racicznicę na ich biomasę (publikacja nr 2) oraz 3) behawioru związanego z otwieraniem i zamykaniem muszli pod wpływem informacji o potencjalnym zagrożeniu takich jak substancja alarmowa (wydzielana przez uszkodzone osobniki własnego gatunku), zapach drapieżnika (płoci *Rutilus rutilus*) i mechaniczne podrażnianie miękkich tkanek małży przez organizmy zasiedlające kolonie racicznicy (w tym przypadku kiełży *Dikreogammarus villosus*) (publikacja nr 3). Eksperymenty terenowe i laboratoryjne oraz badania terenowe wykazały, że racicznica zmienna: 1) przejawiała tendencje do unikania osobników własnego gatunku występujących w dużym zagęszczeniu (aktywnie odczepiając się od dużych druz oraz ograniczając aktywność, kiedy znajdowała się w pewnej odległości od druzy) (pub. 1), 2) preferowała osiadanie na muszlach małży *Anodonta anatina* i *Sinanodonta woodiana* w porównaniu z pozostałymi gatunkami z rodziny Unionidae, a także negatywnie wpływała na biomasę *S. woodiana* (pub. 2), 3) ograniczała rozwarcie muszli w odpowiedzi na substancję alarmową i mechaniczne oddziaływanie kiełży (pub. 3).

Przedstawione wyniki rzucają nowe światło na relacje wewnątrzgatunkowe racicznicy zmiennej. Życie w grupie jest kompromisem pomiędzy ochroną przed szkodliwymi czynnikami środowiskowymi a pogarszającymi się parametrami wody wewnątrz przegęszczonej kolonii racicznicy. Ponadto, znajomość preferencji racicznicy względem różnych gatunków małży Unionidae może mieć znaczenie dla ich ochrony w wodach śródlądowych Polski. Z kolei czynniki środowiskowe, które istotnie modyfikują ruchy muszli racicznicy (podobnie jak zanieczyszczenia antropogeniczne), powinny być brane pod uwagę w kalibrowaniu systemów wczesnego ostrzegania opartych na tym aspekcie behawioru małży w celu uniknięcia fałszywych alarmów.

ABSTRACT

The zebra mussel *Dreissena polymorpha* is regarded as one of the worst invasive species in Europe and North America, which makes it the best-known sessile freshwater bivalve in the world. In Polish waters, it has become a permanent part of the local benthic fauna and therefore is considered as a post-invasive species. Mussels form huge colonies providing shelters and food for many organisms and affect water transparency by efficient filtration. Moreover, there are attempts to use zebra mussels for water restoration and in early warning systems detecting water pollution. Nevertheless, many questions concerning zebra mussel relations with conspecifics and other species, as well as their valve gaping activity still remain unanswered. These issues are strictly related to the mechanisms of forming colonies by zebra mussels and their initial reactions to environmental factors.

The aim of the present study was an experimental evaluation of: 1) relationship of zebra mussels with their conspecifics (publication 1), 2) preferences of zebra mussels for various species of Unionidae mussels and the impact of dreissenid fouling on unionid biomass (publication 2) and 3) valve movement behaviour and its changes in response to potential threat cues, such as the conspecific alarm substance (secreted by injured conspecifics), predator scent (the roach *Rutilus rutilus*) and mechanical irritation by organisms inhabiting mussel colonies (*Dikerogammarus villous*, Gammaridae, Amphipoda, Crustacea) (publication 3).

The field and laboratory experiments and surveys revealed that zebra mussels: 1) tended to avoid conspecifics at high density (actively detached from large druses and reduced their locomotion in the presence of distant conspecifics) (pub.1); 2) preferred to settle on

Anodonta anatina and *Sinanodonta woodiana* compared to other Unionidae species and negatively affected the biomass of *S. woodiana* (pub.2); 3) reduced their valve gaping in response to the conspecific alarm substance and mechanical irritation by gammarids (pub.3).

The presented results put a new perspective on the relations among zebra mussel conspecifics. The life in a colony can be assumed as a compromise between protection against adverse environmental factors and deteriorating water conditions resulting from the increased density. Moreover, the knowledge of specific preferences of zebra mussels for particular Unionidae species may be important for the conservation of native bivalves in Polish inland waters. Finally, biotic environmental factors (in addition to anthropogenic pollutants) turned out to affect valve gaping behaviour of zebra mussels, which should be taken into account during calibration of early warning systems based on mussel activity to avoid false alarms.

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Happy together? Avoidance of conspecifics by gregarious mussels

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Abstract

Zebra mussel *Dreissena polymorpha* is a Ponto-Caspian species invasive in Europe and North America, with great environmental impact. It lives byssally attached to hard substrata in large aggregations, which is often explained by its preferences for conspecifics, though direct evidence for such preferences has been rather limited so far. We studied the reactions of zebra mussels to conspecifics, hypothesizing that they may either be attracted to one another or form aggregations only in the absence of alternative attachment sites. In Experiment 1, we tested mussel tendency to detach from existing druses depending on druse size (2–25 individuals) and substratum type (soft: sand; hard: glass). Mussels detached significantly more often on the hard substratum and from larger druses compared to soft substratum and smaller druses, respectively. This indicates that mussels tended to avoid conspecifics at high density, particularly when alternative substratum was available. In Experiment 2, we tested the responses of single mussels to distant (3 or 15 cm) conspecifics (0, 3, 15 individuals per 2.5 l tank) on the sandy substratum. The presence of conspecifics, regardless of their distance and density, resulted in single unattached mussels staying more often in their initial positions. Mussels did not move preferentially towards or away from the conspecifics. Thus, even on unsuitable substratum mussels were not attracted by conspecifics and probably exhibited an avoidance reaction by reducing their movement. This suggests that dense mussel aggregations are formed due to the lack of available alternative attachment sites rather than due to their preferences for conspecifics.

Key words: active detachment, *Dreissena polymorpha*, movement, mussel aggregations, zebra mussel.

The zebra mussel *Dreissena polymorpha* (Pallas, 1771) has been claimed to be one of the worst invasive species in the world (Gallardo 2014; McLaughlan et al. 2014), generating substantial financial and environmental impacts (Pollick 2013; Prescott et al. 2013; Karatayev et al. 1997; Ricciardi et al. 1996) due to its gregariousness (Kelly et al. 2010; MacIsaac et al. 1992). Throughout the majority of their life, zebra mussels are byssally attached to the substratum. However, metamorphosed individuals are capable of active detachment (Eckroat et al. 1993) and relocation to a better substratum (Toomey et al. 2002). This phenomenon may be a visible avoidance reaction to environmental factors such as light intensity, the presence of conspecific alarm cues, the scent of a predator or water quality (Burks et al. 2002; Toomey et al. 2002; Czarnołęski et al. 2010; Kobak and Ryńska 2014). Active movement of metamorphosed mussels undoubtedly increases their chances of survival (Kobak 2013 for a review) and must be considered in studies aiming at an explanation of their distribution and responses to environmental factors.

Studies to date mainly focused on the practical use of *D. polymorpha* as an efficient filter feeder (Stańczykowska and Lewandowski 1993; Elliott et al. 2008; McLaughlan and Aldridge 2013; Binelli et al. 2014) or bioindicator (Borcherding 2006; Kimbrough et al. 2013). Moreover, the biology, ecology, and less often behavior of this species were also tested to create effective methods of preventing and controlling its invasion (Kobak 2013; Collas et al. 2017). Most of these studies assumed that the zebra

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mussel was extremely gregarious, forming dense 3-dimensional colonies (Burks et al. 2002) and aggregations of individuals byssally attached to one another, called "druses" (Stańczykowska 1964). It has often been suggested that their formation depends on active preferences of zebra mussels for conspecifics (Wainman et al. 1996; Kobak 2006), but none of the conducted studies clearly demonstrated such preferences.

A few studies have suggested that *D. polymorpha* may not exhibit such a high affinity for conspecifics as had been assumed, preferring alternative hard substrata (Kavouras and Maki 2003; Kobak and Ryńska 2014; Tošenovský and Kobak 2016). Perhaps, excessive density may make mussels actively detach from druses and, if possible, move to a more suitable substratum. Certainly, life in a colony is associated with many advantages: protection against predators, drying or hydrodynamics and suitable conditions for reproduction (Okamura 1986). On the other hand, intraspecific competition for food, space and position in the group is higher in a dense colony (Burks et al. 2002; Gascoigne et al. 2005; Wacker and Von Elert 2008; Cubillo et al. 2012). Unsuitable mussel position in the vertical structure of a colony is associated with the greater risk of becoming overgrown by other individuals (Burks et al. 2002; Wacker and Von Elert 2008). This may entail the distortion of their shells and immobilization of syphon parts (Bertness and Grosholz 1985; Cubillo et al. 2012), which finally causes organ failure and death (Griffiths and Hockey 1987; Burks et al. 2002; Czarnołęski et al. 2003). Furthermore, such a colony experiences poorer interstitial water conditions (oxygen depletion, increased waste concentration) (Burks et al. 2002; Tuchman et al. 2004). Thus, it can be assumed that life in a colony of *D. polymorpha* is a compromise between protection and successful reproduction, on the one hand, and deteriorating environmental conditions resulting from increased density on the other hand.

Apart from mussel preferences, another cause of their aggregations may be the limited availability of hard substratum in the environment, where conspecific shells may become the only choice (Stańczykowska 1964). In addition, some observations suggest that locomotor limitations, which become more conspicuous with increasing body size (Uryu et al. 1996; Toomey et al. 2002), affect the life of a sessile mussel. These limitations, stemming from the anatomy and physiology of mature individuals and the fact that they are fouled by younger conspecifics (Burks et al. 2002; Czarnołęski et al. 2003; Wacker and Von Elert 2008), may be responsible for the process of aggregation forming.

Given this complex picture of mussel aggregation forming, it seems to be a paradox that few previous studies focused on the relations between *D. polymorpha* conspecifics and there is no paper which clearly hypothesizes that the zebra mussel may not prefer conspecifics. Thus, we believe the assumption that *D. polymorpha* prefers other individuals needs not only field-based correlational evidence, but also a solid experimental background. Our aim was the detailed examination of reactions of *D. polymorpha* to conspecifics in order to explain the mechanisms of aggregation forming by mussels. This is the first research where a previous conjecture concerning questionable preferences of the zebra mussel to conspecifics has been investigated.

We hypothesized that mussel aggregations were formed due to their active preferences for conspecifics or because of the absence of alternative attachment sites. In our study, we tested mussel responses to conspecifics in two situations: when a mussel is a part of druse or a singleton. Both these situations occur in the field where mussels are exposed to different environmental factors. Druses may be dragged by hydrodynamic forces to new areas and mussels may detach spontaneously to colonize new surfaces appearing in their vicinity (Lauer and Spacie 2003). Thus, mussels are likely to respond to varying conditions by adjusting their position. In the case of mussel preferences for conspecifics, the percentage of detaching and relocating mussels should be independent of the availability of the alternative hard substratum; otherwise, it should be stimulated by the presence of suitable surfaces. Moreover, for mussels physically separated from conspecifics by hydrodynamic forces or transported with pieces of debris (Lewandowski 2001), conspecific signals are likely to constitute an important cue affecting their behavior and enhancing survival. The attractancy (directional movement or increase in activity) of single mussels in the presence of conspecifics would indicate their preferences for living mussel substratum. Finally, based on the results of previous research (Stańczykowska 1964) and knowledge of conditions in dense colonies (Burks et al. 2002; Tuchman et al. 2004), we expected that with the increasing density of conspecifics in the environment, mussels would reduce or reverse their preferences for conspecifics in both experiments. Altogether, mussel responses in our experiments would indicate whether *D. polymorpha* prefers, tolerates or avoids conspecifics at particular abundances.

Materials and Methods

Mussel collection and stocking before the experiments

Dreissena polymorpha individuals were collected by a diver from the Włocławek Reservoir (a dam lake on the lower Vistula River in the central part of Poland, 52°37'04″N 19°24'28″E). They were taken from unionid clams, heavily fouled by this species. Mussels were kept in two 350-l stock tanks at a density of ~8,000 individuals per square meter, which is frequently experienced by this species in the wild. Each tank was supplied with standard aquarium filters, aerators and coolers to sustain an appropriate temperature of 19–20 °C. Mussels were acclimated in the stock tanks for at least one week before the tests and used in the experiments within 5–6 weeks after collection. They were fed with dried *Chlorella* sp. (~2 g per 1,000 mussels every second day).

Experimental conditions

We conducted our experiments in 2.5-l tanks (29/29/3 cm) filled with tap water (1.7-cm layer above the substratum surface) settled and aerated for 6 days before use, at a constant temperature of 19 °C (sustained by air-conditioning) and constant fluorescent light of 52 lx, uniform over all experimental arenas (determined with a luxometer L-20A Sonopan Ltd., Białystok, Poland). The oxygen concentration, saturation and conductivity (measured with a multimeter Multi340i; WTW GmbH, Weilheim, Germany) were similar in all experiments and treatments (oxygen concentration (mean ± *SE*) 8.8 ± 0.1 mg/l, saturation 88.1 ± 1.0%, conductivity: 588 µS/cm). The tanks were not aerated during the experiments to avoid the impact of air bubbles on mussel behavior.

We tested mussels within the length range 18–22 mm. This size falls between medium and large size classes described by Toomey et al. (2002). We chose mussels of this size due to their relatively high motility, which is not reported for larger individuals (Toomey et al. 2002; Kobak and Nowacki 2007). On the other hand, this size was sufficient for marking mussels and tracking their movements by the behavior analysis software (see below). One day before and

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during the experiments, mussels were not fed. Each mussel was used only once.

Experiment 1: Dispersal of mussels from aggregations

This experiment was designed to test the stability of mussel aggregations depending on druse size and availability of alternative substratum.

In order to obtain artificial druses (aggregations consisting of groups of mussels attached to one another), one day before the experiment we placed mussels in aerated 20/30/7 cm tanks filled with a 2-cm layer of fine sand (mean grain diameter ± *SD*: 0.3 ± 0.08 mm), which is not suitable for *D. polymorpha*, due to the lack of attachment possibility (Kobak and Ryńska 2014). The sand for our experiments was collected from the Włocławek Reservoir, dried at 60 °C and kept dry for at least a month after collecting to remove any organisms and/or environmental signals that could affect mussel responses. Groups of 40 mussels were surrounded with cylinders (diameter 7 cm, height 9 cm) made of plastic 1-mm mesh, which is avoided by *D. polymorpha* (Porter and Marsden 2008). The light intensity was 52 lx, corresponding to the experimental conditions. After 24 h, the conditions led to mussels attaching to one another, irrespective of their preferences. We selected single druses consisting of 2, 4, 9, 12, or 25 mussels and carefully placed them in the central part of the experimental tanks. Zebra mussels are known to produce two types of byssal threads: temporary threads used for short-term initial anchoring and permanent threads produced after a longer time (Eckroat et al. 1993). In our study, due to the short time of druse formation, mussels produced the former type of byssus, resulting in clearly lower adhesion strength (Kobak 2006). Using mussels in their initial stage of attachment allowed their responses to the local conditions to be tested: they could stay in a druse or easily adjust their position depending on the test conditions. Handling could affect druse stability by stimulating mussel detachment. However, it equally affected all experimental treatments. A similar destabilization can be also caused by hydrodynamic forces in the field.

The experiment was conducted in tanks (1) filled with a 1-cm layer of fine sand (no alternative substratum for mussels) or (2) directly on the glass tank bottom (alternative hard substratum suitable for mussels, as shown by Kobak and Ryńska 2014). The 25-individual druse treatment was omitted on glass due to the fact that a clear effect of density was visible at lower abundances (see the "Results" section) and because of the difficulties in obtaining such large aggregations. The substrata were put in water 24 h before the tests to allow biofilm development, which makes submerged materials more suitable for mussels (Wainman et al. 1996; Kavouras and Maki 2003). We deployed simultaneously nine tanks with different experimental treatments. We replicated this procedure 35 times, randomizing the position of particular experimental treatments in consecutive trials. Tanks were cleaned and the water and substratum changed between the replicates.

After 24 h of the experiment we determined: (1) the percentage of all individuals that detached from the druse (hereafter referred to as "detached mussels"), (2) the percentage of mussels that detached and moved away, separating themselves physically from the druse, ("separated mussels") and (3) the mean crowding index of mussels after the experiment (Jarman 1974):

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

Where: N_i – the number of individuals in druse i, k – the number of druses. The mean crowding describes the group size experienced by an average individual in the tank or a "typical group size" according to Jarman (1974). Thus, it was an indication of the final druse size after mussel dispersal.

Due to the high heteroscedasticity of the data (resulting from different initial druse sizes), we used nonparametric Kruskal–Wallis tests (separate for each substratum) followed by a post-hoc procedure described by Sokal and Rohlf (1995) to compare the percentages of mussels detached and separated from druses of different sizes, as well as mean crowding index values. Moreover, we compared the aforementioned behavioural responses of mussels between both substrata using sequential Bonferroni-corrected Mann–Whitney U-tests, separate for each druse size.

Moreover, we compared the frequencies of modified druses (with at least one detached or separated individual) in different experimental treatments using a three-way G test of independence with the following factors: (1) druse size (2, 4, 9, or 12 individuals), (2) substratum (sand or glass), and (3) a response variable: druse status (modified or not). Obviously, the probability of druse modification is a simple function of its size, as the probability that at least one individual will detach increases with the number of individuals. Thus, we only used this analysis to check for the difference in mussel behavior between both substrata and the effect of druse size on this difference, testing substratum × druse status and druse size × substratum × druse status interactions. If their results were significant, we ran a series of appropriate sequential-Bonferroni corrected 2×2 G-tests to test the impact of particular conditions on druse stability.

Experiment 2: Movement of single individuals in the presence of conspecifics

This experiment was designed to test the effect of conspecific density and distance to conspecific clusters on the intensity and direction of movements of isolated mussels. In Experiment 1, mussels tended to stay in aggregations more often on unsuitable sandy substratum (see the "Results" section). Therefore, we conducted Experiment 2 using sandy substratum to check whether single, unattached individuals in such conditions would respond to conspecifics constituting potential suitable attachment sites.

