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HIGHLIGHTED STUDENT RESEARCH

Common fear molecules induce defensive responses in marine prey across trophic levels

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Abstract

Predator–prey interactions are a key feature of ecosystems and often chemically mediated, whereby individuals detect molecules in their environment that inform whether they should attack or defend. These molecules are largely unidentified, and their discovery is important for determining their ecological role in complex trophic systems. Homarine and trigonelline are two previously identified blue crab (*Callinectes sapidus)* urinary metabolites that cause mud crabs (*Panopeus herbstii)* to seek refuge, but it was unknown whether these molecules influence other species within this oyster reef system. In the current study, homarine, trigonelline, and blue crab urine were tested on juvenile oysters (*Crassostrea virginica*) to ascertain if the same molecules known to alter mud crab behavior also affect juvenile oyster morphology, thus mediating interactions between a generalist predator, a mesopredator, and a basal prey species. Oyster juveniles strengthened their shells in response to blue crab urine and when exposed to homarine and trigonelline in combination, especially at higher concentrations. This study builds upon previous work to pinpoint specific molecules from a generalist predator's urine that induce defensive responses in two marine prey from different taxa and trophic levels, supporting the hypothesis that common fear molecules exist in ecological systems.

Keywords Blue crabs · Chemical ecology · Induced defense · Oysters · Kairomones

Introduction

Predator–prey interactions are a strong driver of community dynamics, as predator presence stimulates prey adaptations that can result in large ecosystem-wide effects (Hay 1996, 2009; Lima 1998). Mechanisms employed by prey to reduce predation risk include upregulating chemical defenses (Cronin and Hay 1996; Baldwin 1998), behavioral modifications or habitat shifts to avoid detection (Werner and Gilliam 2003; Schmitz 2008; Weissburg et al. 2016), morphological alterations (Brönmark and Miner 1992), changes in life history (Peckarsky 1980; Peckarsky et al.

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This study identifies molecules that induce defensive responses in taxonomically and trophically diverse marine species. Suggesting the groundbreaking idea that there are common fear molecules that induce prey responses across multiple trophic levels. This has widespread impacts on our understanding of sensory ecology and predator–prey interactions in communities. Such as the evolution of these traits and community interactions between trophic levels.

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2001), or some combination of these (Hammill et al. 2010). While evolutionary pressures like predation select for advantageous traits (Lima 1998), considerable investment may be required to implement these strategies, which often come with trade-offs such as lower energy reserves and slower growth (Herms and Mattson 1992; Lima 1998; Nakaoka 2000). Nevertheless, when these defenses are inducible, prey may strike a better balance between defending against predation and the inevitable costs, especially when predators can be sensed reliably and predation risk is variable in space or time (Harvell 1990).

Prey may use several sensory modes to recognize a predator (Weissburg et al. 2014), and substantial evidence supports the hypothesis that many prey rely on detecting a predator's scent (Kats and Dill 1998), particularly when other cues are inaccessible. Organisms in early developmental stages and numerous invertebrates have rudimentary or undeveloped auditory and visual systems, so chemical cues are often their primary source of information about their surroundings (Hay 2009). In aquatic habitats, chemical cues are essential for detecting predators because visual and auditory cues are frequently variable and therefore unreliable due to turbidity, low light, or other physical limitations (Dusenbery 2001; Weissburg et al. 2014). For example, Western toad tadpoles exhibit anti-predator behavior when exposed to chemical, but not visual, cues of several different predators (Kiesecker et al. 1996), indicating that the discovery of these molecules is important for fully understanding the selection pressures that drive prey adaptations and distributions. In addition to reducing predation success, prey responses to chemical cues can lead to a wide range of non-consumptive effects including alterations to predator handling times, increases in basal resources, or changes in competitive ability (Brönmark and Miner 1992; Relyea 2001). Even though it is widely accepted that chemical cues play a significant role in shaping ecosystems, the specific metabolites that modulate predator–prey interactions in aquatic systems remain mostly unidentified (Lass and Spaak 2003), especially for inducible prey defenses (Brönmark and Miner 1992; Smee and Weissburg 2006; Hammill et al. 2010).

Along the eastern and gulf coasts of the United States, the eastern oyster *Crassostrea virginica* is a foundation species and major food source for diverse predators (O'Connor et al. 2008) highlighting the need for oysters to have robust defenses. Although exposure to seawater containing predator exudates causes oysters to develop heavier, stronger shells (Newell et al. 2007; Robinson et al. 2014; Scherer et al. 2016; Scherer and Smee 2017; Ponce et al. 2020) their responses to chemical cues indicative of predation risk may vary substantially depending upon numerous factors such as predator type (Robinson et al. 2014; Belgrad et al. 2023a), predator diet (Scherer et al. 2016, 2017), and temporal variation in risk response (Scherer et al. 2018; Eason et al. 2021). Yet, the bioactive molecules mediating these interactions remain unknown, and the variation in responses to predation risk remains ambiguous.

