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Aggregation Pheromone for an Invasive Mussel Consists of a Precise Combination of Three Common Purines



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#### HIGHLIGHTS

*M. sallei* uses a blend of three common simple purines as an aggregation pheromone

The three purines synergistically induce *M. sallei* larvae to settle

Larvae are highly sensitive to the ratio of purines released by conspecific adults

Common metabolites in precise combinations can act as species-specific pheromones

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### Article

# Aggregation Pheromone for an Invasive Mussel Consists of a Precise Combination of Three Common Purines

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### SUMMARY

Most marine benthic invertebrates have a pelagic larval phase, after which they settle preferentially on or near conspecific adults, forming aggregations. Although settlement pheromones from conspecific adults have been implicated as critical drivers of aggregation for more than 30 years, surprisingly few have been unambiguously identified. Here we show that in the invasive dreissenid mussel *Mytilopsis sallei* (an ecological and economic pest), three common purines (adenosine, inosine, and hypoxanthine) released from adults in a synergistic and precise ratio (1:1.125:3.25) serve as an aggregation pheromone by inducing conspecific larval settlement and metamorphosis. Our results demonstrate that simple common metabolites can function as species-specific pheromones when present in precise combinations. This study provides important insights into our understanding of the ecology and communication processes of invasive organisms and indicates that the combination and ratio of purines might be critical for purine-based signaling systems that are fundamental and widespread in nature.

### INTRODUCTION

Animal aggregation is one of the most striking behaviors in biology that affects many spatial and temporal processes in ecological systems (Toonen and Pawlik, 1994; Parrish and Edelstein-Keshet, 1999). Despite the cost of increased intraspecific competition, for example, for space, food, and oxygen, aggregation has often been viewed as an evolutionarily advantageous state, in which individuals derive the benefits of protection and reproduction (Danchin and Wagner, 2000; Dzierżyńska-Białończyk et al., 2018). Gregarious settlement is a common phenomenon among marine benthic invertebrates, including mussels, barnacles, oysters, and polychaetes. Most benthic marine invertebrates have a pelagic larval phase, after which they settle preferentially on or near conspecific adults, forming aggregations (Toonen and Pawlik, 1994). The transition from a planktonic to a benthic mode of life is generally accepted as a critical point in their life cycle and is fundamental to understanding population and community dynamics (Shikuma et al., 2014). Although many studies have repeatedly implicated a critical role for pheromones from conspecific adults in the induction of larval settlement forming dense aggregates for more than 30 years, surprisingly few settlement pheromones have been isolated and structurally identified (Burke, 1986; Dreanno et al., 2006). The present understanding of aggregation mechanisms and the evolution of aggregation pheromones is limited.

Invasive dreissenid mussels commonly foul submerged structures with typical high-density aggregations and are well-known ecological and economic pests in aquatic ecosystems (Pimentel et al., 2005; Michalak, 2017). These include the zebra mussel *Dreissena polymorpha*, the quagga mussel *Dreissena rostriformis bugensis* in North America and Europe (Michalak, 2017; Stokstad, 2007), *Mytilopsis leucophaeata* in Europe (Kennedy, 2011), and *Mytilopsis trautwineana* in South America (Aldridge et al., 2008). Dreissenids are dioecious with gametes released directly into the water and fertilized externally (Ram et al., 1996). After a brief free-swimning veliger stage, the pediveliger larvae settle and metamorphose to benthic juveniles, which attach to most substrates with secreted byssal threads leading to fouling. The gregarious settlement of dreissenid mussels causes adverse impacts on aquatic systems and serious cost to industries. The introduction of dreissenid mussels into water pipelines in power plants and water treatment plants causes damage worth billions of dollars in the Great Lakes area (Aldridge et al., 2006). Much research has focused on antifouling compounds in preventing invertebrate settlement (Yebra et al., 2004; Almeida and Vasconcelos, 2015; Qian et al., 2015; Martins et al., 2018). For the control of dreissenid mussels, chlorine has been commonly used in pipelines, but there are environmental concerns about this approach (Meehan et al., 2014). Furthermore, at present no practical

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Figure 1. *M. sallei* Adults Release Chemical Cue(s) into Seawater to Induce Conspecific Larval Settlement and Metamorphosis

(A and B) Percentage settlement and metamorphosis of *M. sallei* larvae after 48-h exposure to conspecific adultconditioned seawater (A) and conspecific adult shell-conditioned seawater (B). ACS, adult-conditioned seawater; SCS, shell-conditioned seawater. The preparation of 2-, 4-, and 8-h ACS, and that of 2-, 4-, and 8-h SCS, is described in the Methods.