One day before the experiment, mussels were marked with fast drying red nail polish to allow them to be tracked by the behavior analysis software. The painted mussels were placed for 24 h in temporary tanks with water and light conditions similar to those in the experimental tanks to recover after marking, which was associated with ~2-min. air exposure. Zebra mussels are capable of surviving several days of desiccation, therefore such a short period of air exposure is unlikely to have any long-lasting negative consequences on their health (Ricciardi et al. 1995).

The bottom of the experimental tanks (Figure 1) was covered with a 1-cm layer of fine sand to increase the probability of mussel motion and check their reactions to conspecifics on the unsuitable substratum. Zebra mussels commonly occur on sandy substrata, attached to available hard objects, such as single stones, anthropogenic solid rubbish, or hard-shelled animals (e.g. unionid clams) (Bódis et al. 2013; Garton et al. 2013), thus the conditions in our experiment reflected natural situations taking place in the wild. To prevent mussels from vertical movement, we covered the tank walls with 1-mm plastic mesh (Porter and Marsden 2008). The experimental tanks were divided into two zones: a movement zone, where we placed a single test individual and a signal zone with 3 or 15 conspecifics. These conspecifics served as a signal source, to which the

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test mussel could potentially respond. These zones were separated from each other by a plastic 1-mm mesh barrier (Figure 1). The position of zones relative to the laboratory room was changed in particular trials to avoid bias resulting from some directional factors acting in the room. That said, preliminary observations of the control treatments did not reveal any tendencies for mussel movements in particular directions in the laboratory room.

The experiment consisted of six experimental treatments: (1) control, without conspecifics in the signal zone and a test individual placed 3 cm from the mesh barrier between the zones, (2) control, without conspecifics in the signal zone and a test individual placed 15 cm from the barrier, (3) with three conspecifics in the signal zone and a test individual placed 3 cm from the barrier, (4) with three conspecifics in the signal zone and a test individual placed 15 cm from the barrier, (5) with 15 conspecifics in the signal zone and a test individual placed 3 cm from the barrier and (6) with 15 conspecifics in the signal zone and a test individual placed 15 cm from the barrier. Thus, we could test mussel responses to two conspecific densities and at two distances from the signal source.

The experiment lasted 12 h 10 min., the first 10 min. for recovery after handling followed by 12 h of behavioral observations recorded with a video camera (SNB-6004, Samsung, South Korea) suspended 60 cm above the experimental arena. We deployed simultaneously six tanks with different experimental treatments. We replicated this procedure 30 times, randomizing the position of specific experimental treatments in consecutive trials. We cleaned the tanks and changed the substratum to avoid leaving any potential signals from previous treatments in new replicates.

We determined the percentages of mussels: (1) exhibiting locomotion (relocating from one place to another), (2) exhibiting only non-locomotive movements (e.g. squirming around, swaying, etc.) without relocation, and (3) staying in their initial position without any movement in each experimental treatment. All relocating mussels (group 1) exhibited also non-locomotive movements. Moreover, we applied Noldus Ethovision® XT 10.1 software to determine the following movement characteristics: (1) total distance moved by mussels, (2) total time spent on locomotion, (3) total time spent on non-locomotive movements, and (4) the distance moved towards or away from the signal source.

We compared the frequencies of moving mussels (separately for relocating mussels and for all mussels exhibiting locomotive or non-locomotive movements pooled) in different experimental treatments using a three-way G-test of independence with the following factors: (1) signal strength (3 or 15 mussels), (2) distance from the signal source (close or far), and (3) a response variable: mussel behavior (moving or not moving). All interactions of the response variable with the other factors were tested in both analyses. If their results were significant, we ran a series of appropriate, sequential-Bonferroni corrected 2×2 G-tests to find out which groups of mussels differed from one another in their behaviors.

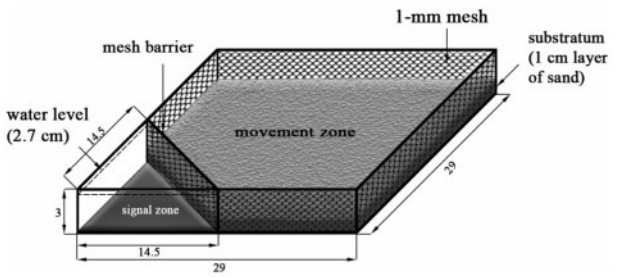

Figure 1. Experimental tank split into two zones, used in Experiment 2. Dimensions are given in centimeter .

In the analysis of movement distance and time, we only considered moving mussels. Thus, we obtained results independent of the frequency of moving individuals, to indicate potential effects of the tested factors on the behavior of those specimens which performed movement. Otherwise, we would duplicate the results of the prior analysis of movement frequency to a large extent. Due to the strong deviations of the data from homoscedasticity and normality assumptions, we compared distances travelled and times spent on moving in all experimental treatments using a nonparametric Kruskal–Wallis test. Moreover, we calculated percentages of distances moved towards and away from the signal source in particular experimental treatments and compared them with a theoretical value of 50%, using sequential Bonferroni-corrected one-sample *t*-tests. A significant departure from 50% would indicate a directional movement. These percentages (arcsine-square root transformed) did not depart significantly from the normal distribution (Shapiro–Wilk test).

Results

Experiment 1: Dispersal of mussels from aggregations

The percentage of mussels detached from druses (Figure 2A) depended on druse size on glass (Kruskal–Wallis test: $\chi^2_3 = 36.4$, $P < 0.001$) and on sand ($\chi^2_4 = 12.9$, $P < 0.012$). According to the post hoc tests, mussel detachment on sand occurred more frequently from 12 to 25-individual druses than from 2 to 4 individual druses. On the hard substratum, the threshold above which the detachment rate increased was between four and nine individuals in a druse. The percentage of mussels separated from druses (Figure 2B) depended on druse size on the hard substratum (Kruskal–Wallis test: $\chi^2_3 = 27.5$, $P < 0.001$) but not on sand ($\chi^2_4 = 4.8$, $P < 0.306$). Similarly to the detachment analysis, mussel separation from 9 to 12 individual druses on glass was greater than that from 2 to 4 individual druses (post hoc tests, Figure 2B). Detachment and separation of mussels were significantly greater on glass than on sand for 12-individual druses (Mann–Whitney U-tests: $z = 4.50$ and 4.35, respectively, $P < 0.001$) and similar on both substrata in all other cases ($z < 2.2$ and $P > 0.05$ after applying the Bonferroni correction).

The mean crowding index after the experiment (Figure 3) depended on initial druse size on glass (Kruskal–Wallis test: $\chi^2_3 = 104.2$, $P < 0.001$) and sand ($\chi^2_3 = 36.4$, $P < 0.001$). On sand, the final druse size increased monotonically with increasing initial druse size up to ~15 individuals for initial 25-individual druses (post hoc tests). On hard substratum, the final druse size reached ~approximately six individuals for initial druses of nine mussels and remained constant for larger initial druse sizes. Video recordings of the experimental trials revealed that groups of mussels were capable of active relocation, which made possible such splits of large druses into smaller aggregations. The mean crowding index was significantly greater on sand than on glass for 12-individual druses (Mann–Whitney U-test: $z = 4.4$, $P < 0.001$) and similar on both substrata in all other cases ($z > -2.1$ and $P > 0.05$ after applying the Bonferroni correction).

The percentage of modified druses (with at least one individual changing its position) differed between substrata depending on druse size (Appendix Figure 1), as shown by significant substratum × druse size × druse status interactions (G-tests: $G_3 = 15.9$ and 22.9 for the detached and separated mussel analyses, $P < 0.001$). As shown by 2×2 G-tests, the detachment and separation of mussels

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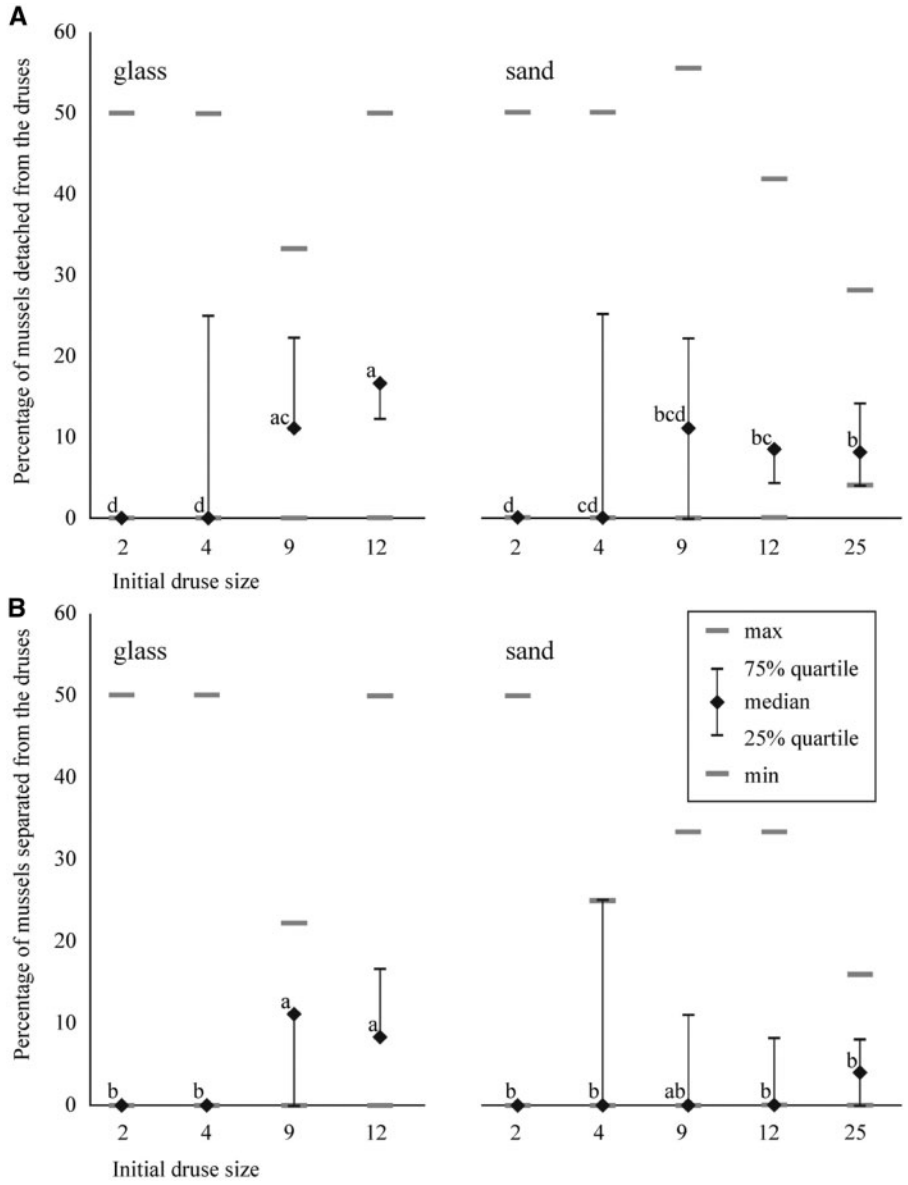

Figure 2. Percentages of mussels which detached from initially formed druses of various sizes (**A**) and mussels which separated themselves from the initial druses (i.e. lost physical contact with the druse) (**B**) in Experiment 1 (mussel dispersal from aggregations on different substrata). Different letters indicate statistically significant differences (determined by post hoc tests following the Kruskal–Wallis test).

from large druses (consisting of nine or more individuals) was greater on the hard substratum than on sand (Appendix Figure 1).

Experiment 2: Movement of single individuals in the presence of conspecifics

Percentages of moving mussels

The percentage of relocated individuals (Figure 4) depended on signal strength (i.e. number of conspecifics in the tank) (G-test: $G_4 = 17.0$, $P = 0.002$), but neither on the distance from the signal source ($G_3 = 1.5$, $P = 0.680$) nor on the interaction between these factors ($G_2 = 1.5$, $P = 0.476$). Pairwise 2×2 G-tests (Figure 4) showed that mussels in the control treatments moved more often (52%) than those exposed to conspecific signals (22%), independent of the signal strength.

The same relationship was observed when locomotive and non-locomotive movements were pooled (G-test: $G_4 = 15.8$, $P = 0.003$, $G_3 = 4.9$, $P = 0.181$, $G_2 = 4.5$, $P = 0.106$ for the signal strength, distance from the signal source and signal strength × distance interaction terms, respectively). Here, mussels in the control treatments moved more often (47%–63%) than those exposed to 15 conspecifics (17%–33%) (Figure 4). The frequency of movement of individuals exposed to three conspecifics was intermediate (30%–43%) and did not differ significantly from the other experimental treatments (Figure 4).

We also checked some qualitative characteristics of mussel behavior associated with these movements by watching the video recordings manually. These observations revealed that the majority of the mussels exhibiting only non-locomotor movements (without relocation) had attempted to burrow into the sand, which was indicated by circular depressions in the sand around the individuals.

Mussel movement parameters

In all the experimental treatments, the test mussels moved on average 10.7 cm ± 16.6 *SD* (range: 0.2–95.2 cm) during 12 h (Figure 5A).

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Distances moved by mussels did not differ significantly among the experimental treatments (Kruskal–Wallis test: $\chi^2_5 = 7.2$, $P = 0.207$), though the mussels exposed at a short distance to the scent of 15 individuals tended to move shorter distances.

No significant differences in the total time spent on locomotion were found among the experimental treatments (Kruskal–Wallis test: $\chi^2_5 = 4.0$, $P = 0.544$), which ranged from 4% to 7% of the total experimental time (Figure 5B). On the other hand, experimental conditions significantly affected time spent by mussels on non-locomotive movements (Kruskal–Wallis test: $\chi^2_5 = 16.0$, $P = 0.007$). The mussels located at a short distance (3 cm) from the signal emitted by three conspecifics spent the least time on non-locomotive movements (3.4% of the total time of the experiment) compared to the other groups. It differed significantly from the times observed in other experimental treatments, ranging from 7.7% to 18.3% (post hoc tests, Figure 5C).

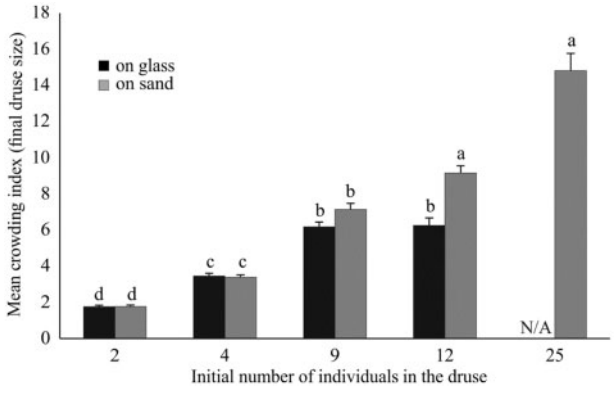

Figure 3. Mean crowding index [the final group size calculated according to Jarman (1974)] after Experiment 1 (mussel dispersal from aggregations on different substrata), depending on druse size and substratum type. Different letters indicate statistically significant differences (determined by post hoc test following the Kruskal–Wallis test). Error bars indicate standard error of the means.

The test mussels did not move preferentially towards or away from the signal source, as shown by non-significant results of the one-sample *t*-tests (Appendix Figure 2).

Discussion

Dispersal of mussels from aggregations

Mussel responses to druse density in Experiment 1 (druse dynamics) did not confirm their preferences for conspecifics, suggested by the previous studies (Wainman et al. 1996; Kobak 2006). We revealed that *D. polymorpha* avoided large druses, particularly when the alternative hard substratum was available. Moreover, mussels detaching from the druses remained in contact with conspecifics in the absence of alternative hard substrata, but otherwise preferred to stay at some distance from one another. We have confirmed earlier preliminary findings by Kobak and Ryńska (2014) and Tošenovský and Kobak (2016), suggesting that zebra mussels do not form druses until forced by the lack of alternative hard substrata, as well as those by Kavouras and Maki (2003) showing that they prefer biofilmed artificial substrata over conspecific shells. This result is against conventional wisdom, which assumes mussel preferences for conspecifics, leading to group formation. On the other hand, Kobak et al. (2009) observed that zebra mussel individuals attached in a direct contact with conspecifics were less likely to detach and move to another site than singletons. However, in the aforementioned study, mussels formed only monolayer aggregations with individuals touching one another's shell, but attached to an alternative hard substratum. Thus, our results suggest that mussels preferred other hard substrata over conspecific shells and the latter were selected only if no choice was possible. It is likely to be associated with the negative impact of a large colony on its members demonstrated in the field by Stańczykowska (1964) and Czarnołęski et al. (2003) as well as in laboratory by Burks et al. (2002) and Tuchman et al. (2004).

The observed reactions of mussels to conspecifics may be important for their distribution in the field. Intentional detachment from a druse and avoidance of a direct contact with a dense colony may contribute to the effective small scale spreading of this invasive species, as well as enhancing the large scale transport of mussels

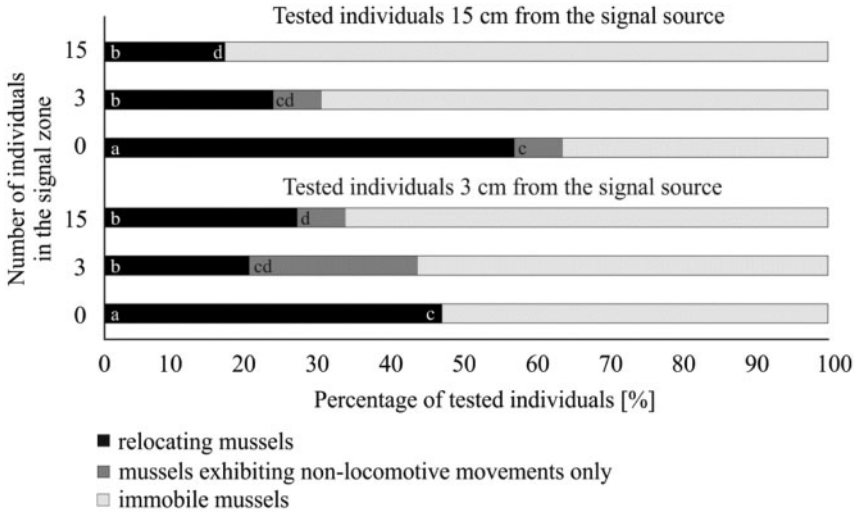

Figure 4. Percentages of mussels that relocated, exhibited only non-locomotive movements and did not move at all in Experiment 2 (mussel movement in the presence of conspecifics). Different letters on the bars indicate statistically significant differences between experimental treatments (2 × 2 G-tests with a sequential Bonferroni correction applied); a and b for relocating mussels, c and d for all moving mussels (with locomotive and non-locomotive movements pooled).