Mud crabs, *Panopeus herbstii*, prey upon oysters, filling a meso-trophic level (i.e., the intermediate prey) between this basal resource and their shared predator, the generalist blue crab *Callinectes sapidus*, which consumes a variety of species including mud crabs and oysters (Tagatz 1968; Laughlin 1982). These smaller crabs detect the presence of danger by interpreting specific metabolites, homarine and trigonelline, in blue crab urine as signs of high predation risk, with cue exposure inducing them to promptly hide instead of forage for food (Weissburg et al. 2016; Poulin et al. 2018). Oysters also respond defensively to this shared predator by strengthening their shells when exposed to exudates from blue crabs (Scherer et al. 2016, 2017), however, the molecules mediating this interaction are unknown. Given that this predator feeds on many types of prey, selection pressure may incentivize predator waste products as reliable cues, and as homarine and trigonelline are known components of blue crab waste excreted via urine, oysters may also associate these chemical cues with danger.

Since oysters and mud crabs coexist in estuarine reefs and are both preyed upon by blue crabs, we investigated whether juvenile oysters respond to the same metabolites as mud crabs and if this response is concentration dependent. Although mud crabs and oysters are taxonomically distinct and occupy different trophic levels, selection pressure from predation risk by blue crabs may have prompted prey to evolve responses to the same predator cues, specifically in the waste products of this generalist predator. Thus, we hypothesize that common fear molecules exist in environments with heavy predation pressure from generalist consumers. Given that homarine and trigonelline are seemingly ubiquitous in the marine environment (Boysen et al. 2021; Dawson et al. 2020; Heal et al. 2021; Núñez-Pons and Avila 2015), and known blue crab urine metabolites that reduce mud crab foraging, thus lowering their risk of predation (Weissburg et al. 2016; Poulin et al. 2018), this study probed whether both molecules also induce oysters to defend themselves by making stronger shells. Testing known ecologically relevant molecules is an essential step for a deeper comprehension of marine ecosystem dynamics, especially because chemical cues are known to regulate biological phenomena (Harborne 2001; Ferrari et al. 2010; Hay 2014; Pohnert et al. 2007). Therefore, it is imperative that these bioactive cues be identified to better predict how chemistry influences multi-trophic level interactions, and in turn, prey evolution.

Methods

We performed a series of experiments to test how the effects of known metabolites in the urine of a generalist predator affected morphological defenses in one of their prey. We examined the effects of blue crab urine and two molecules, homarine and trigonelline, on oyster shell morphology as these molecules are known to be present in blue crab urine and influence antipredator behavior in another species. We then performed dose response experiments to ascertain the relevant concentrations that elicited responses and confirm if concentration was also important for oysters in governing the strength of their response to predation risk. All biological experiments were performed at the Dauphin Island Sea Lab in Dauphin Island, AL, USA over three years (July–Sept. 2020, June–Aug. 2021, and Aug–Oct. 2022). The predator cue bioassay was performed July–Sept. 2020, the homarine and trigonelline dose response experiment was performed June–Aug. 2021, and the blue crab urine dose response was performed August–Oct. 2022.

Predator cue bioassay (July–Sept. 2020)

For the predator cue bioassay, oyster spat were exposed to one of the following cues in individual aquaria: blue crab urine (mud crab or oyster diet), trigonelline (24.6 µM), homarine (15.1 µM), or trigonelline (24.6 µM) + homarine (15.1 µM). Additionally, we used natural seawater taken directly from the Gulf of Mexico (settled for 3 days to remove sediment) as a negative control and predator water, water from a tank with actively foraging blue crabs, as a positive control.

Diploid oyster larvae were purchased from the Auburn University Shellfish Laboratory and settled onto 4.5 cm × 4.5 cm marble tiles to become oyster spat. For one week after settlement, spat on tiles were caged and kept in 1250 L mesocosms with natural flowing seawater from the Gulf of Mexico at a flow rate of 20 L/min. This time was necessary for spat to grow and become firmly attached to the tiles for subsequent experiments.