(C) Percentage settlement and metamorphosis of *M. sallei* larvae after 48-h exposure to three factions of mantle cavity fluid from conspecific adults. Preparation of the three fractions is described in the Methods. EtOAc-fr, the ethyl acetate fraction; butanol-fr, the n-butanol fraction; Aq-fr, the aqueous fraction; Control, filtered (0.22  $\mu$ m) seawater. Results are shown as mean  $\pm$  SD (n = 3). Asterisk denotes significant difference compared with the control (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, Dunnett's test).

technology is available for inhibiting the gregarious settlement and invasion of dreissenid mussels in open waters (Molloy et al., 2013). Research on aggregation mechanisms of dreissenid mussels may shed light on developing environment-friendly and effective methods for their control. Although the formation of aggregates by adults of dreissenid mussels and factors that affect this process have been previously studied (Dzierżyńska-Białończyk et al., 2018; Tošenovský and Kobak, 2016), there is still very little understanding of the biochemical mechanisms of gregarious settlement of dreissenid mussels.

The Caribbean false mussel *Mytilopsis sallei* (Recluz, 1849) (Figure S1), a close relative of *D polymorpha* and *D. rostriformis bugensis*, was introduced into the Pacific via the Panama Canal (Morton, 1981). This dreissenid mussel has wide environmental tolerance, grows rapidly, matures fast, and has a high fecundity, contributing to its success as an invasive species (Morton, 1981, 1989). *M. sallei* has been found in Australasia, East Asia, and India (Willan et al., 2000; Wong et al., 2011; Cai et al., 2014). Large aggregations of *M. sallei* cause serious fouling problems on monsoon drains, concrete walls, floating rafts, aquaculture facilities, and other submerged artificial structures. Here, we hypothesized that *M. sallei* adults could release aggregation pheromone to induce settlement of conspecific larvae. The aim of the present study was to investigate whether such a pheromone exists in *M. sallei* adults and to attempt to isolate such an aggregation pheromone by bioassay-guided fractionation to determine its chemical structure. This work may provide insights into our understanding of the population dynamics and ecology of invasive dreissenid mussels and allow the development of methods for their control.

### **RESULTS AND DISCUSSION**

### An Aggregation Pheromone Exists in M. sallei

To confirm the existence of the conspecific cue(s), we examined larval settlement and metamorphosis of *M. sallei* in response to conspecific adult-conditioned seawater (ACS). Larval settlement and metamorphosis

Bioassay	Cue	Response	df between Groups	df within Groups	F Value	p Value
Effect of conditioned seawater	ACS	Settlement	3	8	55.035	0.000
		Metamorphosis	3	8	22.839	0.000
	SCS	Settlement	3	8	1.724	0.239
		Metamorphosis	3	8	2.303	0.154
Effect of MCF	EtOAc fraction	Settlement	3	8	8.514	0.003
		Metamorphosis	3	8	0.385	0.814
	Butanol fraction	Settlement	3	8	3.047	0.070
		Metamorphosis	3	8	0.771	0.568
	Aqueous fraction	Settlement	3	8	8.500	0.003
		Metamorphosis	3	8	12.458	0.001
Effect of subfractions obtained during the bioassay-guided fractionation of MCF	F1	Settlement	4	10	8.859	0.003
		Metamorphosis	4	10	4.400	0.026
	F2	Settlement	4	10	6.100	0.009
		Metamorphosis	4	10	1.150	0.388
	F3	Settlement	4	10	7.597	0.004
		Metamorphosis	4	10	10.750	0.001
	F3-1	Settlement	4	10	0.849	0.526
		Metamorphosis	4	10	0.885	0.507
	F3-2	Settlement	4	10	0.827	0.537
		Metamorphosis	4	10	0.512	0.729
	F3-3	Settlement	4	10	2.226	0.139
		Metamorphosis	4	10	1.726	0.220
	F3-4	Settlement	4	10	7.264	0.005
		Metamorphosis	4	10	16.469	0.000
	F3-4-1	Settlement	4	10	0.394	0.809
		Metamorphosis	4	10	0.310	0.865
	F3-4-2	Settlement	4	10	13.676	0.000
		Metamorphosis	4	10	9.342	0.002
	F3-4-3	Settlement	4	10	18.015	0.000
		Metamorphosis	4	10	12.375	0.001
	F3-4-4	Settlement	4	10	3.438	0.052
		Metamorphosis	4	10	1.314	0.329
	F3-4-3-1	Settlement	4	10	15.886	0.000
		Metamorphosis	4	10	11.452	0.001
	F3-4-3-2	Settlement	4	10	32.947	0.000
		Metamorphosis	4	10	17.717	0.000
	F3-4-3-3	Settlement	4	10	5.857	0.011
		Metamorphosis	4	10	1.521	0.269