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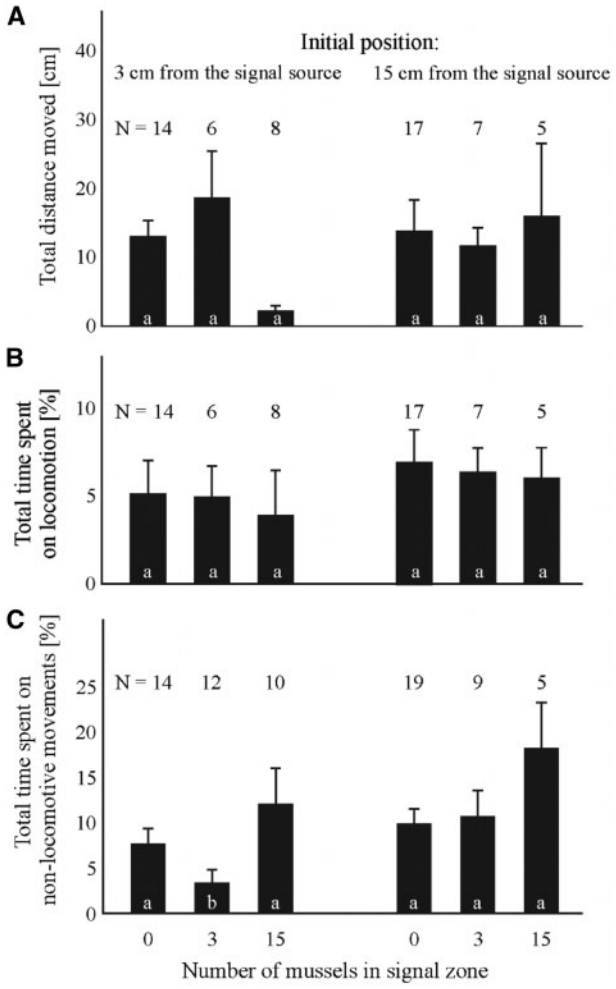

Figure 5. Total distance moved (**A**), time spent on locomotion (**B**) and time spent on non-locomotive movements (**C**) by mussels depending on their distance from the signal source and signal strength in Experiment 2 (mussel movement in the presence of conspecifics). *N* indicates the numbers of analyzed mussels (only translocating/moving individuals were used in the analysis and to calculate the means). Error bars indicate standard error of the means. Different letters on the bars indicate statistically significant differences (determined by post hoc tests following the Kruskal–Wallis test).

attached to drifting debris or boat hulls (Minchin et al. 2003) by increasing their penetration of new areas and thus the probability of their colonization (Collas et al. 2017).

Movement of single individuals in the presence of conspecifics

Character of stimulus

The nature of the conspecific cue observed in our study might be either chemical or physical, but the latter seems much less likely. As the signal donor mussels in Experiment 2 were located behind a dense mesh barrier, the test mussels had no physical contact with conspecifics and siphonal currents are clearly too weak (Ertman and Jumars 1988) to be detectable though the mesh from the distance tested in our design. Thus, given the fact that responses of zebra mussels to conspecific chemical signals were also observed in other studies (Kobak 2001; Kobak and Ryńska 2014), we assume that mussels in our experiments responded to chemical stimuli.

Percentages of moving mussels

Mussels in the presence of conspecifics clearly reduced their activity, regardless of the adverse substratum on which they were located and the lack of any shelter. A similar reduction in locomotor activity of *D. polymorpha* has been observed in response to stress factors, such as the presence of predators, alarm cues or light (Toomey et al. 2002; Kobak and Nowacki 2007; Naddafi and Rudstam 2013). On the other hand, Commito et al. (2014, 2016) observed a different behaviour of marine *Mytilus edulis*, which quickly formed aggregations even on hard substrata and irrespective of any predation cues. Perhaps, clumping is more important for marine bivalves, experiencing heavy hydrodynamic forces (Bell and Gosline 1997) and facing much more diverse predators (Reimer and Tedengren 1997). The behavior of mussels observed in our study may indicate their "passive" avoidance reaction to other individuals. An indirect reason for such activity reduction could be the fact that mussels were unable to move directionally in response to conspecific cues. As the probability of accidental encounters with other individuals during random movements increases with density, the activity reduction in response to conspecific signals seems to be the best solution if such encounters are undesirable.

Mussel movement parameters

The individuals placed near 15 conspecifics (thus potentially experiencing the strongest signal) displayed a clear, though non-significant tendency for a substantial decrease in travelled distance and a significant increase in non-locomotor movement, revealing their ability to estimate the strength of conspecific signals. The non-locomotor movement seems to be associated with preparation for attachment in the current position, as shown by visual examination of the video recordings. The increase in bivalve byssogenesis in the presence of conspecifics is a known phenomenon (Uryu et al. 1996; Kobak 2006) and up to now has been considered as a reaction showing preferences of mussels to conspecifics. Our study suggests that non-locomotive movement of mussels can also be the effect of "passive avoidance": mussels experiencing a high density of conspecifics and being unable to move directionally reduced their locomotion and searched for the nearest suitable substratum to attach.

Apparently, the intraspecific relations of *D. polymorpha* are the result of interactions among a number of factors varying in different environments, including the presence of predators (Kobak and Kakareko 2009) and strong water currents (Tošenovský and Kobak 2016), which may stimulate aggregation forming. Nevertheless, our study has shown that conspecific avoidance reactions, as well as the availability of hard substratum, are among the major forces forming these relationships.

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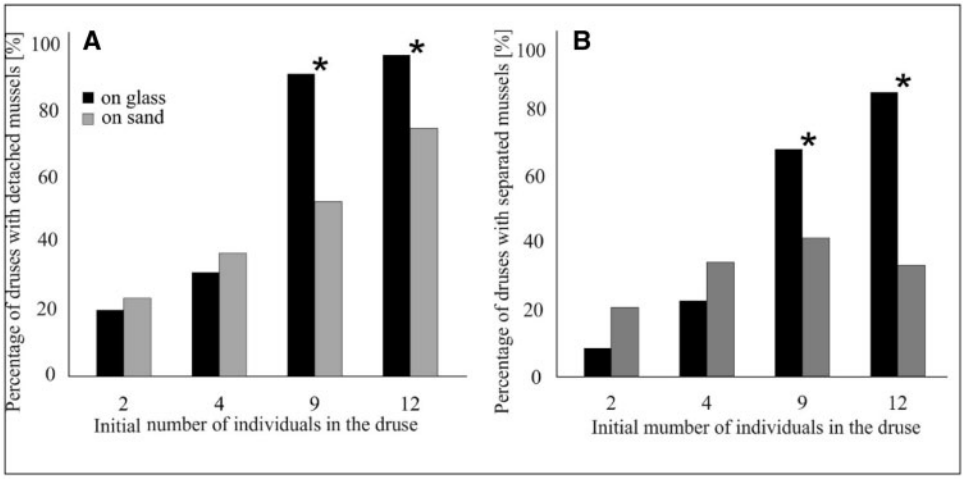
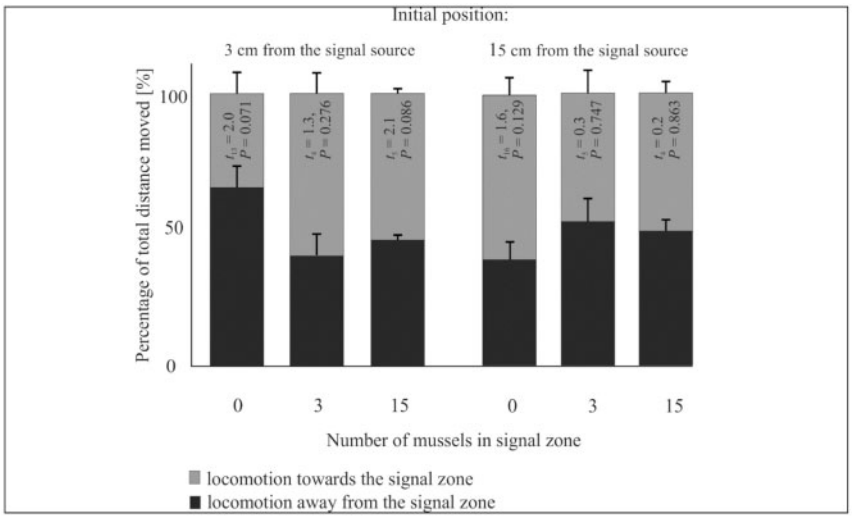


Appendix Figure 1. Percentages of druses changed by (**A**) detachment and (**B**) separation (i.e., detachment and losing physical contact with the druse) of mussels in Experiment 1 (mussel dispersal from aggregations on different substrata). Asterisks indicate statistically significant differences between substratum types for a given druse size (2 × 2 G-tests with a sequential Bonferroni correction applied).



Appendix Figure 2. Percentages of distances moved towards and away from the signal source in Experiment 2 (mussel movement in the presence of conspecifics). The results of one-sample *t*-tests comparing the percentages with the theoretical value of 50% (denoting the lack of directional movement) are shown on the bars. Error bars indicate standard error of the means.

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Mechanisms and impact of differential fouling of the zebra mussel *Dreissena polymorpha* on different unionid bivalves. *Freshwater Biology, 2018, 63: 687-699*

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ORIGINAL ARTICLE

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Mechanisms and impact of differential fouling of the zebra mussel *Dreissena polymorpha* on different unionid bivalves

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Abstract

1. Macrobiofouling is an important phenomenon in the aquatic environment, resulting in economic losses and environmental changes, including negative impact on hard-shelled animals. A freshwater invasive byssate bivalve, the zebra mussel *Dreissena polymorpha* (Dreissenidae), strongly affects bivalves from the family Unionidae by fouling their shells. We tested potential mechanisms explaining variable fouling of different unionids (four native species: *Anodonta anatina*, *A. cygnea*, *Unio pictorum*, *U. tumidus* and the non-native *Sinanodonta woodiana*) by the zebra mussel.
2. We conducted a field survey (unionids collected at a sandy and muddy site in a dam reservoir), field experiment (unionids exposed without sediments in suspended baskets to dreissenid settlement) and laboratory experiment (a multiple choice test with and without the possibility of unionid burrowing into sandy sediments).
3. In the survey, zebra mussel density was highest on *A. anatina* and *S. woodiana*, intermediate on *U. pictorum* and lowest on *A. cygnea* and *U. tumidus*. In the field experiment, *A. anatina* and *S. woodiana* were more fouled than *Unio* spp. In the laboratory, zebra mussels less often attached to *U. pictorum* and, when unionids could burrow themselves, also to *S. woodiana*. However, no unionid species was positively selected in the presence of stone substrata.
4. The percentage of zebra mussels attached directly to unionids (compared to those attached to conspecifics) in the field survey was negatively related to the overall zebra mussel biomass. Zebra mussel fouling negatively affected the biomass of an allopatric *S. woodiana* on muddy bottom.
5. Dreissenids overgrow different unionid species to variable extent, not only due to the differences in their exposed surface area, but, as our experimental results show, also because of their active substratum selection. Moreover, mussels seem to prefer unionid surface over conspecific shells, the latter being fouled only when overall dreissenid fouling is heavy. As unionids often constitute the main source of hard substratum for zebra mussels in waterbodies, the species composition of a unionid assemblage may affect dreissenid success by offering them variable substratum quality.

KEYWORDS
macrobiofouling, mussel recruitment, substratum preferences, substratum selection, Unionidae

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1 | INTRODUCTION

Macrobiofouling of hard substrata by sessile organisms, such as algae, bivalves, polychaetes, barnacles, ascidians, sponges and cnidarians is an important phenomenon in the aquatic environment (Khalaman, 2001; Prendergast, 2010), causing severe economic losses due to the overgrowth of hydrotechnical appliances and contributing to habitat formation by gregarious ecosystem engineers (Sousa, Gutiérrez, & Aldridge, 2009; Terlizzi & Faimali, 2010). Hard-shelled animals, including mobile taxa, also suffer from macrofouling (Ďuriš, Horká, & Petrusek, 2007; Strayer & Malcom, 2007; Yohannes, Ragg, Armbruster, & Rothhaupt, 2017) and have developed numerous chemical and mechanical defences against this process (Bers, Prendergast, Zürn, Head, & Thomason, 2006; Bers et al., 2010; Hirota, Okino, Yoshimura, & Fusetani, 1998).

The zebra mussel *Dreissena polymorpha* (Dreissenidae) is one of the most important freshwater invasive fouling species, exerting a strong impact on invaded ecosystems in Europe and North America (Karatayev, Burlakova, & Padilla, 1997; Sousa, Novais, Costa, & Strayer, 2014). Apart from increasing water transparency by filtration, as well as affecting the composition and diversity of zoobenthos by habitat forming, it also affects large freshwater bivalves from the Unionidae family (Kelly, Herborg, & MacIsaac, 2010). Zebra mussels overgrow unionid shells, mainly aggregating near their posterior, siphonal part, exposed above sediments to enable water exchange in the mantle cavity (Bódis, Tóth, & Sousa, 2014; Lewandowski, 1976; Sousa, Pilotto, & Aldridge, 2011). Fouling groups of dreissenids commonly reach high biomasses, even exceeding those of their hosts, which deteriorates unionid locomotion, burrowing, respiration and/or feeding, finally leading to their death when the fouling is high (Ricciardi, Whoriskey, & Rasmussen, 1996; Schloesser, Nalepa, & Mackie, 1996; Strayer & Smith, 1996). Moreover, juvenile zebra mussels may attach between the valves of a unionid mussel, resulting in its permanent opening. Impossibility of the complete valve closure increases the risk of predator attacks and parasite infestation (Mackie, 1991).

On fine sediments, unionid mussels are an important source of hard substratum (Schloesser et al., 1996; Sousa et al., 2011; Strayer & Smith, 1996). This implies their high importance for the successful development of dreissenid populations and potential conservation problems for unionids, often already endangered by habitat loss, pollution, climate change and/or excessive harvesting (Lopes-Lima et al., 2017, 2018; Ricciardi & Rasmussen, 1999). Reports on the outcome of the interactions between dreissenids and unionids vary from heavy extirpation of the latter (Ricciardi et al., 1996; Schloesser et al., 1996) to co-existence with no signs of decline (Burlakova, Karatayev, & Padilla, 2000; Lewandowski, 1976). Nevertheless, most of the studies point to some negative effects of fouling on unionid condition, abundance and/or occupied range (Bódis et al., 2014; Sousa et al., 2011; Strayer & Malcom, 2007).

Several field studies have reported differential dreissenid fouling and impact on various unionid species (Schloesser et al., 1996; Sousa et al., 2011; Strayer & Smith, 1996). However, given their correlational nature, it is still difficult to distinguish among potential mechanisms of these differences among species. Unionid size and their burrowing behaviour seem to be key factors driving zebra mussel fouling (Bódis et al., 2014; Sousa et al., 2011). Nevertheless, active dreissenid selection or avoidance cannot be unambiguously excluded without experimental evidence.

In this study, we checked various potential mechanisms explaining variable fouling of different unionid species by dreissenids, as well as the impact of dreissenids on fouled mussels. We combined a field survey, field experiment and laboratory experiment (with and without the possibility of unionid burrowing) to obtain an overall picture of the phenomenon in the wild (with all potential factors present) and to test separate effects of particular factors under controlled conditions. The combined results of the three parts of our study allowed to test the following hypotheses: (1) various unionid species would be differently fouled by zebra mussels (Lewandowski, 1976; Sousa et al., 2011). This selection would be based on specific unionid features, origin (sympatric or allopatric) and/or burrowing, (2) Zebra mussels would discriminate between unionid shells and stones, indicating that the shells are preferred substratum or are only selected in the absence of alternatives, (3) Zebra mussels would form druses (clumps of conspecifics attached to one another) on unionid shells irrespective of their density, indicating that conspecific shells are preferred sites for mussel settlement (as shown by Wainman, Hincks, Kaushik, & Mackie, 1996 and marine examples, e.g. Tamburri, Zimmer-Faust, & Tamplin, 1992; Commito et al., 2014). Alternatively, they could form druses only when the exposed unionid surface is no longer available. This would indicate that conspecifics are selected only when no alternatives are available, as suggested by Tošenovský and Kobak (2016) and Dzierżyńska-Białończyk, Skrzypczak, and Kobak (2018), (4) Finally, unionid biomass would be negatively correlated with dreissenid fouling.

2 | METHODS

2.1 | Field survey

We performed a field survey to investigate the intensity of fouling and its effects on different unionid species under natural conditions, in the presence of all potential modifying factors (unionid burrowing, active selectivity of zebra mussels and their variable survival on different unionid species). This constituted the background knowledge for designing the field and laboratory experimental studies, separating different factors and fouling mechanisms. We hypothesised that zebra mussels would be more abundant, larger and more often attached directly to unionid shells (rather than to conspecifics) on more suitable unionid species.