After 1 week, we moved the spat tiles into closed aquaria (without flowing seawater) to test oyster shell changes in response to our treatments. Only tiles with at least 15 spat were used. Tiles were tied together with high-density polyethylene fishing line with the side containing oyster spat facing outwards. Three tile pairs were placed in each aquarium, ensuring that every tile pair was upright to maintain good water flow around the spat. An intact, sun-bleached adult oyster shell was also placed into each aquarium for spat tile pairs to lean on so that they maintained an upright position to avoid smothering. Aquaria were filled with 2 L of natural seawater (with the exception of the predator water control, which received 1.5 L seawater + 0.5 L predator water). Seawater was supplemented with either Instant Ocean salt or deionized water to reach 20 ppt (± 2 ppt). Each aquarium was aerated with filtered air, covered with a lid to reduce evaporation, and stored outside under a covered pavilion in a water bath containing ambient flow-through seawater to regulate temperature. Aquarium aeration via airstone provided circulation within the system and because of the small volume (2L) of each aquaria, circulation pumps were unnecessary. Spat were fed Reed Mariculture Instant Algae Shellfish Diet 1800 (a proprietary, non-living mixture of *Isochrysis*, *Pavlova, Tetraselmis*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*). At the start of the experiment, spat were fed 0.5 mL twice daily, but we increased this amount to 1 mL twice daily as spat grew larger. Complete water changes and aquarium cleanings were performed twice weekly to deter accumulation of ammonia, nitrates, food waste, and changes in pH, and 1 mL of predator chemical cues (i.e., blue crab urine, solutions of trigonelline, homarine, or a combination) were added to the aquaria immediately after water changes. The pH and salinity of each aquarium was recorded before water changes, and these physical factors did not cause concern at any point in the experiment (SI Appendix, Table S1). Predator water was created by housing 6 blue crabs (carapace width 12–17 cm) in a 238 L volume mesocosm of recirculating filtered natural seawater (50% water changes performed every 1–2 weeks) and feeding each crab an adult oyster (length 6–7 cm). Three to five hours after feeding crabs, 500 mL of this water was added to each positive control aquarium.

Homarine (15.1 µM) and trigonelline (24.6 µM) solutions used to induce oyster spat were prepared at natural concentrations found in urine of blue crabs fed an Eastern oyster diet (Poulin et al. 2018), and a later analysis revealed that our chosen experimental concentration of trigonelline occurs at the upper limit of natural concentration ranges in blue crab urine from our population (SI Appendix, Fig. S1). Homarine (Santa Cruz Biotechnology Company) and trigonelline (Toronto Research Chemicals) were dissolved in deionized water to create individual stock solutions of each chemical, which were aliquoted and frozen at −80 °C to avoid repeated freezing and thawing of the chemical mixture. On days designated for cue addition, the stock solution aliquots were diluted to the experimental concentration (15.1 µM homarine and 24.6 µM trigonelline) and the trigonelline + homarine combination treatment was diluted and combined to achieve the same final concentration of each individual chemical solution (15.1 µM homarine + 24.6 µM trigonelline) to most realistically mimic urine. 1 mL of solution was then immediately added to each corresponding replicate

aquarium. After chemical cue addition, final concentrations in aquaria were 7.55 nM homarine and 12.3 nM trigonelline. These concentrations fall within the range in which these molecules have been detected in the natural environment (Muslin 2017; Dawson et al. 2020; Heal et al. 2021; Boysen et al. 2021; Rasyid 2021) and should be comparable to the exposure oyster spat would experience in a wild habitat.

Predator urine was collected from blue crabs fed two different diets, oysters or mud crabs. Blue crabs were collected from crab pots near Dauphin Island, AL, USA and housed in 1250 L mesocosms flowing with natural seawater. Crab size ranged from 11 to 19 cm, measured from spine to spine on the widest part of the carapace. Crabs were starved for 2–3 days and then were each fed either one adult oyster (~6–7 cm length) or ~5 g of frozen mud crabs every 48 h to standardize diets. Mud crabs used for feeding were collected from either Priest's Landing, Skidaway Island, GA (31°57′44.89"N, 81° 0′48.22"W) or the North Inlet Estuary, SC (33°21′52"N, 79°10′03"W). Crabs were maintained on these diets for one week prior to the urine extraction regimen to ensure all extracted metabolites were from the specified diets and that crabs were not undergoing any starvation stress that could affect the chemistry of their urine. Urine was collected from individuals twice a week. Crabs were cooled to quiescence, then a 23 gauge-needle was inserted approximately 2 mm into the nephropore and urine was extracted with gentle vacuum suction (< 10 psi) into clean glass vials. Urine used for the experiment was clear or yellow in color and foamy; urine was discarded if it appeared cloudy or bluish-gray in color, as this indicated contamination with hemolymph. Urine was frozen at –80 °C immediately after collection. Urine from blue crabs fed an oyster diet was collected from 161 crabs, each crab provided 4.08 ± 3.11 mL per collection, and urine was extracted from them 2.2 ± 1.0 times. Urine from blue crabs fed a mud crab diet was collected from 102 crabs; each crab provided 5.04 ± 4.07 mL per collection and was used for extraction 2.0 ± 1.0 times. We later combined the urine of different individuals into nine mixtures using the fewest individuals possible, where each mixture was considered a biological replicate (SI Appendix, Table S2). These mixtures were then partitioned into 1 mL aliquots and stored at –80 °C until use.