Table 1. ANOVA Results for the Effect of Chemical Cues on Larval Settlement and Metamorphosis of *M. sallei* 

(Continued on next page)

Bioassay	Cue	Response	df between Groups	df within Groups	F Value	p Value
Effect of purine compounds	Ado	Settlement	6	14	13.991	0.000
		Metamorphosis	6	14	16.123	0.000
	Ino	Settlement	6	14	26.119	0.000
		Metamorphosis	6	14	18.028	0.000
	Нур	Settlement	6	14	13.604	0.000
		Metamorphosis	6	14	18.028	0.000
	Ade	Settlement	6	14	1.127	0.396
		Metamorphosis	6	14	0.673	0.673
	Xan	Settlement	6	14	2.497	0.074
		Metamorphosis	6	14	0.220	0.964
Effect of dilution of ACS	Different dilutions of ACS	Settlement	4	10	10	0.000
		Metamorphosis	4	10	10	0.000
Synergistic effect of Ado, Ino, and Hyp	Mixture of Ado, Ino and Hyp, and individual components	Settlement	5	12	12	0.000
		Metamorphosis	5	12	12	0.000
Effects of different ratios of Ado, Ino, and Hyp	Different ratios of Ado, Ino and Hyp	Settlement	34	70	70	0.000
		Metamorphosis	34	70	70	0.000

#### Table 1. Continued

ACS, adult-conditioned seawater; SCS, shell-conditioned seawater; MCF, mantle cavity fluid; Ado, adenosine; Ino, inosine; Hyp, hypoxanthine; Ade, adenine; Xan, xanthine; EtOAc, ethyl acetate; df, degree of freedom.

were both significantly induced by ACS (Figure 1A, Table 1). The three treatments using ACS prepared by placing *M. sallei* adults in seawater for 2, 4, and 8 h, respectively, all showed inducing activity. However, treatments using shell-conditioned seawater prepared by placing empty shells of *M. sallei* adults in seawater for 2, 4, and 8 h, respectively, had no significant impact on larval settlement and metamorphosis compared with the control (Figure 1B, Table 1). These findings demonstrated that *M. sallei* adults could release chemical cue(s) to induce conspecific larval settlement and metamorphosis, and that the cue(s) were not derived from the shell of the adult *M. sallei*. We hypothesized that the inducing cue(s) in ACS were derived from mantle cavity fluid (MCF), as MCF is released with exhalant current from adults (Zimmer and Butman, 2000). MCF was successively partitioned with ethyl acetate and n-butanol, and the resultant three fractions, including the residual aqueous fraction was active in inducing larval settlement and metamorphosis (Figure 1C, Table 1), further confirming the existence of waterborne inducing cues.

### M. sallei Uses Three Common Simple Purines as Aggregation Pheromone

The active aqueous fraction was then subjected to bioassay-guided fractionation by ultrafiltration and column chromatography, which gave three pure active compounds (1, 2, and 3) (Figure 2). They all significantly induced settlement and metamorphosis of *M. sallei* larvae (Table 1). Based on analysis of their electrospray ionization mass spectrometry and nuclear magnetic resonance spectral data (Supplemental Information), compounds 1, 2, and 3 were identified as the purines, hypoxanthine (Hyp), inosine (Ino), and adenosine (Ado), respectively (Chenon et al., 1975; Saladino et al., 2006; Abou-Hussein et al., 2007; Ghose, 2009). This study identifies purines as pheromones for larval settlement or metamorphosis of a marine invertebrate. To further confirm the activity of compounds for larval settlement of *M. sallei* under hydrodynamic conditions, as in the natural environment, we performed larval bioassays using Ado (as an example of inductive purines) in a racetrack flume with seawater at a flow rate of 10.8–16.2 L min<sup>-1</sup> (Figure 3A). More than twice the number of *M. sallei* larvae were found to settle on substrates, which slowly released





Results are shown as mean  $\pm$  SD (n = 3). Asterisk denotes significant difference compared with the control (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, Dunnett's test).