We conducted the survey in July 2016 in the middle part of the Włocławek Reservoir located on the lower Vistula River in central Poland (52°37′03″N, 19°19′37″E) (Figure 1a, b). It is a large (area: 75 km^2; capacity: 400 million m^3), shallow (average depth: 5.5 m;

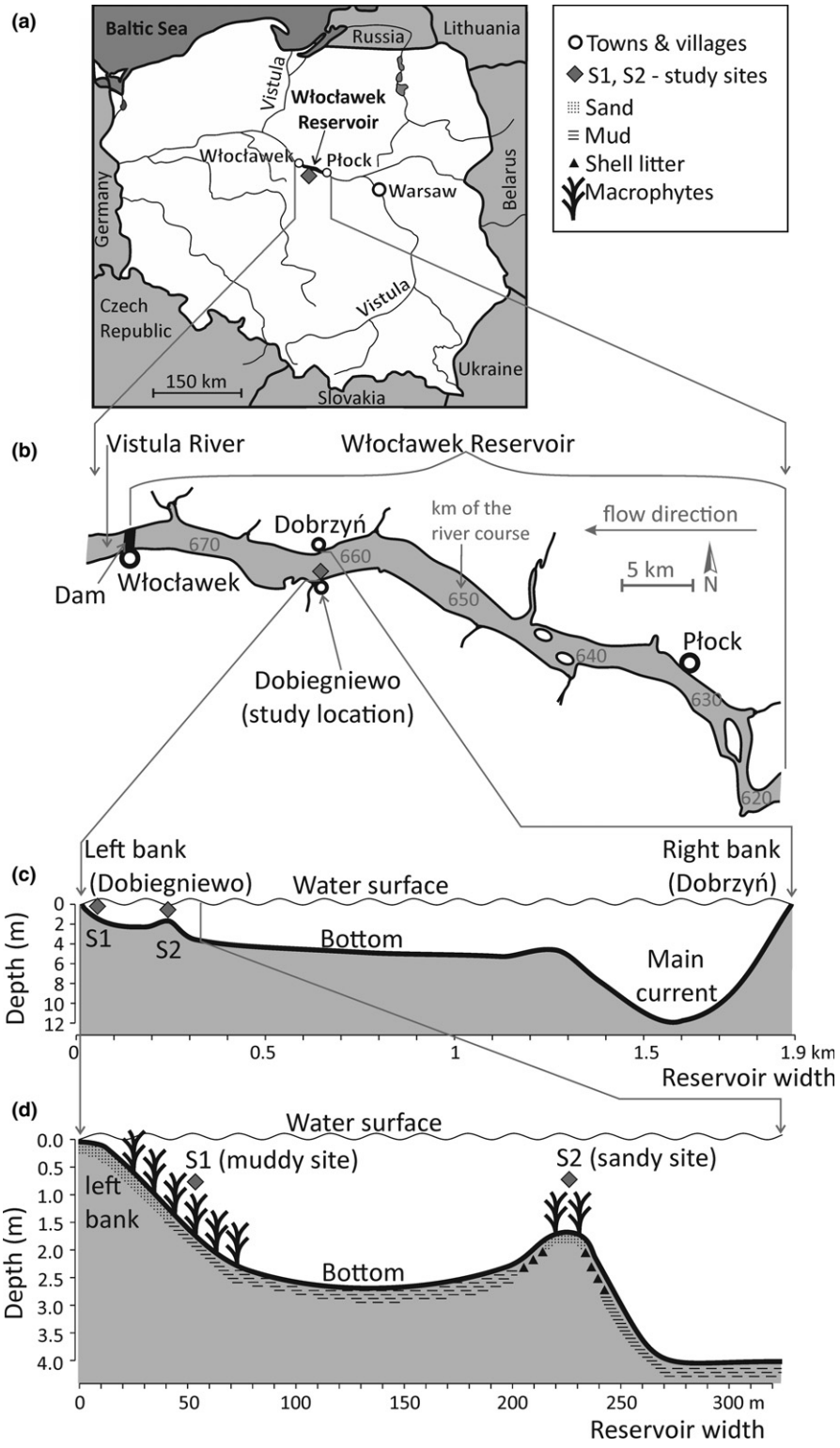

FIGURE 1 The location of sites for the field studies and collecting animals for the experiments. The position of the reservoir in Poland (a), the longitudinal view of the reservoir (b), the cross-sectional profile of the reservoir at the study location (c), the close-up of the part of the cross-sectional profile containing the study sites S1 and S2 (d). Sampling sites are indicated by grey diamonds

max.: 15 m) eutrophic lowland dam reservoir with a short water retention time (4–5 days) and mean water flow of 900 m^3/s. During normal operation of the hydroelectric power plant located at the dam, the amplitude of water level fluctuations is *c.* 0.5 m (Poznańska, Kobak, Wolnomiejski, & Kakareko, 2010).

The width of the reservoir at the sampling location is *c.* 2 km. The main current is located *c.* 200 m from the right bank, leaving a wide lenitic zone along the left bank (Figure 1c). Unionids were sampled at two sites located in the lenitic part of the reservoir (Figure 1d). Site 1, placed at *c.* 50 m from the left bank, was *c.* 2 m deep and had muddy sediments. Site 2, located *c.* 220 m from the left bank, was *c.* 1.5 m deep and had sandy bottom overgrown by submerged macrophytes (mainly *Myriophyllum spicatum* (Haloragaceae), *Potamogeton nodosus*, *Potamogeton pectinatus* and

Potamogeton perfoliatus (Potamogetonaceae)). At both sites, unionids were the only abundant hard substrata available for zebra mussel settlement apart from macrophytes, few stones and solid waste objects (Czarnecka, Poznańska, Kobak, & Wolnomiejski, 2009). This sampling design allowed us to compare dreissenid fouling on unionid mussels on two different bottom types, occupied by different unionid assemblages (Table 1). Sand was clearly more suitable for dreissenids and for most of the unionid species due to the difficulties in staying on the surface of loose muddy sediments. Therefore, we expected higher zebra mussel fouling on sandy bottom, although their impact on unionids might be stronger on the overall more demanding muddy site.

Unionid mussels were sampled by a SCUBA diver, who randomly selected 10 spots within the area of *c.* 30–35 m^2 at each site and collected all individuals within a radius of 40 cm (0.5 m^2) around them. Thus, mussels were collected at random but then we added randomly selected extra individuals from less common species to the analysed dataset to balance the numbers of all species within each site (Table 1). On the shore, we removed all zebra mussels from each unionid and determined the following variables: (1) taxonomic identity of Unionidae mussels (five species: *Anodonta anatina*, *Anodonta cygnea*, *Unio tumidus*, *Unio pictorum* and *Sinanodonta woodiana*) using the keys by Kołodziejczyk and Koperski (2000) and Piechocki and Wawrzyniak-Wydrowska (2016), (2) the fresh unionid biomass with shell to the nearest 0.1 g (using a scales WPT 3/6, Radwag, Radom, Poland), (3) the number of dreissenids attached directly to the unionid shell surface and those attached to conspecifics, (4) the fresh biomass of dreissenids (with shells) fouling the unionid mussel. We photographed unionids and zebra mussels to determine their lengths (and heights in the case of unionids). We calculated unionid shell surface areas using regression equations based on the length and height of their shells. These equations were established on the basis of shell surface areas of 10–12 individuals of each species, determined by wrapping them with aluminium foil and measuring the areas of the stretched pieces of foil. We carried out these measurements using IMAGEJ 1.51k software (W.S. Rasband, National Institutes of Health, USA, https://imagej.nih.gov/ij). We returned all unionid mussels except invasive *S. woodiana* to the reservoir immediately after processing.

To check the intensity of fouling of various unionid species by zebra mussels, we analysed dreissenid density (number per unit shell area, log-transformed) using a general linear model (GLM) with (1) bottom type (sand or mud) and (2) unionid species (5 levels) as categorical between-subject factors, (3) interaction between (1) and (2) (to check whether mussel preferences depended on the bottom type) and (4) unionid biomass (log-transformed) as a continuous variable (to check whether mussels discriminated among unionids of various sizes).

To check whether zebra mussels exhibited variable preferences for conspecific or unionid shells, depending on unionid species, we analysed the percentage of mussels attached directly to unionid shells, assuming that its higher value would point to the selectivity for unionid surface. We analysed this variable using a generalised linear mixed model (GLMM) with a binomial distribution and logit link function, including (1) bottom type and (2) unionid species as categorical between-subject fixed factors, (3) interaction between (1) and (2) (to check whether zebra mussel distribution on the shells of various species changed with the bottom type), as well as (4) zebra mussel biomass per unit unionid shell area (log-transformed) as a continuous variable and (5) unionid individual as a random factor. We included the continuous variable to check whether the dreissenid distribution on the shell depended on the intensity of their fouling (the higher biomass indicates the greater number and/or sizes of the fouling mussels, i.e. fouling of a greater surface area).

To check whether the size of fouling dreissenids depends on unionid species or their position in a colony, we analysed mean dreissenid length (log-transformed) attached to particular unionid individuals in the field survey using a GLM with (1) bottom type and (2) unionid species as categorical between-subject factors, (3) mussel position (directly on the unionid or on the conspecific surface) as a within-subject factor (as we expected that early settlers, attaching directly to unionid shells, could be larger than those attached to conspecifics), (4) interactions between (1), (2) and (3) (as we expected that various unionid species can differently affect zebra mussel size depending on the bottom type and mussel position), (5) zebra mussel biomass per unit unionid shell area (log-transformed) as a continuous variable (to check whether dreissenid size was limited by the intensity of their fouling) and (6) interaction between (3) and (5) (to check whether the difference in size between mussels attached directly to unionids and to conspecifics changed with fouling intensity). We included only zebra mussels from unionids having dreissenids

TABLE 1 Numbers and lengths of unionid mussels in the field survey and field experiment

	Field survey						Field experiment	
	Percentages in the field		Numbers of analysed mussels		Mean length (mm ± *SD*)			
Species	Sand	Mud	Sand	Mud	Sand	Mud	Numbers of analysed mussels	Mean length (mm ± *SD*)
A. anatina	29	35	25	15	74 ± 18.5	76 ± 12.0	15	60.9 ± 8.3
A. cygnea	0	14	0	11		119 ± 6.8		
S. woodiana	26	12	22	8	78 ± 17.2	84 ± 24.2	15	72.4 ± 21.9
U. pictorum	27	14	23	9	79 ± 12.8	69 ± 7.4	13	64.5 ± 7.2
U. tumidus	18	26	25	11	58 ± 7.3	72 ± 10.9	12	61.4 ± 7.0

attached both directly to their shells and to the conspecific surface in this analysis.

To check whether fouling zebra mussels affected their unionid hosts, we analysed unionid biomass (log-transformed) in the field survey using a GLM with (1) bottom type and (2) unionid species as categorical between-subject factors as well as (3) unionid length (log-transformed to linearise the size-biomass relationship) and (4) zebra mussel biomass per unit unionid biomass (log-transformed) as continuous variables. This analysis allowed us to check whether zebra mussel fouling affected unionid biomass after controlling for the unionid length (i.e. whether heavily fouled unionids had lower biomasses than less fouled individuals of the same size). Thus, we included these two continuous variables and interactions involving them in the model. Here, the intensity of dreissenid fouling was related to the unionid biomass, rather than surface area, as this was more likely to correspond to the strength of the potential effect of fouling mussels on a unionid individual.

We transformed the data for the GLM analyses (as indicated above) to counteract violations of normality and homoscedasticity assumptions, checked with Shapiro–Wilk and Levene tests, respectively. We further examined significant effects with sequential Bonferroni-corrected post hoc procedures: Fisher LSD tests or paired contrasts for the GLM and GLMM analyses, respectively. We carried out all the analyses using SPSS 24.0 (IBM Inc.).

2.2 | Field settlement experiment

This study was complementary to the field survey. Its purpose was to check whether the variable susceptibility of different unionid species to zebra mussel fouling, observed in the survey, would persist in the absence of sediments, that is when burrowing is not possible. Such a result would indicate an active selection and/or avoidance of different unionids by settling zebra mussel larvae, driven by the quality of shell properties and/or siphonal currents (potentially enhancing filter-feeding, as suggested by Pilotto, Sousa, and Aldridge (2016)). Therefore, we tested the settlement of zebra mussel larvae on unionids exposed without the possibility of burrowing into sediments.

On the 18 of July 2016, we deployed five plastic openwork baskets (diameter: 300 mm, height: 250 mm, square holes and spaces between the holes: 5 mm, Figure 2) at the muddy site used in the above-mentioned survey (Figure 1). The baskets were attached to an anchor fixed at the bottom with a 0.5-m rope and to a submerged float with another 0.5-m rope, which resulted in their upright arrangement 0.5 m above the bottom and *c.* 1.5 m below the surface, as well as in the stable horizontal position of their bottoms. Another float attached to the anchor with a longer rope was used to signal the location on the surface (Figure 2).

We introduced twelve unionid mussels to each basket (three individuals of *A. anatina*, *S. woodiana*, *U. pictorum* and *U. tumidus*, collected immediately before the experiment and cleaned of the attached zebra mussels and remnants of their byssal threads) without any sediments. The shell surface areas of all species did not differ from one another (one-way ANOVA on log-transformed data: $F_{3,\ 57} = 0.8$, $p = .483$). The total shell side area of 12 mussels in the basket was *c.* 3 times smaller than the area of the basket bottom. Thus, all the mussels rested separately on the basket bottom, did not cover one another and were equally exposed to dreissenid settlement. We did not use *A. cygnea* in this experiment due to the problems with the survival of this species and difficulties with finding individuals of the same size as that of the other species. The baskets were closed from the top with solid lids, so dreissenid larvae could enter inside through the holes in the walls.

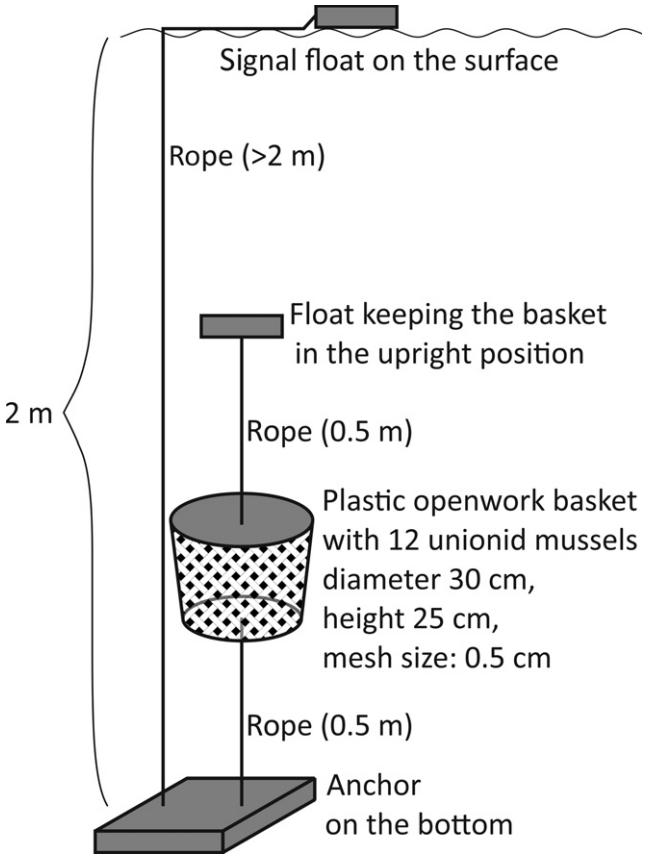

FIGURE 2 The design of the field experiment. Five replicate baskets were exposed in the reservoir depicted in Figure 1

We retrieved the baskets on the 28 of September and determined the following variables: (1) the numbers of settled zebra mussels (>1 mm) (only on living unionids, see Table 1), (2) the lengths and heights of the unionids (to calculate their shell areas), (3) the unionid biomasses. We returned all unionid mussels except invasive *S. woodiana* to the reservoir immediately after processing.

We analysed dreissenid density (individuals per shell area, log-transformed) and mean size (log-transformed) on unionids in the field experiment using a general linear model (followed by sequential Bonferroni-corrected Fisher LSD tests) with (1) unionid species as a fixed factor, (2) basket as a random factor, included to avoid pseudoreplications (as each basket contained a group of unionids), and (3) unionid biomass (log-transformed) as a continuous variable included to check the effect of unionid size.

2.3 | Laboratory fouling experiment

This study was designed to test preferences of juvenile metamorphosed zebra mussels crawling over the bottom, which can also contribute to adult dreissenid distribution by selecting particular microhabitats (Kobak & Nowacki, 2007). Similarly to our field studies, we discriminated between the effect of unionid burrowing, protecting them from fouling, and active selectivity of zebra mussels, by varying the amount of the bottom sediments.

We tested the fouling of juvenile zebra mussels (length: 5–10 mm) on various species of unionids in 10-L tanks (bottom: 240 × 240 mm, height: 200 mm) filled with sandy sediments and continuously aerated tap water (used after at least 24 hr of aeration). Water conditions (means ± *SD*) during the experiment were as follows: temperature 18 ± 0.9°C, conductivity: 580 ± 20 μS/s, oxygen concentration: 8.1 ± 0.8 mg/L, pH: 8.2 ± 0.2 (measured with a multimeter Multi340i, WTW GmbH, Weilheim, Germany). We collected zebra mussels and unionids shortly before the experiment at the location of the field studies described above and cleaned them of the attached mussels and remnants of their byssal threads. We placed single individuals of each unionid species (*A. anatina*, *S. woodiana*, *U. pictorum* and *U. tumidus*) in the experimental tank in random positions. Additionally, we introduced a unionid-shaped stone (pre-exposed in water for a week to allow biofilm development) to compare its quality as a substratum with unionids. Thus, each tank contained five different hard substrata. All the hard substrata in a single tank had equal length with a tolerance of ±1 cm. In this experiment, equalising linear dimensions was more important than surface areas, as crawling mussels encounter potential substratum objects at the water-bottom interface, differently than planktonic larvae settling in the field onto the entire shell surface. The mean length (±*SD*) of the unionids and stones was 73 ± 10.6 mm. We did not use *A. cygnea* in this experiment as it was impossible to find the sufficient number of specimens small enough to match them with the individuals of the other species. After 24 hr, when the unionids established their positions in the tanks, we introduced 30 juvenile zebra mussels to the central part of the tank. The experiment consisted of two treatments: (1) with a 5-cm sand layer, into which the unionids could burrow, and (2) with 1-cm sand layer, making the unionid burrowing impossible, but preventing the zebra mussels from attaching to the tank bottom. After 24 hr, we counted zebra mussels attached to particular objects in the tank. We replicated this experiment 24 times, each time using new unionid and zebra mussel individuals.

To check preferences for or avoidance of particular objects (stones or various unionid species) offered to zebra mussels, we compared mean percentages of mussels attached to these objects (calculated taking into account all mussels that attached to the objects present in the tank) with a theoretical value of 20%, assuming the random distribution of mussels among the five object types. We used sequential Bonferroni-corrected one-sample Wilcoxon signed rank tests for this comparison, as the data strongly violated the normality assumption (checked with a Shapiro–Wilk test).