In this bioassay, all treatments and controls had 9 replicates. The experiment was performed July 2020 through Aug. 2020, for a total of 48 days before being disrupted due to heavy storms in Dauphin Island, AL. At the completion of the experiment, spat from each aquarium were randomly selected for assessment of shell strength. Approximately 20 oysters were crushed from each aquarium (distributed across tile pairs), except for one predator water replicate (15 oysters) and one trigonelline replicate (19 oysters) due to high mortality. Individual spat width was measured for each crushed oyster to 0.01 mm using a Vernier digital caliper. The force required to crush oysters was measured to the nearest 0.1 N using a Kistler 5995 charge amplifier and Kistler 9207 force sensor following standard protocol (Robinson et al. 2014). Crushing force was divided by spat width to produce a size-standardized metric of shell strength (i.e., standardized crushing force, N/mm) because larger individuals typically have a stronger shell as a byproduct of their size (Fig. S2). Standardized crushing force measurements for oysters within the same aquarium were averaged. Some measurements were removed from analysis if the oysters were found dead or if the crushing device malfunctioned, resulting in fewer spat contributing to an aquarium average. Some aquaria suffered high mortality and were unusable for analysis. When the experiment concluded, 9 replicate aquaria per treatment were used except for the predator water positive control ($n = 7$) and trigonelline + homarine ($n = 8$) treatments.

Homarine and trigonelline dose response experiment (June–Aug. 2021)

An experiment was designed for the following year (June–Aug. 2021) to test whether oysters respond to homarine, trigonelline, and both cues combined in a dose-dependent manner. Experimental set up and aquaria maintenance followed almost identical protocols to those used in 2020. Stock solutions for homarine (7.4 mM) (Santa Cruz Biotechnology Company), trigonelline (6.6 mM) (Toronto Research Chemicals), and homarine + trigonelline were prepared in liquid chromatography mass spectrometry (LCMS) grade water, aliquoted to avoid freezing and thawing the solutions more than once, and stored at − 80 °C. Serial dilutions for each cue were prepared in deionized water the same day they were added to their respective aquaria (SI Appendix, Table S3). Concentrations were determined by calculating half-log steps encompassing the natural concentrations of homarine and trigonelline found in the urine of blue crabs fed an oyster diet (SI Appendix, Fig. S1). On the day of cue addition, serial dilutions were performed with deionized water and using a micropipette (for volumes under 5 mL) and 10 mL graduated cylinder (for volumes greater than 5 mL). Mixtures were vortexed for 10 s and manually agitated for 10 s before continuing with the serial dilution. Dilutions for treatments of individual compounds and the homarine + trigonelline treatments were prepared at the same half-log concentrations (SI Appendix, Table S3). The same stock solutions were used to prepare both the individual compound and homarine + trigonelline treatments. All serial dilutions were done in tandem. Once chemical mixtures were made, they were stored in glass bottles, and 1 mL of each solution was pipetted into the appropriate experimental aquaria with a clean pipette tip. Chemical solutions were added after water changes, which were performed twice weekly. This

experiment also included a seawater control group, which received the same care (without cue addition) as all other experimental aquaria.

The preparation and maintenance of the homarine and trigonelline dose response experiment was identical to the previous predator cue bioassay except aquaria received four tile pairs per aquarium instead of three. The experiment was conducted for 56 days (8 weeks), and at the completion of the experiment tile pairs were removed from their aquaria, measured, and crushed according to the same methodology from the predator cue bioassay. There were four replicate aquaria per concentration, and within these replicate aquaria, we took an average of 17 crushed oysters. Replicates with high mortality (less than 6 spat alive) were excluded from statistical analyses. In total, 14 aquaria from the homarine dose array, 16 aquaria from the trigonelline dose array, and 17 aquaria from the trigonelline + homarine dose array were included in statistical analysis (SI Appendix, Table S3).

Urine dose response experiment (July–Oct. 2022)