### Figure 3. Adenosine Induced Larval Settlement of *M. sallei* under Flow Condition

(A) Experimental apparatus used in this study, with seawater in the racetrack flume at a flow rate of 10.8–16.2 L min<sup>-1</sup>. (B–D) Number of settled larvae on the treated substrates that slowly released adenosine (Ado) and control substrates after 12 h (B), 24 h (C), and 48 h (D). Results are shown as mean  $\pm$  SD (n = 3). Asterisk denotes significant difference compared with the control (\*p < 0.05, Student's t test).

Ado compared with the controls (Figures 3B–3D), demonstrating the inducing activity of Ado for larval settlement under flow conditions.

Purines as signal molecules have been reported in fish and polychaetes. In fish, purines such as ATP, ADP, AMP, and Ado evoke attraction or feeding (appetitive) responses, whereas hypoxanthine 3-N-oxide can evoke avoidance or alarm (Wakisaka et al., 2017; Shamchuk et al., 2018). In polychaetes, the purine metabolites uric acid and Ino serve as the sperm- and egg-release pheromones, respectively, and were the first identified gamete-release pheromones in marine invertebrates (Zeeck et al., 1998a, 1998b). Here we demonstrate the use of purines for intraspecific communication in the phylum Mollusca. Purines are common metabolites in all organisms. Utilizing already existing purine metabolites as pheromones might be more energetically favorable than producing entirely new molecules. However, as suggested in a recent study (Shamchuk et al., 2018), the role of purines as chemical communication molecules in animals is substantially underestimated.

As Hyp, Ino, and Ado are purine metabolites, we wondered whether there were other active purine metabolites in *M. sallei* MCF. By using liquid chromatography-mass spectrometry, five purine metabolites, namely, Hyp, Ino, Ado, xanthine (Xan), and adenine (Ade), were detected in MCF of *M. sallei* (Figure 4A). However, unlike the significant inducing activity exhibited by Hyp, Ino, and Ado for *M. sallei* larval settlement and metamorphosis, Xan and Ade showed no significant effects (Figure 4B, Table 1). This finding further confirmed the efficiency of the bioassay-guided fractionation procedure for identification of active compounds used here.





(A) Detection by liquid chromatography-mass spectrometry of purines in the mantle cavity fluid (MCF) of adults. (B) Chemical structures of the purines detected in MCF, and percentages of settlement and metamorphosis of *M. sallei* larvae after 48-h exposure to each purine compound. Xan, xanthine; Hyp, hypoxanthine; Ino, inosine; Ado, adenosine; Ade, adenine. Results are shown as mean  $\pm$  SD (n = 3). Asterisk denotes significant difference compared with the control (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, Dunnett's test).

#### Pheromone Components Synergistically Induce Larval Settlement and Metamorphosis

To serve as effective pheromone signals in the natural environment, Hyp, Ino, and Ado should be released into seawater by *M. sallei* adults at effective concentrations. As ACS was active for conspecific larval settlement and metamorphosis, whether the three purines were released into ACS and responsible for its inducing activity was determined as follows. Figure 5A showed that when ACS was diluted with seawater, the inducing activity of ACS decreased with increasing dilution. A 5-fold dilution of ACS (5-d ACS) was the highest dilution to show inducing activity. Ado, Ino, and Hyp were found to be present in the 5-d ACS with concentrations of 8, 9, and 26 nM, respectively (Figures 5B and S2B), which were much lower than the lowest effective concentration of each compound when tested individually (0.5  $\mu$ M for Ado, 0.5  $\mu$ M for Ino, and 1.0  $\mu$ M for Hyp, Figure 4B), indicating that there might be a synergistic effect of these three purines on *M. sallei*. To determine whether there was a synergistic effect, the response of larval settlement and metamorphosis to a mixture of the three purines (8 nM for Ado, 9 nM for Ino, and 26 nM for Hyp) was investigated, and compared with the response to individual compounds under the same concentrations of each compound separately in the mixture. The 5-d ACS was used as positive control. Interestingly, the

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#### Figure 5. Pheromone Components Synergistically Induce Larval Settlement and Metamorphosis

(A) Percentage of larval settlement and metamorphosis in response to different dilutions of adult-conditioned seawater (ACS). Filtered seawater was used as control.