3 | RESULTS

3.1 | Field survey

Only one *A. cygnea* individual was totally free of zebra mussels. All other unionid mussels were overgrown by 1–556 zebra mussels (median: 61, mean: 84) of the maximum weight of 107 g (median: 14 g, mean: 20.5 g). The maximum number and biomass of zebra mussels were found on two *S. woodiana* individuals from the sandy site.

Zebra mussel density depended on the bottom type (GLM: $F_{1,\ 136} = 85.6$, $p < .001$, see Table S1 for the full results), with greater fouling per unit shell surface found at the sandy site. Zebra mussel density was also affected by the unionid species (GLM: $F_{4,\ 136} = 23.6$, $p < .001$). *Anodonta anatina* and *S. woodiana* were the most fouled species, *A. cygnea* and *U. tumidus* bore the lowest zebra mussel densities whereas *U. pictorum* was intermediate (Figure 3). The effect of unionid biomass on mussel fouling was non-significant (Table S1).

Approximately 40% of zebra mussels were attached directly to the unionid shells, whereas the remaining individuals attached to conspecifics to form multilayer aggregations. The percentage of zebra mussels attached directly to unionids was negatively related to the biomass of zebra mussels per unit unionid surface area (GLMM: $F_{1,\ 136} = 111.0$, $p < .001$, see Table S2 for the full results). At low dreissenid biomasses (<0.2 g per 10 cm^2), zebra mussels attached exclusively to unionid shells (Figure 4).

Moreover, after controlling for the variable zebra mussel biomass, the percentage of mussels attached directly to unionid shells depended on an interaction between unionid species and bottom type in the GLMM ($F_{3,\ 136} = 4.9$, $p = .003$). At the sandy site, the percentage of mussels attached directly to the unionid surface was lower on *Unio* spp. than on *A. anatina* and *S. woodiana*, whereas on the muddy bottom it was lower on *A. cygnea* than on the other species (Figure 5).

Zebra mussel length ranged from 2.8 to 33.7 mm (mean: 11.4 mm, median: 11.3 mm). The position of mussel attachment (unionid or conspecific shell) affected their size, but this relationship depended also on the biomass of the zebra mussels attached to the unionids, resulting in a significant interaction between these factors (GLM: $F_{1,\ 115} = 7.2$, $p = .008$, see Table S3 for the full results). At lower zebra mussel biomasses, the individuals attached to conspecifics were smaller than those attached directly to unionids, whereas at higher biomasses this difference was not significant (Figure 6).

After controlling for the variable biomass of zebra mussels, larger dreissenids (irrespective of their position) occurred on *A. cygnea* than on the other species (Figure 7), as shown by a significant species effect in the GLM (GLM: $F_{4,\ 115} = 3.4$, $p = .012$).

After controlling for unionid length, zebra mussel fouling significantly affected unionid biomass. This relationship depended on bottom type and unionid species, as shown by significant interactions between these factors in the GLM (GLM: bottom type × dreissenid biomass: $F_{1,\ 127} = 5.0$, $p = .027$, unionid species × dreissenid biomass: $F_{4,\ 127} = 4.1$, $p = .004$, see Table S4 for the full results). Linear regressions of unionid biomass on their length and fouling zebra

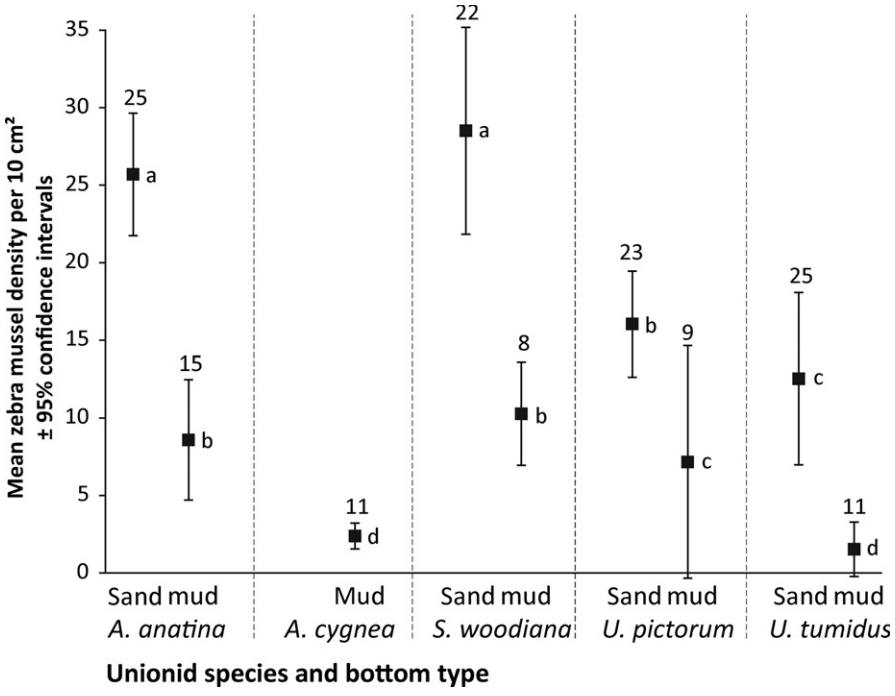

FIGURE 3 Mean (±95% confidence intervals) densities of zebra mussels (per 10 cm^2 of unionid shell) on various unionid species sampled from different substrata. The bars labelled with the same letters indicate the lack of significant differences between particular means. The numbers above the bars indicate the numbers of analysed unionids

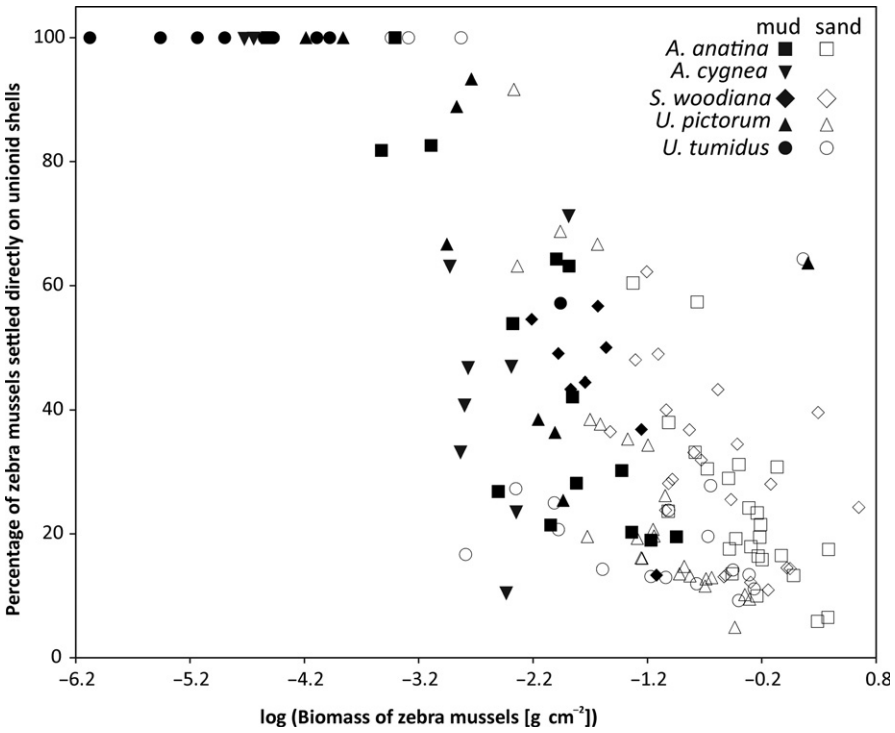

FIGURE 4 Relationship between the percentage of zebra mussels attached directly to the unionid shell and zebra mussel biomass (per 1 cm^2 of unionid shell). See Figure 3 for the numbers of analysed unionids

mussel biomass carried out separately for each bottom type and unionid species (Table S5) revealed that fouling significantly reduced the biomass of *S. woodiana* on the muddy bottom (Figure 8). Thus, more heavily fouled mussels of that species had lower biomasses than their less fouled conspecifics of the same length.

3.2 | Field experiment

Zebra mussel settlement on various unionid species ranged from 0.40 to 1.20 individuals per 10 cm^2 shell area. The effect of unionid species on zebra mussel density was significant (GLM: $F_{3,\ 46} = 3.6$, $p = .021$, see Table S6A for the full results), *Unio* spp. being less fouled than *A. anatina* and *S. woodiana* (Figure 9). Zebra mussel length (mean ± *SD*: 3.44 ± 1.72 mm, range: 1.00–13.54 mm) did not depend significantly on unionid species nor biomass (Table S6B).

3.3 | Laboratory experiment

Approximately 56% of zebra mussels attached to unionids and stones offered to them in the experimental tanks. The remaining

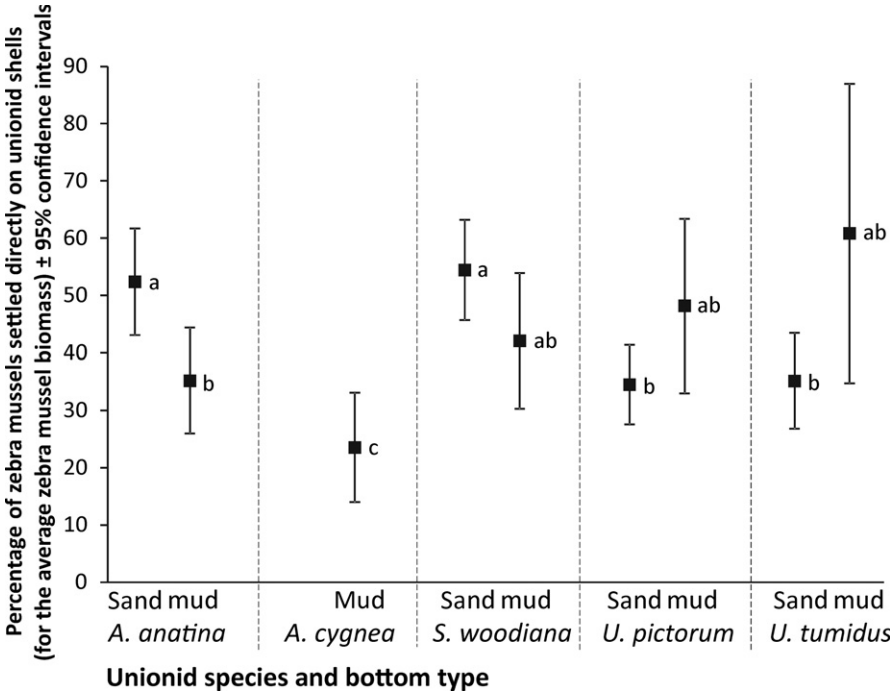

FIGURE 5 Mean (±95% confidence intervals) percentages of zebra mussels attached directly to the shells of various unionid species sampled from different substrata. The values are predicted by the GLMM for the mean biomass of zebra mussels on the unionid (1.9 g per 10 cm^2 of unionid shell). The bars labelled with the same letters indicate the lack of significant differences between particular means. See Figure 3 for the numbers of analysed unionids

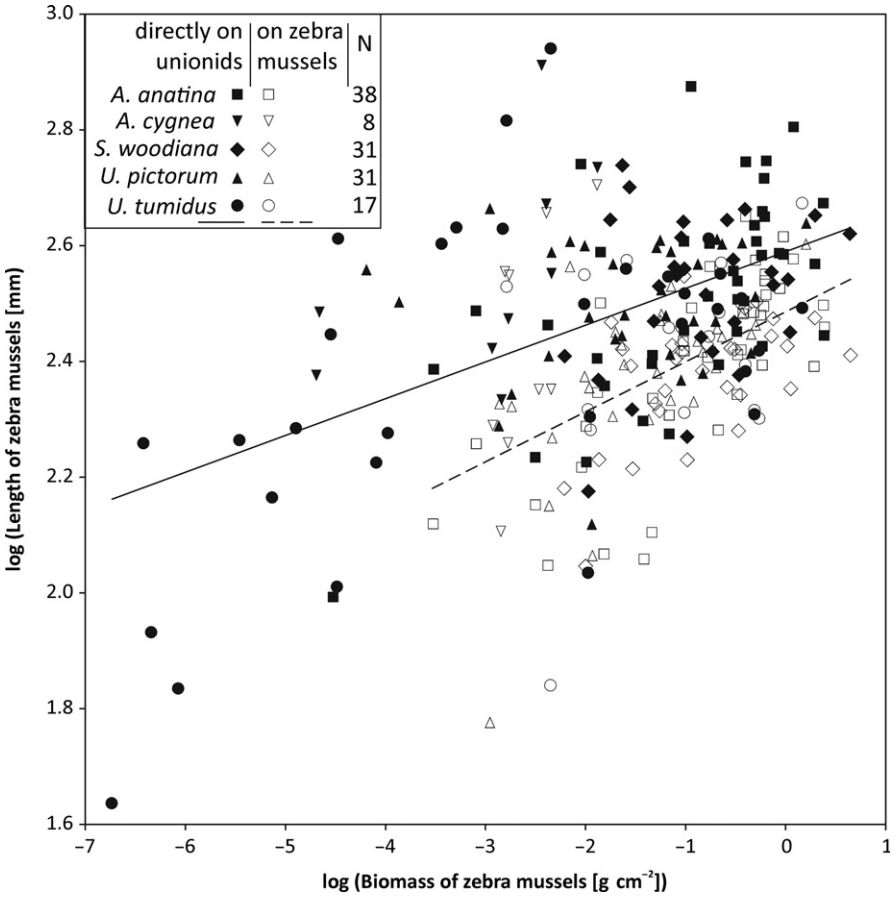

FIGURE 6 Relationships between the length of zebra mussels attached directly to unionid shells or to conspecifics, and zebra mussel biomass (per 1 cm^2 of unionid shell). *N* values indicate the numbers of analysed unionids. Only unionids having zebra mussels both directly on their shells and attached to conspecifics have been included

individuals moved and attached to the glass walls of the tanks. When unionids could burrow into the sediments, zebra mussels attached less often than expected on the basis of a theoretical random distribution to *S. woodiana* and *U. pictorum* (Figure 10a). The percentage of individuals attached to *A. anatina* tended to be higher than expected, although this effect was non-significant after applying the Bonferroni correction (Figure 10a). When unionids were forced to stay on the sediment surface, zebra mussels attached less often only to *U. pictorum* (Figure 10b). We found no significant positive selection for any substratum type in this experiment.

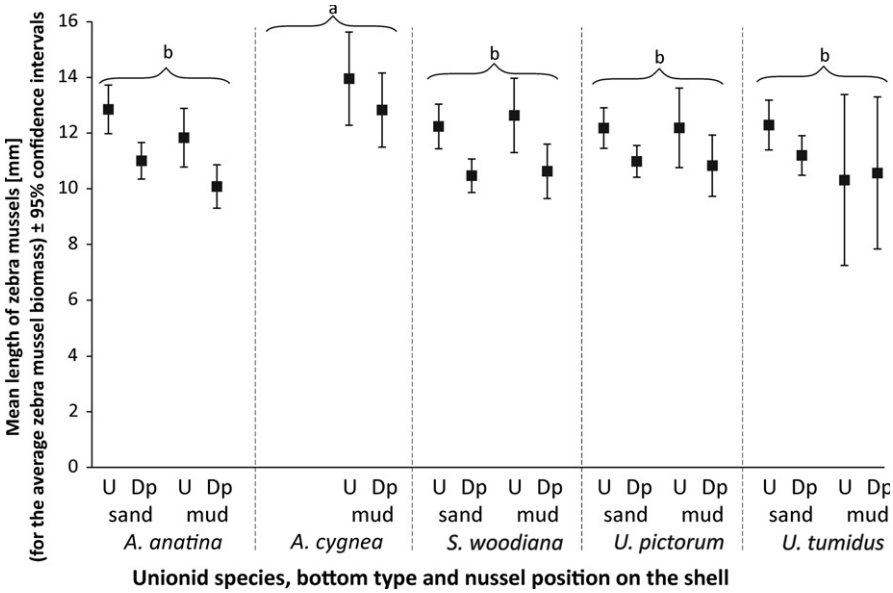

FIGURE 7 Mean (±95% confidence intervals) lengths of zebra mussels attached directly to unionid shells (U) or to conspecifics fouling various unionid species (Dp) on different substrata. The values are predicted by the GLM for the mean biomass of zebra mussels (3.1 g per 10 cm^2 of unionid shell) and back-transformed from the log transformation used for the analysis. The bars labelled with the same letters indicate the lack of significant differences between particular means. See Figure 6 for the numbers of analysed unionids

4 | DISCUSSION

4.1 | Dreissenid fouling on different unionid species

In our field survey, *A. anatina* and *S. woodiana* were more fouled than *Unio* spp. and *A. cygnea*. This difference could result from: (1) dreissenid selectivity, (2) variable dreissenid survival, (3) variable burrowing depth of unionids, exposing different shell areas to fouling. Higher fouling of *Anodonta* sp. compared to *Unio* sp. was also observed by Sousa et al. (2011) and Lewandowski (1976), who attributed this difference to variable burrowing depth (Sousa et al., 2011) or the occurrence of *Unio* sp. in shallower areas, less susceptible to zebra mussel settlement (Lewandowski, 1976). The effect of burrowing depth has been supported by Bódis et al. (2014), who found no differences in fouling intensity among unionid species after controlling for shell area. However, the better settlement of dreissenids on *A. anatina* and *S. woodiana* compared to *Unio* spp. in our field experiment (in the absence of sediments) confirmed the importance of mechanisms (1) and/or (2). As the experiment was relatively short-timed, its results were mainly based on events taking part during and immediately after the settlement, which suggests the stronger effect of site selection than survival. Moreover, zebra mussels attached to *A. cygnea* and *U. pictorum* (relatively poorly fouled in our study) were found to be in a good physiological condition by Hörmann and Maier (2006) and Pilotto et al. (2016), supporting hypothesis (1) rather than (2). The effect of mussel selectivity was also confirmed by our laboratory treatment with no possibility of unionid burrowing, where post-attachment events were completely excluded. On the other hand, some differences among species, such as the lower dreissenid fouling on *U. tumidus* than on *U. pictorum*, were observed in the survey only, suggesting that the effect of burrowing in the sediments was also important.