A blue crab urine dose response experiment was designed to determine whether differences in oyster shell strengthening could be fully explained when considering the complete blend of blue crab urine cues. The experiment was conducted for 56 days (8 weeks) from Aug. 2022 to Oct. 2022. Blue crabs were collected from crab pots near Dauphin Island, AL and stored in the same facility and conditions as described for the predator cue bioassay (2020). Urine collection methods remained the same as for previous experiments (2020 predator cue bioassay) except urine was pooled from all crabs for this experiment. All crabs for this experiment were fed an oyster diet (i.e., one adult oyster (~6–7 cm in length) twice weekly). Crab were kept in aquaria for a one-week acclimation period before beginning a urine extraction regimen. Urine was collected twice weekly from 22 crabs that ranged 13–18 cm in size and each crab produced 1.31 ± 0.18 mL. Urine dose concentrations were determined based on the average concentrations of homarine and trigonelline quantified in blue crab urine in the 2020 predator cue bioassay (homarine, 13 ± 21 µM; trigonelline, 3.6 ± 6.9 µM). It was assumed that 1 mL of pooled blue crab urine contained these concentrations, and doses were adjusted by half-log steps until approximated concentrations of homarine and trigonelline spanned four orders of magnitude (SI Appendix, Table S4). Though initial experimental design was based on an assumed concentration of homarine and trigonelline, all urine doses were reported as urine volume divided by the total volume of seawater within an experimental aquaria. For the six lowest doses, 1 mL aliquots of blue crab urine were prepared via serial dilution. The two highest doses received 5.00 mL and 1.25 mL aliquots of pure urine, respectively. All aliquots were frozen at −80 °C to be used on the day of cue addition.

The preparation and maintenance of the urine dose response experiment was identical to the previous homarine and trigonelline dose response experiment. At the completion of the experiment tile pairs were removed from their aquaria, measured, and crushed according to the same methodology from the predator cue bioassay. There were five replicate aquaria per concentration, and within these replicate aquaria, we took an average of 32 crushed oysters.

Quantification of homarine and trigonelline

All chemical analyses were performed at the Georgia Institute of Technology, Atlanta, GA, USA. Concentrations for stock solutions of trigonelline and homarine prepared for the predator cue bioassay (summer 2020) and quantitative standards were determined using nuclear magnetic resonance (NMR) spectroscopy (Bharti and Roy 2012). For preparation of standards, trigonelline and homarine were dissolved in LCMS grade water. Both stock solution and standard samples were prepared for quantitative NMR spectroscopic analysis by combining 138 µL sample, 46 µL phosphate buffer in D_2O and 16 µL D_2O. A caffeine standard (0.00949 mg) was dissolved in 138 µL LCMS grade water, 46 µL phosphate buffer in D_2O and 16 µL D_2O. A benzene-d6 NMR insert capillary tube was used as the reference standard. Spectroscopic data were acquired using a Bruker Avance IIIHD 800 MHz NMR spectrometer and processed in MestReNova 11.0.4. All calculations were done in Microsoft Excel (Office 365).

Standard solutions previously quantified using NMR spectroscopy were used for mass spectroscopic quantification of trigonelline and homarine in multiple samples: 24 blue crab urine mixtures (12 oyster diet and 12 mud crab diet) from the predator cue bioassay (summer 2020) and stock solutions of trigonelline and homarine prepared for the dose response experiment (summer 2021). For preparation of urine samples, 20 µL of urine was added to 180 µL of methanol, solutions were vortexed for mixing, centrifuged at $19{,}980 \times g$ to pellet precipitate, and the supernatant was transferred to clean new vials. Serial dilutions of samples and standards were prepared using 90% aqueous methanol so that concentrations for homarine and trigonelline fell within the nanomolar range. All standards were injected in triplicate, as were dilutions prepared for the dose response curve stock solutions. These stock solutions were diluted to two concentrations within the linear range of the curve, each dilution was injected three times, and final concentrations were determined by averaging data from all six injections. Concentrations of analytes in urine were determined from a single injection. Peak areas were integrated using Xcalibur Version 4.3.73.11 (Thermo Fisher Scientific) and linear

regression analyses were performed in GraphPad Prism 9.3.0 for Windows to generate standard curves. Concentrations for urine samples and stock solutions interpolated using standard curves were converted to natural concentration in micromolar using Microsoft Excel for Windows.

Data for standards and urine samples were acquired using a Vanquish ultraperformance liquid chromatography setup coupled to a high-resolution accurate mass Orbitrap ID-X Tribrid mass spectrometer (Thermo Fisher Scientific). A Waters Corporation ACQUITY UPLC BEH Amide column (2.1 × 150 mm, 1.7 µm particle size) was used for chromatographic separation. Analytes were eluted using 4:1 water/acetonitrile with 10 mM ammonium formate and 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) using the following gradient program: 0 min 5% A; 0.5 min 5% A; 8 min 60% A; 9.4 min 60% A; 9.5 min 5% A; 12 min 5% A. The flow rate was set at 0.40 mL/min, the column temperature was set to 40 °C, and the injection volume was 1 µL.