(B) Concentrations of adenosine (Ado), inosine (Ino), and hypoxanthine (Hyp) in the 5-fold dilution of ACS determined by high-performance liquid chromatography.

(C) Percentage of larval settlement and metamorphosis of *M. sallei* in response to 8 nM Ado, 9 nM Ino, 26 nM Hyp, and mixture of the three purines. Filtered seawater was used as a negative control. The 5-fold dilution of ACS (5-d ACS) was used as a positive control. Results are shown as mean  $\pm$  SD (n = 3). Asterisk denotes significant difference compared with the negative control (\*\*p < 0.01, \*\*\*p < 0.001, Dunnett's test).

blend of the purines produced a significant response, whereas each individual component showed no inducing activity under the concentration tested in this bioassay (Figure 5C, Table 1), which strongly suggests a synergistic effect of Ado, Ino, and Hyp in ACS. By comparing the effective concentration of the mixture and that of the individual components, the mixture was 38.5- to 62.5-fold more potent than the individual compounds. Furthermore, the synthetic mixture and the 5-d ACS were equally effective at inducing settlement and metamorphosis.

#### **Pheromone Components Function in a Precise Ratio**

As Ado, Ino, and Hyp are common metabolites found in aquatic environments (Cunliffe, 2015), we wondered how M. sallei larvae can distinguish conspecific adult-derived purine signals from background levels. The use of a blend of pheromone components that act synergistically has also been reported in insects (Meier et al., 2016), although there are few published examples for marine organisms (Li et al., 2018). Usually the ratio of the pheromone components is highly species specific in insects, allowing effective communication among related sympatric species (Symonds and Elgar, 2008). For example, two Helicoverpa (moth) species use the same sex pheromone components (Z)-11-hexadecenal and (Z)-9-hexadecenal in nearly reverse ratios, 100:2 is used by Helicoverpa armigera and 6:100 is used by Helicoverpa assulta, thus ensuring segregation in nature (Wang et al., 2005). To determine whether M. sallei larvae had specific preference for particular ratios of Ado, Ino, and Hyp, thirty-three treatments with different ratios but with the same total concentration of compounds (50 nM) were prepared using simplex lattice mixture design (Figure 6A) (50 nM was chosen because the sum of concentrations of Ado, Ino, and Hyp in the effective 5-d ACS was close to 50 nM). These mixtures were then tested for larval response to settlement and metamorphosis. Results showed that different ratios produced quite different larval responses (Figures 6B-6E, Table 1). Twenty-five mixtures had no significant effect on settlement and metamorphosis. Six treatments with ratios of Ado, Ino, and Hyp of 0:4:1, 1:3:1, 0:1:4, 5:1:19, 1:5:19, and 1:1:3, gave a significant inducing activity (Figures 6B and 6C). The highest rates of settlement and metamorphosis were observed with a ratio of 1:1:3, which, interestingly was similar to the ratio of 8:9:26 (1:1.125:3.25) for Ado, Ino, and Hyp in natural ACS (Figures 5B, 6D, and 6E). The finding that M. sallei larvae are highly sensitive only to a particular ratio of Ado, Ino, and Hyp suggested that M. sallei larvae may have evolved species-specific responses to this ratio.

We further measured the ratios of these three purines in the conditioned seawater of the mussel *Perna viridis* and the oyster *Crassostrea angulata* (Figures S2B and S2C), two molluscan species with ecological niches similar to *M. sallei*, and found that the ratio of Ado, Ino, and Hyp in ACS was 6:6:1 for *P. viridis* and 1:10:5 for *C. angulata*. The clear difference of these ratios and that of *M. sallei* (1:1.125:3.25) supports the hypothesis that *M. sallei* larvae recognize conspecific adult-derived pheromones from background levels of purines, by only responding to a particular ratio of pheromone components. The other noteworthy finding was that two ratios of Ado, Ino, and Hyp (20:4:1 and 20:1:4) significantly inhibited settlement of *M. sallei* (Figure 6B), suggesting that even under the same total concentration of compounds, the larval



### Figure 6. Specific Ratios of Adenosine, Inosine, and Hypoxanthine Induce Conspecific Larval Settlement and Metamorphosis

(A) Thirty three different ratios of Ado, Ino, and Hyp were designed based on simplex lattice mixture design. The test levels used for each purine compound were designed as 0, 1/25, 1/5, and 1. Fractions of each level were used, and the sum of contributory fractions for each ratio treatment was one. The positions of the coded levels are shown in the triangle, and the corresponding ratios are shown in the table on the right. All treatments were at the same total concentration of compounds (50 nM). Scale bar, 2 nM (i.e., 1/25 of total concentration).