In our laboratory experiment, *U. pictorum* was the least selected species, irrespective of burrowing. This suggests the negative effect of its shell surface quality on dreissenids. Antifouling properties of bivalve shells, associated with their microtopography and chemical composition, were detected in blue mussels *Mytilus* sp. (Mytilidae), protecting itself against marine biofouling (Bers et al., 2006, 2010). Nevertheless, it should be noted that in our field survey *U. tumidus* was less fouled than *U. pictorum*, whereas dreissenids recruiting onto unionids in our field experiment did not differentiate between these species. Perhaps, crawling mussels studied in the laboratory had better capabilities of active substratum selection compared to planktonic post-veligers, being the main source of fouling mussels in the field. It should also be noted that the difference between both *Unio* species observed in our field survey (Figure 3), occurred mainly on the muddy substratum (with a marginally non-significant substratum × species interaction, Supporting information Table S1). This substratum was not tested in the experimental part of our study, which could also cause the discrepancy between the results.

Another mechanism of variable dreissenid fouling on different unionid species can be differences in unionid filtration rates (Kryger & Riisgård, 1988). Zebra mussels can benefit from unionid feeding currents (as suggested by Pilotto et al., 2016), but, on the other hand, planktonic mussel larvae can be ingested by large bivalves (Welker & Walz, 1998). However, Kryger and Riisgård (1988) showed that the most fouled species in our study (*A. anatina*) had an intermediate filtration rate, positioned between the two least fouled *Unio* species. This suggests that unionid filtration did not substantially affect zebra mussel fouling in our study.

Sinanodonta woodiana seemed to be partly protected against mussel fouling due to its burrowing into the substratum, as shown by our laboratory study. However, our longer time field studies showed this was inefficient over longer periods, making individuals of this species one of the most fouled substrata. This confirms the ability of zebra mussels to attach to a wide range of hard substrata (Garton, McMahon, & Stoeckmann, 2013), including novel materials in their environment. Thus, the recent spread of *S. woodiana* (Cichy, Urbańska, Marszewska, Andrzejewski, & Żbikowska, 2016; Douda,

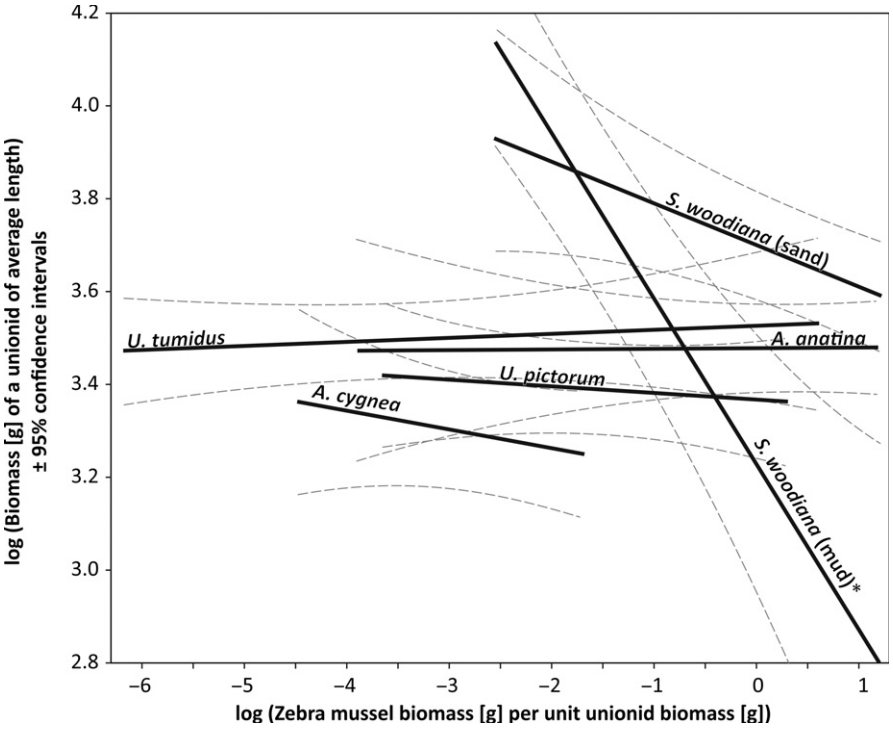

FIGURE 8 Relationship between the unionid biomass and the biomass of fouling zebra mussels (per 1 g of unionids). The values are predicted by the GLM for the mean unionid length (74 mm) and the actual range of zebra mussel biomasses found on each unionid species and shown with 95% confidence intervals. The values for *S. woodiana* are shown separately for each substratum, whereas the common lines are shown for the other species as they did not exhibit any responses to the presence of zebra mussels irrespective of the substratum type. Significant relationships between unionid and zebra mussel biomass are indicated with asterisks. Full regression equations are given in Table S5. See Figure 3 for the numbers of analysed unionids

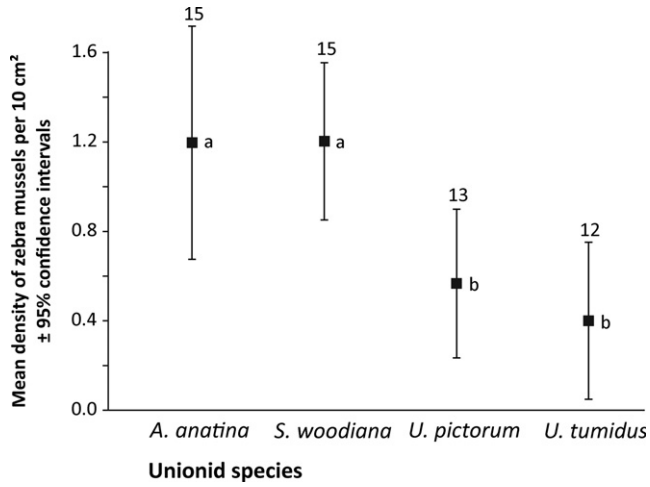

FIGURE 9 Mean (±95% confidence intervals) density of zebra mussels settling on the shells of various unionid species in the field experiment. The bars labelled with the same letters indicate the lack of significant differences between particular means. The numbers above the bars indicate the numbers of analysed unionids

Vrtílek, Slavík, & Reichard, 2012) is likely to provide zebra mussels with additional substratum resources.

Dreissenid size in our field survey did not correspond to their substratum selectivity. Only individuals from *A. cygnea* were significantly larger than those collected from the other unionids. This could result from the lack of settlement in the recent period (zebra mussels from *A. cygnea* being older) or better growth of mussels at lower density. However, the general positive relationship between the dreissenid size and density observed in our survey points against the latter hypothesis, as dreissenid size was not limited by their density.

To summarise, *U. pictorum* seemed to be the species selected by dreissenids less often than other unionids. After a longer exposure, *U. tumidus* also became less fouled than other species. *Anodonta anatina* was the most fouled species, regardless of its burrowing, whereas *S. woodiana* exhibited some burrowing protection, though inefficient over a longer period. *Anodonta cygnea*, in contrast to its congener, appeared to be less prone to dreissenid fouling.

4.2 | Dreissenid fouling on unionid, conspecific and stone substrata

In laboratory, no unionid species were positively selected over the neutral stone substratum. In contrast, Toczylowsky and Hunter (1997) and Lewandowski (1976) observed higher zebra mussel fouling on unionids than on stones. Moreover, dreissenids were found to have a better physical condition (Pilotto et al., 2016) and faster growth (Hörmann & Maier, 2006) on unionids than on rocks. These findings suggest a positive effect of the living bivalve substratum on fouling mussels, for example through filtration facilitated by unionid siphonal currents (Pilotto et al., 2016). Dreissenids can also benefit from the ability of unionids to stay at the sediment surface and thus avoid siltation. Perhaps, this positive effect of unionids did not appear in our short time laboratory experiment. On the other hand, Jokela and Ricciardi (2008) have shown that dreissenids fouled unionids more often on fine-grain substrata, whereas the increase in substratum particle diameter was negatively correlated with the fouling of unionid shells. Thus, large non-living objects seem to be selected over living bivalve substrata. Nevertheless, the importance of unionids for zebra mussel fouling as the hard substratum source in soft bottom habitats, common in lowland lakes and reservoirs, is still large.

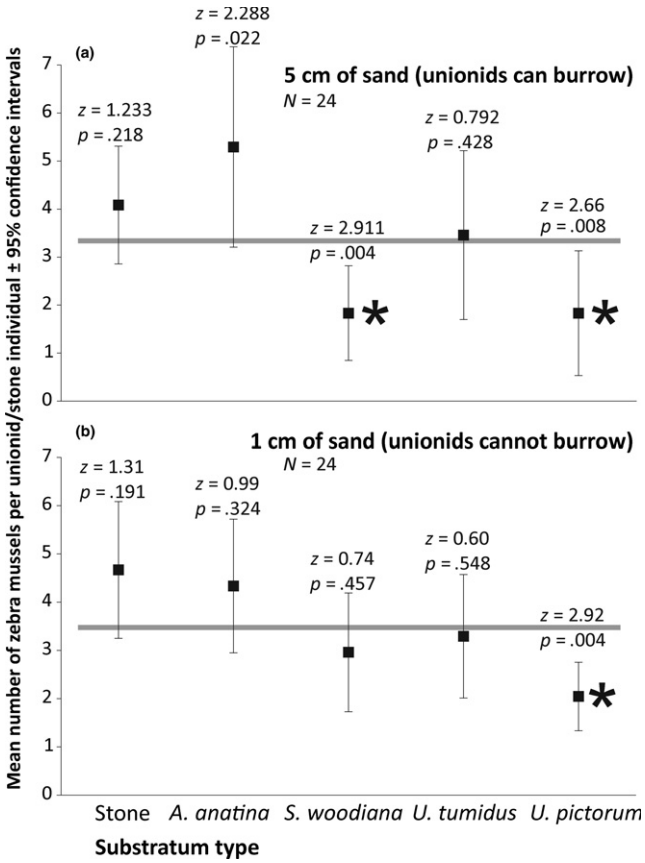

FIGURE 10 Mean (±95% confidence intervals) numbers of zebra mussels attached to the shells of various unionid species or stones in the laboratory experiment. Asterisks indicate significant departures (checked by one-sample Wilcoxon signed rank tests shown above the bars) from the random distribution with equal numbers of mussels on each substratum (shown by the horizontal line). *N* values indicate the numbers of replicates

The percentage of dreissenids attached directly to unionid shells in our field survey was lower on the less fouled species (*Unio* spp. on sand and *A. cygnea*). This might be associated with the smaller available surface exposed by deeply burrowing *Unio* spp. individuals, compared to *Anodonta* and *Sinanodonta* sp. (Bódis et al., 2014). Alternatively, particularly in the case of *A. cygnea*, this may also suggest the active avoidance by zebra mussels, which relatively more often attached to conspecifics. The negative relationship between dreissenid density and percentage of mussels attached directly to unionid shells indicated that mussels preferred unionid shell surface over conspecifics and fouled it as long as it was available, whereas conspecific shells were used only when the choice was difficult or impossible.

Avoidance of conspecifics by zebra mussels, in contrast to their commonly claimed preferences for own species (Wainman et al., 1996), was observed by Dzierżyńska-Białończyk et al. (2018). Together with our present observations, this suggests that druses of dreissenids attached to one another are formed due to the scarcity of alternative hard surfaces, rather than because of their real substratum preferences. This differs from many marine species, such as mytilids and oysters, preferentially recruiting on conspecifics (Commito et al., 2014; Tamburri et al., 1992). Perhaps, due to greater diversity of predators and stronger hydrodynamics, benefits of aggregated life are more important in the marine environment.

4.3 | Consequences of mussel fouling to unionids

In our study, *S. woodiana* sampled from the muddy substratum was negatively affected by fouling. This species is native to eastern Asia and has had no contact with zebra mussels until its arrival in the 1960s (Watters, 1997; Douda et al., 2012). In Poland, *S. woodiana* has been known since the 1980s only from artificially heated areas (Kraszewski, 2007) until recently, when it started to appear in natural waters (Cichy et al., 2016), including our study site. Similarly, American unionids, having no contact with dreissenids until the 1980s, seem to be more affected by biofouling (Ricciardi, 2003; Ricciardi et al., 1996; Strayer & Malcom, 2007) than European species (Burlakova et al., 2000; Lewandowski, 1976; Marescaux et al., 2016). Thus, that is why *S. woodiana* could be more sensitive to zebra mussel fouling. On the other hand, zebra mussels co-existed in Europe before the last glaciation, with the ancestors of native unionids (Stańczykowska, 1977). Furthermore, both taxa have co-occurred for the recent 200 years, which gave them a chance for co-evolution. Moreover, the muddy bottom is more demanding for the most of unionids due to their sinking in the watery substratum and irritating their gills by easily resuspending particles. Thus, the negative impact of fouling dreissenids could add to that of the less suitable substratum, resulting in the biomass decrease in *S. woodiana*.

Nevertheless, Sousa et al. (2011) and Bódis et al. (2014) observed a negative impact of zebra mussels on the condition of European unionids, measured as glycogen content. Very suitable food conditions in the highly eutrophic Włocławek Dam Reservoir and its fast water exchange (Poznańska et al., 2010) could have reduced the competition for food between fouling mussels and unionids in our study. This situation is different from that in Lake Balaton, studied by Bódis et al. (2014). This lake has a low chlorophyll content in the water column (Balogh, Vláčilová, G.-Tóth, & Serfőző, 2018), indicating poor food conditions for bivalve filter feeders.

It should be noted that wet biomass used in our analysis can also include water stored by mussels in their mantle cavities, potentially increasing the unexplained data variability and making detection of significant relationships more difficult. Thus, we cannot exclude that subtle effects of zebra mussels on other unionid species, detectable by more sensitive methods, such as glycogen measurements (Sousa et al., 2011), also existed in our study.

5 | CONCLUDING REMARKS

Our study has shown that *D. polymorpha* actively discriminates among various unionid species (fouling *A. anatina* and *S. woodiana* more often than *Unio* spp. and *A. cygnea*), regardless of their burrowing. Taxonomic identity of their unionid hosts can affect dreissenid

density, distribution on the shell and, to a lesser extent, size. Moreover, fouling by zebra mussels can negatively influence unionid biomass, particularly of an allopatric species (*S. woodiana*). Thus, the presence of unionid bivalves and taxonomic composition of their assemblages may shape living conditions for dreissenids on sandy and muddy bottoms. In particular, the likely future spread of *S. woodiana* can increase the substratum availability for dreissenids due to the large size of this unionid and its susceptibility to fouling.

Several crucial questions have arisen in the course of our study, including the nature of unionid traits shaping their susceptibility to dreissenid fouling (e.g. shell surface quality, filtration activity, potential antifouling defences) and factors potentially modifying interactions among the bivalves (e.g. trophic status, siltation, season). Given the widespread distribution and ecological and economic impacts of zebra mussels, as well as the threatened status and conservation importance of unionids, relationships between these taxa should be further tested using field and/or laboratory experiments.

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What scares a mussel? Changes in valve movement pattern as an immediate response of a byssate bivalve to biotic factors. *Hydrobiologia, 2019, 841: 65-77*

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PRIMARY RESEARCH PAPER

What scares a mussel? Changes in valve movement pattern as an immediate response of a byssate bivalve to biotic factors

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Abstract Responsiveness to biotic factors is crucial for the survival of sessile aquatic animals. They cannot escape from danger, but developed a number of defences against predation, usually delayed in time. We checked the initial defence of the freshwater byssate zebra mussel, *Dreissena polymorpha*, associated with valve gaping. We tested the effect of chemical signals: fish predator scent (the roach *Rutilus rutilus*), conspecific alarm cue and a mixture of both, as well as a mechanical stimulus: the presence of an amphipod (*Dikerogammarus villosus*) mechanically irritating mussels. The alarm cues and amphipod presence made mussels spend more time with closed/narrowly open valves, which can be related to decreasing detection probability by reduced infochemical excretion and/or protecting soft tissues in the presence of an imminent threat. In contrast, reactions to the predator scent alone were much weaker. Moreover, the fish scent mixed with alarm substance induced weaker responses than the alarm substance alone. Thus, the fish infochemical might mask the presence of the alarm cue components, potentially benefiting the predator. A variety of defences exhibited by mussels demonstrates the importance of the predation cue type (direct/indirect, chemical/mechanical, originating from conspecifics/predators/mixed) for the behaviour of sessile animals.

Keywords Valve gaping · *Dreissena polymorpha* · Predation cues · Activity · Induced defence

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Introduction

Aquatic sessile species are ecologically and economically important as ecosystem engineers, fouling community members and nuisance for submerged equipment (Khalaman, 2001; Sousa et al., 2009). Their responses to environmental factors differ from those exhibited by mobile animals. With no or limited relocation capabilities they can react by withdrawing into their shells or shelters and modifying their activity (Reimer & Tedengren, 1997; Kobak et al., 2010; Naddafi & Rudstam, 2013). On the other hand, prolonged activity is crucial for their survival as most of them are filter-feeders, relying on constant pumping of water into their filtering structures (Gosling, 2003).

One of the factors most affecting animal behaviour is the presence of predators (Lima & Dill, 1990;

Ferrari et al., 2009). Common defensive reactions of animals are active avoidance (Lima & Dill, 1990; Wisenden & Millard, 2001; Jermacz et al., 2015) and/or taking an appropriate shelter (Schmidt-Drewello et al., 2016). However, sessile organisms developed more sophisticated and less conspicuous responses. Depending on evolutionary constraints and predator hunting modes, they can more strongly adhere to the substratum (Shin et al., 2008; Cheung et al., 2009), reduce feeding rates (Naddafi et al., 2007), develop harder shells (Czarnołęski et al., 2006), produce toxins (Hill et al., 2005) and/or select sheltered sites during their mobile phase of life (Bryan et al., 1998; Dahms et al., 2004). These responses may vary depending on the quality of the predation cue, ranging from predator kairomones (depending on their diet and hunger level) to alarm substances produced by wounded conspecifics (indicating a more direct danger: a currently foraging predator) (Smee & Weissburg, 2006; Large et al., 2012; Jermacz et al., 2017). Furthermore, prey reactions vary with time, different measures being induced immediately after the detection of predation cues and during the chronic exposure to predator pressure (Reimer & Tedengren, 1997; Turner et al., 2006).