Statistical analyses

For the predator cue bioassay, a one-way ANOVA was performed on the data ($n = 9$ for all treatments except predator water, $n = 7$, and trigonelline + homarine, $n = 8$), followed by an uncorrected Fisher's Least Significant Difference (LSD) test for pairwise comparisons of each treatment and the seawater negative control (Fig. 1). Simple linear regression analyses were done to assess the relationship between chemical cue concentration (i.e., trigonelline, homarine, or trigonelline + homarine) in blue crab urine used for the predator cue bioassay and standardized crushing force (Fig. 2 and S4). One urine sample was excluded from the trigonelline analyses because the measured concentration was below the limit of quantitation. Additionally, quantified concentrations of homarine and trigonelline in blue crab urine were regressed against each other using simple linear regression (Figs. S2 and S3). All predator cue bioassay regression analyses were done for the individual diets (i.e., oyster or mud crab) and both diets combined.

For the pure cue (Fig. 3, $n = 4$ per concentration) and urine dose response (Fig. 4, $n = 5$ per concentration) experiments, cue concentration was regressed against standardized crushing force using semilog line regression where X is log and Y is linear. Concentrations of homarine and trigonelline above those naturally found in blue crab urine were excluded from analyses of the pure chemical dose response (SI Appendix, Table S3). A nested one-way ANOVA was used to compare cue concentrations for dose response curves where the slope of the linear model was not significantly non-zero, and an uncorrected Fisher's LSD test was used for pairwise comparisons of cue concentrations to seawater (i.e., Fig. 3A).

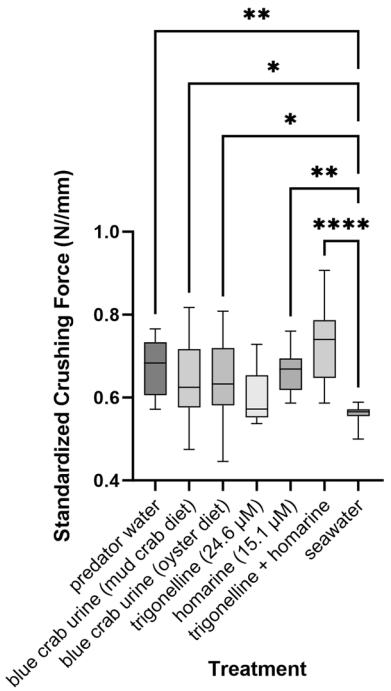

Fig. 1 Oyster spat were exposed to predator water ($n = 7$), blue crab urine ($n = 9$ per diet type), pure chemical compounds homarine or trigonelline ($n = 9$), a combination of homarine + trigonelline ($n = 8$), or seawater ($n = 9$). Their shells were crushed and the measured force was normalized to shell width (i.e., standardized crushing force). A one-way ANOVA followed by an uncorrected Fisher's LSD test compared the means of all treatments to the seawater control. Predator water, blue crab urine of both diets, homarine (15.1 µM) and homarine + trigonelline induced significantly stronger oyster shells than seawater. Trigonelline (24.6 µM) did not significantly induce stronger shells. This box-and-whisker plot shows the median of each treatment, the upper and lower quartiles, and the minimum and maximum bounds for each treatment. Treatments are denoted as significant with * at $P \leq 0.05$, ** at $P \leq 0.01$ and **** at $P \leq 0.0001$

Values plotted on the x-axes for the trigonelline + homarine analyses (Figs. 2c and 3c) were generated by adding the concentrations of these molecules together. Considering their sum as a single independent variable allowed for the assessment of trigonelline + homarine as a blend. All statistical analyses were performed using GraphPad Prism 9.3.0 for Windows.

Results

Oyster shell strengthening induced by predator cues

Predator chemical cues induced oysters to strengthen their shells (Fig. 1; ANOVA, $F(6, 53) = 3.97$, $P = 0.002$). Homarine (15.1 µM) (Fisher's LSD, $P = 0.008$, $n = 9$) and predator water (Fisher's LSD, $P = 0.005$, $n = 7$) significantly induced