(B and C) Percentage of larval settlement (B) and metamorphosis (C) of *M. sallei* in response to different ratios of Ado, Ino, and Hyp. Filtered seawater was used as a negative control (NC). The 5-fold dilution of adult-conditioned seawater was

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#### Figure 6. Continued

used as a positive control (PC). Results are shown as mean  $\pm$  SD (n = 3). Asterisk denotes significant difference compared with the negative control (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, Dunnett's test). (D and E) Mixture contour plots show the effect of different ratios of Ado, Ino, and Hyp on settlement (D) and metamorphosis (E) of *M. sallei* larvae. Star shows the measured ratio of Ado:Ino:Hyp in ACS, i.e., 1:1.125:3.25.

response to certain mixtures could change from induction of settlement to its prevention. The discovery that changing the ratio of the pheromone components can result in the opposite observed effect is reported here. It suggests a potential method for interfering with the gregarious settlement of *M. sallei* and other dreissenid mussels by simply changing the ratio of components in an aggregation pheromone blend.

Purines have received increasing attention as intracellular and intercellular signaling messengers (Massé et al., 2007; Idzko et al., 2014; Verkhratsky and Burnstock, 2014). Sensitivity to purines is widespread across prokaryotes, plants, and animals. The purinergic signaling system is not only ancient in evolution but also omnipresent across species and tissues, involved in highly diverse functions (Verkhratsky and Burnstock, 2014). This signaling system is essential in living organisms because it mediates numerous cellular processes, including neurotransmission, neuromodulation, immune responses, cell proliferation, differentiation and death in development, regeneration, wound healing, cancer, and aging (Burnstock, 2012). Purines as signaling molecules in internal tissues of organisms are now widely accepted. Purinergic communication between individuals has also been found in a few organisms such as fish and polychaetes as mentioned above. As purines are most likely to occur as mixtures both in internal tissues and external environments (Shamchuk et al., 2018), our finding that the combination and ratio of purines is critical for communication in aquatic mussels has significant implications for the study of purinergic signaling in general.

We have shown that *M. sallei* MCF contains three purines, Ado, Ino, and Hyp, which can induce conspecific larval settlement and metamorphosis. Our discovery that the aggregation pheromone of *M. sallei* consists of a synergistic blend of these three purines, most active in a specific ratio allows us to begin to understand how different species of marine bivalve can communicate effectively in the same environment. We further suggest that a blend of Hyp, Ino, and Ado at the optimum ratio induces *M. sallei* larvae to settle and is a critical driver for the development of dense aggregations of this bivalve in the natural environment. Similar pheromone-driven aggregation mechanisms are likely to exist in other invasive dreissenid mussels. Throughout the chemical ecology literature, pheromone molecules are commonly reported to be unique compounds (Wyatt, 2014). However, this work indicates that further study of simple common metabolites that can act as species-specific pheromones when present in specific combinations, like a chemical combination lock, is warranted. In addition, the discovery of settlement inhibition by specific mixtures of purines suggests approaches to the prevention of marine biofouling.

### **Limitations of the Study**

Our results suggested that *M. sallei* adults release a blend of three purines as the aggregation pheromone to induce settlement and metamorphosis of conspecific larvae. However, the mechanism of the synergetic effect of the three purines, Ado, Ino, and Hyp, on settlement of *M. sallei* larvae is unknown. Moreover, whether this pheromone plays an important role in *M. sallei* aggregation in the complex and dynamic natural environment remains to be confirmed, although we have demonstrated that Ado could induce larval settlement of *M. sallei* under flow conditions in laboratory. Further study will be needed to address these issues.

#### **METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

#### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.08.022.

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### **AUTHOR CONTRIBUTIONS**

J.H., D.F., P.S., M.H., and C.K. designed research. J.H., D.F., Q.D., Y.Q., Z.W., and Q.F. performed research. J.G.B. provided constructive suggestions. All authors wrote the paper.

### **DECLARATION OF INTERESTS**

The authors declare no competing interests.

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