Moreover, sessile species are exposed to the impact of mobile non-predatory organisms occupying their colonies in great numbers (Botts et al., 1996). Being attached to the substratum, they have limited capability of avoiding scraping and tickling by specimens moving over their bodies, which can negatively affect their activity and condition, sometimes resembling the effect of predation cues (Kobak et al., 2012). Both predators and cohabiting organisms form a natural biotic environment of sessile organisms. In the field, various stimuli may create conflicting situations (when protection against one danger increases exposure to another) or act synergistically, optimizing responses to different risk sources (Wudkevich et al., 1997; Beggel et al., 2016). Therefore, the knowledge how they respond to these factors is crucial for understanding their functioning, impact on the environment and relationships with other species.

One of the most common freshwater sessile species is the zebra mussel, *Dreissena polymorpha* (Pallas, 1771). Although a hard shell defends it from most of its potential predators, it constitutes a considerable diet item for several fishes (Prejs et al., 1990; Naddafi & Rudstam, 2013). In the presence of predators, mussels increase their attachment (Kobak et al., 2010; Naddafi & Rudstam, 2013), aggregation (Naddafi & Rudstam, 2013) and shell strength (Czarnołęski et al., 2006; Naddafi & Rudstam, 2014). These changes increase predator handling costs (Prejs et al., 1990; Czarnołęski et al., 2006), but develop over a relatively long time (> 4 days) (Kobak & Kakareko, 2009; Kobak et al., 2012; Naddafi & Rudstam, 2013, 2014). Quite similar responses of zebra mussels (increase in attachment strength, reduction in upward movement) to the presence of gammarid amphipods occupying mussel colonies were also observed (Kobak et al., 2012). These animals are not considered a direct threat to mussels, but they may mechanically irritate mussel soft tissues and affect their responses to other environmental factors. The unresolved question is whether and how *D. polymorpha* reacts to these nuisance cues immediately after the signal reception. To answer this question, we checked mussel valve movements. They are associated with all life activities of mussels, such as nutrition, respiration, reproduction, excretion and signal reception (Gosling, 2003; García-March et al., 2008). Their feeding efficiency is directly related to the degree of valve gaping, regulating the pumping rate (Jørgensen et al., 1988; Newell et al., 1998; Riisgård et al., 2003; Saurel et al., 2007). In optimal environmental conditions, a mussel spends most of the time with open valves (Jørgensen et al., 1988; Kramer & Foekema, 2001). Thus, prolonged disturbance in valve opening may negatively affect mussel condition. In urgent stress situations, one of the fastest mussel reactions is valve closing (Borcherding, 2006), which reduces its contacts with the environment: both in terms of intaking potentially harmful substances (Rajagopal et al., 1997) as well as releasing chemical and mechanical cues that could be used by enemies to locate the sender (Antoł et al., 2018). Thus, reduction in valve gaping can be a potential defensive response regardless of the predator hunting mode.

In the present study, we checked short-time valve movement responses of *D. polymorpha* to predation cues and physical presence of non-predatory organisms occupying mussel beds. We tested mussel reactions to the kairomone of the roach *Rutilus rutilus* (Linnaeus, 1758). This fish is an efficient molluscivore (Prejs et al., 1990) and defensive responses of *D. polymorpha* to its kairomones have been found in previous studies (e.g. Naddafi et al., 2007; Kobak et al., 2010). The fish were fed with mussels or neutral

food (chironomid larvae) to check for the potential impact of dietary cues in predator exudates (Chivers et al., 1996; Wisenden, 2015). Predation is connected with alarm substances released by crushed prey; thus, we also used this stimulus in our study. We hypothesized that all these chemical stimuli would decrease time spent with widely open valves and frequency of valve movements, which would be beneficial in terms of "chemical hiding" from a predator (Antoł et al., 2018). We also intended to check whether a mixture of predation cues (conspecific alarm substance, predator dietary cues and/or kairomone) would increase synergistically the magnitude of mussel reactions. Additionally, we checked the effects of the presence of a Ponto-Caspian gammarid *Dikerogammarus villosus* (Sowinsky, 1894), occupying *D. polymorpha* colonies (Devin et al., 2003). We supposed that gammarids might irritate soft parts of siphons and mantle surface and finally induce valve closing similarly to the aforementioned predation cues. If so, we intended to compare the relative strength of responses to predation and gammarid cues.

Materials and methods

Animal collection and housing

All experiments were conducted under laboratory conditions, using *D. polymorpha*, roach *R. rutilus* and gammarids collected from the Włocławek Reservoir (a dam lake located on the lower Vistula River, Central Poland) from a depth of ca. 2 m (flow velocity < 10 cm/s, Gierszewski, 2006) in October 2015 (temperature 10 °C). *D. polymorpha* and *D. villosus* occupying mussel colonies were sampled from the reservoir bottom by a diver together with their substrata (unionid bivalves). *D. polymorpha* were removed from the unionids in situ and the later were immediately returned to the environment. Roach were captured by electrofishing, using a certified device IUP-12 (Radet, Poznań, Poland). Other fish were released after capture and recovered within a few minutes. Fish collection and use in the experiments were conducted under permit from the Local Ethic Committee (University of Science and Technology, Bydgoszcz, Poland) no. 21/2015. Animals were transported to the laboratory (2 h transport time) in 10-l aerated plastic containers. All animals were kept for 2 weeks in single-species stock aquaria (all mussels: 350 L, 3 fish per 200 L, all gammarids: 150 L) supplied with aquarium filters and aerators. The water in aquaria had a constant temperature of 18 °C (sustained by air-conditioning). Mussels were fed every second day with 2 g of dried *Chlorella sp*. per 1000 individuals (Kilgour & Baker, 1994). Fish were fed according to the experimental treatment (see below) and gammarids were fed every day with 4 g of frozen chironomid larvae. Ca. 30% of the water volume was changed on weekly basis and on the occasion of water collection for the purpose of the experiment (see below).

Experimental design

All tested mussels were collected from the top layer of druses formed on unionid shells to minimize the risk of their weak condition. Mussels were detached from their natural substrate by severing their byssal threads with a scalpel. 24 h before the experiments, mussels (length: 18–22 mm) were marked by a thin layer of a low-weight red plasticine (determined as the fastest and hence least invasive labelling method in preliminary trials), attached to the posterior part of one of the valves (Fig. 1). This mark allowed the behaviour analysis software to follow the video-recorded valve movements. Marked mussels were fastened to glass microscope slides with unreactive fast-binding methyl acrylic glue by the ventral surface of the non-marked

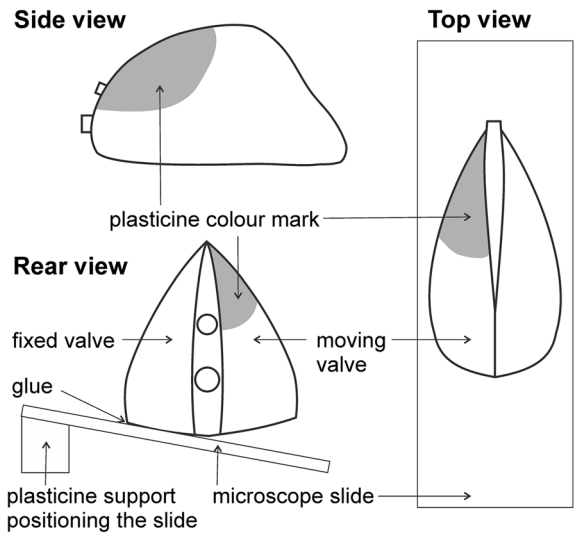

Fig. 1 Experimental setup

valve to prevent them from relocating during the experiment (Fig. 1). In the wild, mussels are often immobilized by overgrowing conspecifics and spend most of their life attached to the substratum; thus, this situation is not unusual for them. All processed individuals were left to recover in a 150-l stock tank for 24 h in the same conditions as those in experimental tanks. Only mussels which produced byssus threads after this time were used in the experiments.

The experiments were conducted in glass beakers (diameter: 140 mm, height: 72 mm) filled with 700 ml of water. A microscope slide with a single mussel was placed in the beaker and positioned so that the mussel was located in a horizontal plane. The experimental beakers were cleaned, water changed and new individuals used for each replicate. Experimental treatments were replicated 19–25 times. This variability depended on the availability of suitable mussels (byssally attached after marking and widely open at least once during the entire experimental period) and number of successful trials (without technical errors, such as accidental detachment of the plasticine marker during the trial). Individuals that did not extend their siphons were also removed from the analysis as it could result from their poor physical condition or injury during the pre-experimental handling. After the visual inspection of the recordings ca. 5% of all trials were rejected due to the aforementioned reasons including only few cases of individuals that did not open their valves.

Mussels were placed in the beakers filled with water containing a particular stimulus (see below) and exposed for the next 2 h 15 min. The first 15-min. period was considered as an acclimation time and excluded from the analysis. Disturbances resulting from handling during the stimulus application at the beginning of the experiment could interrupt mussel behaviour and in consequence responses to examined factors. During the next 2 h mussel valve movements were recorded from the top by video cameras (SNB-6004, Samsung, South Korea).

The experiment consisted of seven treatments: (1) the conspecific alarm substance, (2) the scent of roach (kairomone) fed with chironomid larvae, (3) the scent of roach fed with *D. polymorpha* (kairomone and conspecific dietary cues), (4) the alarm substance and scent of roach fed with chironomids, (5) the alarm substance and scent of roach fed with mussels, (6) the presence of *D. villosus* and (7) control treatment without any cues. The alarm substance was a colourless filtered mixture of water and manually crushed mussels in volumetric proportion of 4:7. One ml of the alarm substance was added to the experimental beaker filled with control water to obtain the active concentration according to Toomey et al. (2002). The scent of the roach was applied by adding 700 ml of water (i.e. the entire beaker volume) from a 200 L stock tank, in which three fish, 220 mm in length, had been incubated for 2 weeks, to the experimental beakers. The fish were fed once a day with 3 g of chironomid larvae or 3 *D. polymorpha* per individual. They were starved for 24 h before collecting water for the experiment to avoid the presence of signals from living mussels (not consumed yet) in the test. The control water was pre-conditioned for 2 weeks in the same tank as that used to incubate the fish, but devoid of mussels and predators. In the gammarid treatment, three *D. villosus* individuals (15–18 mm in length) were placed in the experimental arena and allowed to move freely among the mussels.

Water temperature (sustained by air-conditioning) during the experiments was 18 °C. Illumination (necessary due to video recording) provided by incandescent light was 65 lx. Oxygen concentrations (measured with a multiparameter benchtop meter, inoLab Multi 9620 IDS, WTW, Poland connected with an optical sensor FDO 925, WTW, Poland) before and after the trials were 8.95 ± 0.04 and 8.85 ± 0.08 mg/l (mean ± SD), respectively, and did not differ among the experimental treatments.

Data analysis

We used Noldus Ethovision XT 10.1 software to quantify mussel behaviour on the basis of the recorded videos. Every second, we determined the position of the colour mark located on the movable mussel valve, relative to a constant point fixed on the other valve. Then, each valve position was expressed as the percentage of the maximum valve gaping observed for a given individual. We found no significant differences among the treatments in the maximum valve gaping (in mm) observed for each individual, showing that they all exhibited their maximum gaping at some time during the exposure. Moreover, we found no correlation between maximum valve gaping and mussel size (within the range used in the experiments). This justifies the use of the percentage gaping as the

optimum response variable. The obtained dataset was further processed with a custom-made application written in Visual Basic for Microsoft Excel to detect gradual changes in valve position (opening and closing movements) and stable periods spent at particular valve gaping ranges, as well as their parameters (rate, amplitude, duration) during the experiment. We considered two valve movements as separate when they were separated by a stable period lasting for at least 5 s. We examined the following response variables: (1) Average valve gaping during the experiment; (2) Percentage of time spent by mussels with: (i) widely open valves (> 80% of the maximum valve gaping) (ii) narrowly open valves (< 20%) and (iii) totally closed valves (for better understanding of the average valve gaping value); (3) Number of valve opening movements. Visual inspection of the recordings indicated that mussels did not extend their siphons at valve gaping below 20%. Such a detailed approach can potentially lead to detection of subtle mussel reactions to environmental factors, undetectable with methods using only binary valve states (closed/open shell). All these parameters were calculated separately for the first and second hour of the experiment in order to reflect the potential impact of time on mussel responses to experimental factors.

We applied a General Linear Model analysis to test the effect of experimental treatments on the average valve gaping and percentages of time spent by mussels in particular gaping ranges. The models included two fixed factors: treatment (7 levels: various predation or gammarid cues and control) and experiment duration (repeated measures factor: hour 1 and 2) as well as their interaction. Normality and homoscedasticity were checked with Shapiro–Wilk and Levene tests, respectively, and percentage time data were log-transformed to meet these assumptions. A Tukey HSD test was used as a post hoc procedure for significant main effects of treatment and a sequential Bonferroni-corrected Fisher LSD test was applied for pairwise comparisons when an interaction was significant.

Numbers of valve opening movements were compared using a Generalized Linear Model (Poisson distribution, log-link function) including treatment, time (repeated measures) and their interaction. Sequential Bonferroni-corrected pairwise contrasts were used as a post hoc procedure.

The analyses were carried out using SPSS 25.0 statistical package (IBM Inc.).

Results

Average valve gaping during the experiment varied from 2 to 72% (Fig. 2) and significantly depended on experiment duration (valve gaping in the second hour of the experiment was by 10% higher than in the first hour) and on the cues (General Linear Model: Table 1A). Significantly lower valve gaping was observed in the presence of gammarids (2–3%) and the alarm substance alone (39–43%) relative to the control treatment (53–72%).

The presence of gammarids, fish scents and/or alarm signals significantly affected the time spent by mussels with totally closed, narrowly open (0–20%) or widely open (> 80%) valves (General Linear Model: Table 1B–D). Time spent by mussels with totally closed valves varied depending on experiment duration (a significant treatment x time interaction). The highest total closure times were observed in the presence of gammarids (34–48% of the test duration), as well as, to a lesser extent and only during the first hour of the test, in the presence of the alarm substance alone (2.5%) and in the control treatment (1.8%) (Fig. 3A). The presence of fish scents (both diets) together with the alarm substance reduced the time spent with totally closed valves (0.1–0.2%) compared to the treatments with both these stimuli applied separately (0.5–2.5%).

Time spent by mussels with narrowly open valves (< 20%) depended on treatment only (General Linear Model: Table 1C). Mussels were narrowly open for a longer time in the presence of gammarids (95% of test duration) and the alarm substance alone (12%) (Fig. 3B). The shortest times spent with narrowly open valves were exhibited by mussels exposed to the fish scents (both diets) mixed with the alarm substance (1.7–3.1%). Values observed in these treatments differed significantly from the corresponding treatments with the alarm substance absent (4.8–6.0%).

Time spent with widely open valves (> 80%) depended on treatment only (General Linear Model: Table 1D) and was markedly lower in the presence of gammarids (0.1%) and the alarm substance alone (12%) (Fig. 3C). Moreover, this parameter was significantly lower in the presence of the alarm substance and the scent of fish fed with mussels (21%) than in the control treatment (38%).

The number of valve opening movements varied strongly among individuals, ranging from 1 to 816 per

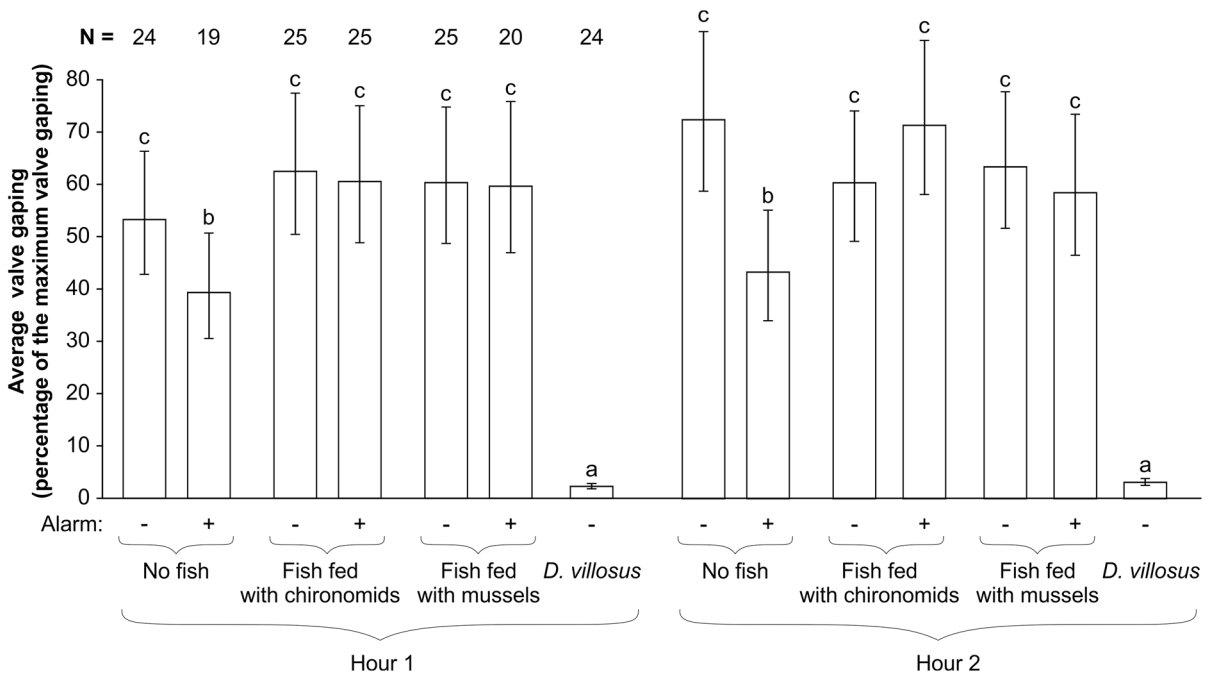

Fig. 2 Average valve gaping of zebra mussels responding to the presence and diet of predators (fish), conspecific alarm substance (present: +, absent: −) and gammarid (*D. villosus*) cues. Presented values are means predicted for significant effects by the General Linear Model (± 95% confidence intervals). Treatments labelled with the same letter above the bar did not differ from one another with regard to the average valve gaping (within each experimental hour) (see Supplementary Table S1 for the full results of the post hoc analysis). N indicates the number of replicates