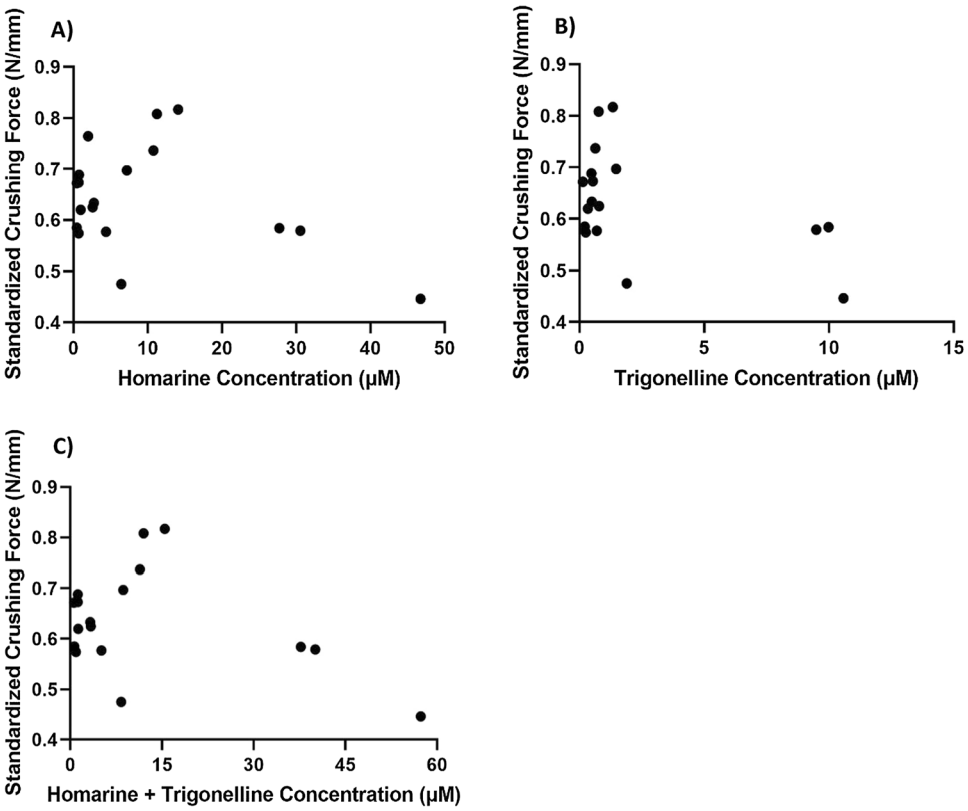

Fig. 2 Linear regression analyses of chemical cue concentrations quantified for blue crab urine from the predator cue bioassay. Models suggest that homarine (**A**), trigonelline (**B**) and their combined concentrations (**C**) were not reliable indicators of induced oyster shell strengthening for oysters exposed to blue crab urine. Analyses were performed for combined diets: **A** homarine concentration ($F(1, 16) = 2.44$, $P = 0.14$), **B** trigonelline concentration ($F(1, 15) = 4.47$, $P = 0.052$), and **C** trigonelline + homarine concentration ($F(1, 15) = 2.44$, $P = 0.14$). Oyster spat were exposed to one of 17 crab urine mixtures (oyster diet, $n = 8$, mud crab diet, $n = 9$), crushed, and crushing force was standardized by oyster shell width. Each point is an average of 20 oyster spat. Data were excluded for concentrations below the limit of quantification