Table 1 Analyses of the zebra mussel valve movement parameters in response to the presence and diet of fish, conspecific alarm substance and gammarid (*D. villosus*) cues

Response variable	Effect	*df*	MS	*F*	*P*
A. Average valve gaping (% of the maximum valve gaping) (GLM)	Treatment	6, 154	66.2	175.50	< 0.001
	Experiment duration	1, 154	1.2	6.69	0.011
	Interaction	6, 154	0.2	1.30	0.258
B. Time spent with totally closed valves (GLM)	Treatment	6, 154	164.0	68.79	< 0.001
	Experiment duration	1, 154	11.0	4.35	0.039
	Interaction	6, 154	7.8	3.10	0.007
C. Time spent with narrowly open valves (< 20% of the maximum gaping) (GLM)	Treatment	6, 154	79.2	31.14	< 0.001
	Experiment duration	1, 154	6.8	3.44	0.066
	Interaction	6, 154	3.9	1.96	0.075
D. Time spent with widely open valves (> 80% of the maximum gaping) (GLM)	Treatment	6, 154	203.1	132.69	< 0.001
	Experiment duration	1, 154	< 0.1	0.02	0.895
	Interaction	6, 154	1.7	1.36	0.234
E. Number of valve opening movements (GLZ)	Treatment	6, 302		15.31	< 0.001
	Experiment duration	1, 302		1.19	0.277
	Interaction	6, 302		0.57	0.751

Experiment duration (the first or second hour of the trial) is included in the models as a repeated measures factor

GLM general linear model, *GLZ* generalized linear model

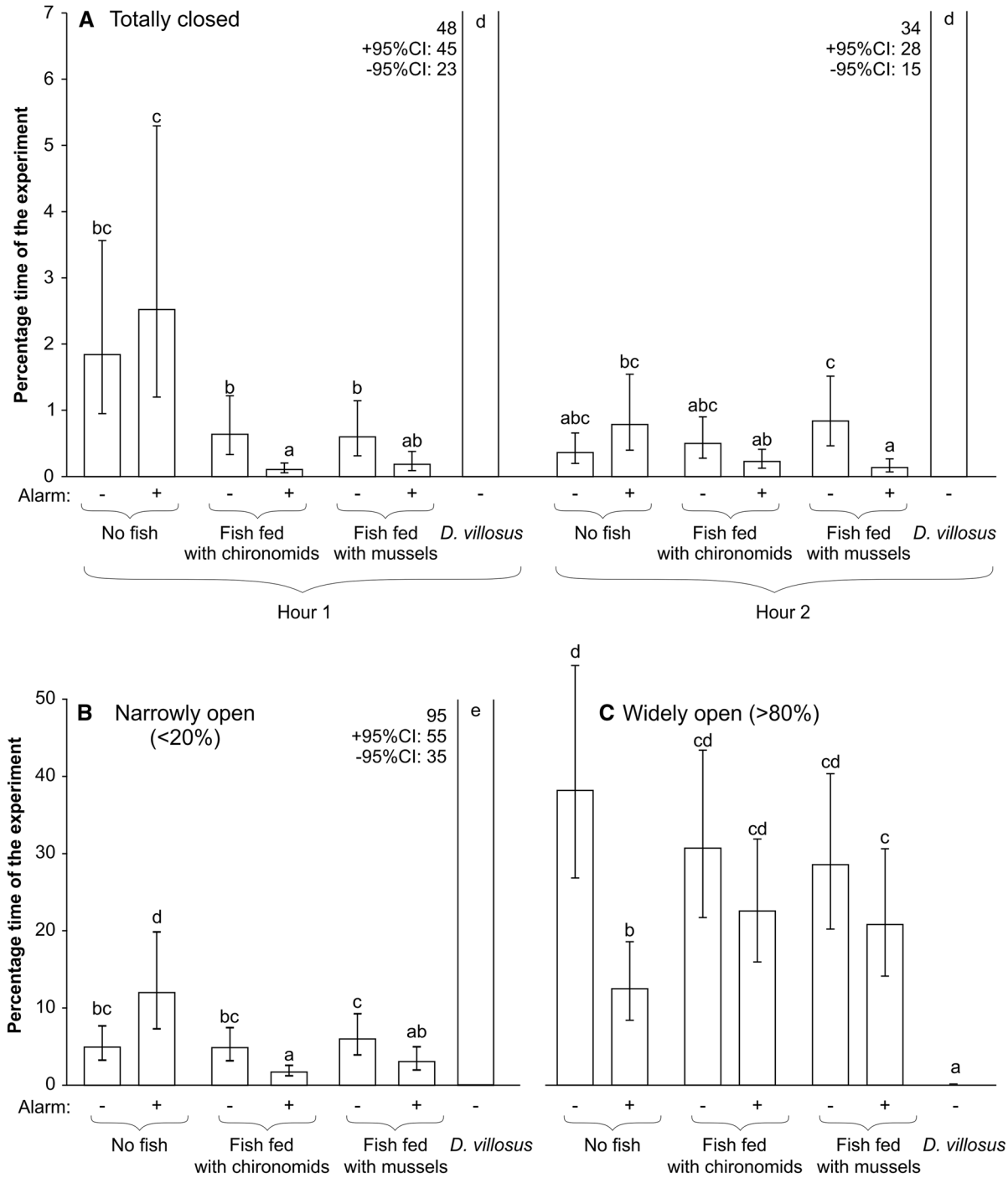

Fig. 3 Percentage time spent by zebra mussels at particular gaping ranges during exposure to the presence and diet of predators (fish), conspecific alarm substance (present: +, absent: −) and gammarid (*D. villosus*) cues. Presented values are means predicted for significant effects by the General Linear Model (± 95% confidence intervals). Treatments labelled with the same letter above the bar did not differ from one another with regard to the time spent at the particular valve gaping range (within each experimental hour, if separated) (see Supplementary Tables S2–S4 for the full results of the post hoc analyses)

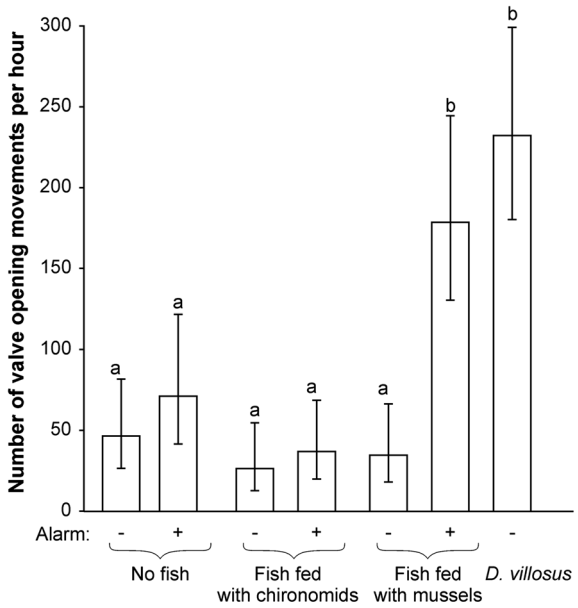

Fig. 4 Numbers of valve opening movements exhibited by zebra mussels responding to the presence and diet of predators (fish), conspecific alarm substance (present: +, absent: −) and gammarid (*D. villosus*) cues. Presented values are means predicted for significant effects by the Generalized Linear Model (± 95% confidence intervals). Treatments labelled with the same letter above the bar did not differ from one another (see Supplementary Table S5 for the full results of the post hoc analysis)

h. This parameter significantly depended on experimental treatment, but not on duration of the test (Generalized Linear Model: Table 1E). The mussels tested in the presence of gammarids (232 movements/h), as well as those exposed to the mixture of the scent of fish fed with mussels and the alarm substance (179 movements/h) were generally more active than in the other treatments (35–71 movements/h) (Fig. 4).

Discussion

The most influential factors in our study turned out to be the physical presence of *D. villosus* [which naturally occurs in *D. polymorpha* colonies (Devin et al., 2003)] and the alarm substance excreted by injured conspecifics. Mussel responses to these factors were of similar character; however, the effect of the mechanical stimulus was much greater than that of the chemical predation cue. *D. polymorpha* exposed to gammarids spent most of the time with totally closed or narrowly open valves (< 20%), which resulted in the very narrow average valve gaping during the entire experiment. Visual inspection of the video recordings revealed that these strong behavioural reactions were likely connected with the protection of siphons and other exposed soft tissues against mechanical irritation by *D. villosus* appendages. The longer-term impact of *D. villosus* on *D. polymorpha* was reported by Kobak et al. (2012), who observed that mussels in the presence of amphipods increased their attachment strength and reduced upward movement. Moreover, *D. villosus* is considered to be a voracious and aggressive carnivore (Kley & Maier, 2005; Bacela-Spychalska et al., 2013). These features are associated with its direct and indirect negative impact on other amphipods (Bij de Vaate et al., 2002; Devin et al., 2003; MacNeil et al., 2013; Jermacz et al., 2015) and even fish fry (Platvoet et al., 2009). However, it has not been demonstrated to attack *D. polymorpha* directly, except one observation of gammarids feeding on mussel byssal threads (Platvoet et al., 2009). Thus, it is unlikely that *D. villosus* could pose a direct predatory threat to mussels. Nevertheless, taking into account the results of the present study, we assume that a prolonged exposure to its high density may negatively affect *D. polymorpha* condition.

In contrast to *D. villosus*, the presence of the alarm substance in the environment definitely indicates a direct and immediate danger, i.e. the proximity of a foraging predator (Wisenden, 2015). In our study, mussels exposed to this cue significantly reduced their average valve gaping and time spent with widely open valves (> 80%). This behaviour does not defend them during a direct attack of a shell-crushing predator, but can prevent detection by limiting the amount of infochemicals released from the exposed mantle surface and siphonal currents generated during filtration (Weissburg et al., 2002; Smee & Weissburg, 2006; Antoł et al., 2018). Similarly to our results, Robson et al. (2007, 2010) revealed that another bivalve species, *Mytilus edulis* Linnaeus, 1758 in the presence of predation risk (alarm cue) demonstrated smaller gape angles. Moreover, in the presence of the alarm cue, mytilids closed their valves more rapidly and became less responsive to food. Strong behavioural responses to alarm cues have also been described for many freshwater animals (Wisenden & Millard, 2001; Wisenden et al., 2001; Dalesman et al., 2006; Smee & Weissburg, 2006), indicating the

importance of this factor in shaping animal behaviour. Predator foraging activity can be triggered by chemical signals from metabolically active mussels (Czarnołęski et al., 2011), which further supports the protective value of valve gaping reduction.

The reduced valve gaping of *D. polymorpha* exposed to the alarm cues, found in our study, corroborates previous observations, revealing that byssate mussels during the first few hours of exposure to predation cues reduce their attachment (Reimer & Tedengren, 1997) travelled distances (Toomey et al., 2002; Czarnołęski et al., 2010) and respiration rates (Antoł et al., 2018). All these responses lead to decreased probability of detection of mussels by predators (Antoł et al., 2018). However, after the initial activity reduction, mussels exposed to predation cues were found to relocate more than in the control treatment (Kobak & Ryńska, 2014) and attach more strongly to substratum (Reimer & Tedengren, 1997; Kobak et al., 2010), indicating the importance of time factor for shaping animal responses to danger cues.

For most of the analysed valve movement parameters we did not reveal any specific effects of the predator (the roach *R. rutilus*) scent itself, separated from conspecific alarm cues, regardless of the differences in the roach diet. In a previous experimental study, Kobak et al. (2010) demonstrated that mussels were capable of recognizing the scent of roach (fed with neutral chironomid diet) and in response to this stimulus attached more strongly to the substratum, though the effect appeared after 6 days of exposure. These findings show that mussels can adjust their antipredator strategy depending on the exposure time and proximity of the danger. Their immediate responses to direct dangers (currently hunting predators indicated by the presence of the conspecific alarm cue) involve activity decrease aimed at hiding the prey presence from the predator (Reimer & Tedengren, 1997; Czarnołęski et al., 2010; Antoł et al., 2018). When a danger is indirect (a non-foraging predator releasing only kairomones) and/or exposure lasts for a longer time, different mechanisms become involved, increasing the mussel security through stronger attachment (Reimer & Tedengren, 1997), aggregation (Kobak et al., 2010) and/or development of thicker shells (Czarnołęski et al., 2006; Naddafi & Rudstam, 2014). Changes in valve movements seem to be the first behavioural reaction exhibited immediately after detecting predatory cues, but only in the case of a direct threat (as the alarm substance in our experiments). The alarm cue in the direct vicinity may be treated as a sign of an immediate danger by mussels, with no time to employ more sophisticated and time-consuming defence mechanisms. On the other hand, valve gaping reduction in response to indirect and/or long-term predation danger would be too costly, limiting respiration and feeding (Jørgensen et al., 1988), whereas the presence of some numbers of predators in the environment is a natural situation in the wild.

In our study, mussels exposed to the alarm cues spent more time with totally closed or only narrowly open valves (< 20%) which was probably insufficient for effective filtration (Jørgensen et al., 1988; Newell et al., 1998; Robson et al., 2010), as at this degree of valve opening they were unable to extend their siphons (personal observation). This narrow valve opening could be related to a compromise between hiding and sustaining respiration (impossible during the complete closure) or to periodic "testing the external environment" in a stress situation. The latter hypothesis is also supported by another reaction of mussels exposed to alarm cues in our study: the increased number of valve opening movements. This seems useful, as completely closed mussels have impaired sensory contact with the external environment and, being unable to sustain filter-feeding and aerobic respiration, bear severe costs of non-consumptive predation effects. Thus, although *D. polymorpha* can stay closed even for several days protecting themselves against unfavourable environmental conditions (Rajagopal et al., 2010), it is beneficial if they can quickly detect a positive change in their environment and return to their normal activity as soon as possible.

Another interesting phenomenon observed in our study concerns the reactions of mussels to the scent of fish mixed with the alarm substance and/or dietary alarm cue. We supposed these separate predation cues to act synergistically and elicit stronger mussel responses than each of them applied separately (Dalesman et al., 2006). In contrast to this expectation, the compound stimulus (i.e. the conspecific alarm substance combined with predator scent) made mussels spend less time with the totally closed or narrowly open valves compared to the treatments with separate alarm cue and fish scents. On the other hand, mussels exposed to the mixture of the alarm cue and the scent of a predator fed with conspecifics still showed the

increased number of valve movements, similar to that exhibited by the individuals exposed to the alarm cue alone. Nevertheless, the response of mussels to the mixed predation cue was clearly weaker than that observed in the presence of the alarm substance alone and actually more similar to the results of the control treatment. Taking into account the fact that we always used the same amount of the alarm cue, the change in its effect on mussel behaviour must have been caused by the presence of fish infochemicals. Perhaps, the predator might be able to mask the components of alarm cues (including those originating from its faeces containing the remnants of digested mussels). It should be noted that, in the first hour of experiment, the control mussels tended to be totally closed for a longer time than those exposed to the fish scents. This might point to the fact that they were still adapting to the experimental conditions, whereas this effect did not occur in the presence of fish scents, potentially supporting our hypothesis on their masking effect on stressors influencing mussel behaviour. Anyway, further experimental evidence is needed to disentangle this observation. A similar phenomenon was suggested by Wisenden (2015) but only for dietary alarm cues (postingestion cues released by the predator, the northern pike *Esox lucius*) of the ruffe (*Gymnocephalus cernuus*) (Maniak et al., 2000). Similarly, Feminella & Hawkins (1994) observed that tadpoles of the tailed frog (*Ascaphus truei*) were unable to detect the presence of their predator, the shorthead sculpin (*Cottus confusus*) fed with conspecific tadpoles, whereas other predator species were recognized. It is not known, however, whether the tadpoles can respond to the scent of the conspecific alarm cues alone. Thus, to our knowledge, our work is the first to suggest an interaction between predator infochemicals and alarm cues released by wounded prey resulting in the weakening of the prey response. Definitely, this phenomenon deserves further research to explain its mechanisms.

Conclusion

We have shown that biotic environmental factors, such as predation alarm cues and mechanical irritation by mobile dwellers of a mussel bed, may significantly affect *D. polymorpha* valve movements. This points to potential negative non-consumptive effects on mussels, disrupting their normal filtration and respiration activity. Due to the common occurrence of both stimuli in the wild, we can expect that they may have a real influence on the functioning of mussel colonies, including their filtration (which varies with the degree of valve opening, Jørgensen et al., 1988; Newell et al., 1998; Saurel et al., 2007), being one of the most important aspects of their environmental impact. The decrease in filtration efficiency in the presence of predators was demonstrated by Naddafi et al. (2007). Thus, colony filtration rates may be overestimated if these factors are not taken into account.

Our study together with the existing literature (Naddafi et al., 2007; Shin et al., 2008; Cheung et al., 2009; Czarnołęski et al., 2010; Kobak et al., 2010; Naddafi & Rudstam, 2013; Antoł et al., 2018) shows that sessile organisms are capable of fine-tuning their responses to specific situations involving variable predation pressure, taking multiple factors, such as the vicinity and condition of a predator as well as duration of exposure into account. Although unable to escape from environmental dangers and having limited possibilities of selecting their location, they can use a variety of behavioural responses to avoid detection by predators and/or to make their handling more difficult.

Additionally, our findings show that in behavioural studies on mussel valve movements special attention should be paid to subtle, non-binary valve reactions, which can reveal responses invisible when only two valve states (open/closed) are considered. Thus, our study may also be important for the development of early warning systems detecting environmental pollution on the basis of mussel valve movement patterns (Kramer et al., 1989; Borcherding, 2006; Robson et al., 2007; Redmond et al., 2017). Such non-target factors as predation pressure should be taken into account when calibrating these systems to avoid false alarms.

Several questions raised by our present study still await answers to be given by future research, the most important being valve movement responses to the studied factors over a longer time scale (e.g. after several days of exposure) and the mechanism behind the observed reduction in the effect of the alarm cue in the presence of fish.

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