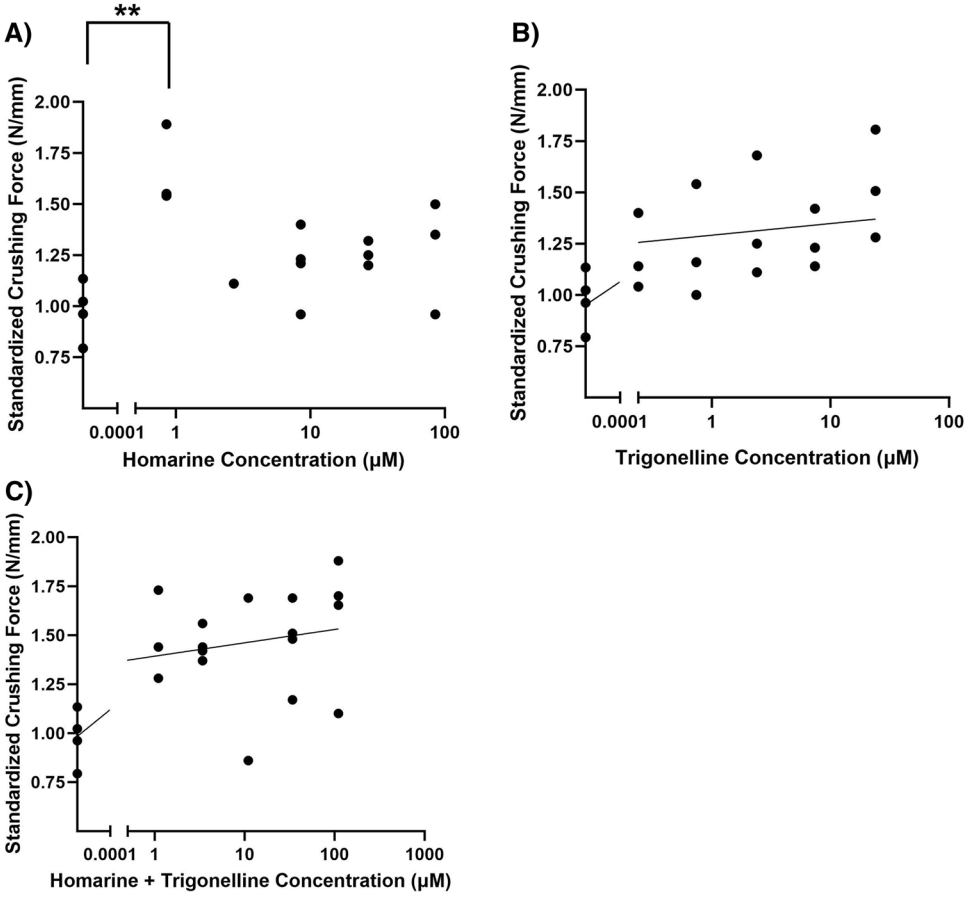

Fig. 3 Dose response curves for oyster spat exposed to solutions of homarine (**A**), trigonelline (**B**), or a combination of homarine and trigonelline (**C**), with concentrations spanning five orders of magnitude. The slopes of B and C non-linear regression analyses are significantly non-zero ($P = 0.005$ and $P = 0.002$, respectively) indicating that standardized crushing force increases with higher concentrations of chemical cue. The non-linear regression analysis for A was not significantly non-zero ($P = 0.063$), however, the 0.84 μM homarine dose was significantly different from seawater (Fisher's LSD pairwise comparison, $P = 0.001$). Seawater controls are plotted on the *y* axes since the concentrations of chemical cue in these treatments is considered to be 0 μM. Each point is an average of 6–17 oysters. Crushing force was standardized by oyster shell width

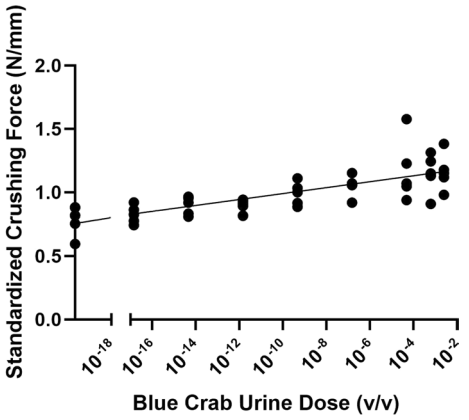

Fig. 4 A dose response curve for oyster spat exposed to urine of blue crabs fed exclusively oysters. Eight different urine concentrations were tested spanning several orders of magnitude. Urine potency increased significantly with concentration (non-linear regression, $F(1, 43) = 44.0$, $P < 0.0001$, $R2 = 0.5785$). Urine doses along the *x*-axis are the total volume of undiluted blue crab urine applied to the experimental aquaria. Each point is an average of 32 oyster spat and crushing force was standardized by oyster shell width. A one-way ANOVA revealed that the lowest potent urine volume is $1.50 \times 10–7$ ($P = 0.012$)

oyster shell strengthening when compared to the seawater control; however, trigonelline (24.6 µM) did not (Fisher's LSD, $P = 0.27$, $n = 9$). Despite trigonelline not having an effect on its own at 24.6 µM, oysters made significantly stronger shells when exposed to a mixture of trigonelline (24.6 µM) + homarine (15.1 µM) (Fisher's LSD, $P < 0.0001$, $n = 8$). Furthermore, mud crab and oyster diet blue crab urine treatments were both significantly potent (mud crab diet, Fisher's LSD, $P = 0.034$, $n = 9$; oyster diet, Fisher's LSD, $P = 0.033$, n = 9).

Homarine and trigonelline in blue crab urine do not fully explain induced oyster defenses

For the quantification of chemical cues within natural blue crab urine mixtures, concentrations of homarine and trigonelline were quite variable, spanning several orders of magnitude. Homarine concentrations ranged from 0.4 to 65 µM and trigonelline from 0.1 to 22 µM, respectively (SI Appendix, Fig. S1). It was hypothesized and later confirmed that higher concentrations of homarine quantified in blue crab urine correlated positively with higher concentrations of trigonelline ($F(1, 21) = 246$, $P < 0.0001$, $R^2 = 0.92$; SI Appendix, Fig. S4). This relationship was also significant when the two blue crab diets were analyzed separately (mud crab diet, $F(1, 10) = 68.3$, $P < 0.0001$, $R^2 = 0.87$; oyster diet, $F(1, 9) = 150$, $P < 0.0001$, $R^2 = 0.94$; SI Appendix, Fig. S3). Additionally, concentrations of homarine ($F(1, 16) = 2.44$, $P = 0.14$), trigonelline ($F(1, 15) = 4.47$, $P = 0.052$), and homarine in combination with trigonelline ($F(1, 15) = 2.44$, $P = 0.14$) in blue crab urine trended negatively with standardized crushing force but not significantly (Fig. 2). This was also true when diets were analyzed separately (SI Appendix, Figs. S4).

Oyster response to cue concentration is dose-dependent

Results from the predator cue bioassay justified an evaluation of oyster response to a wider range of concentrations for pure chemical cues: trigonelline, homarine, and homarine + trigonelline (Fig. 1). Trigonelline potency increased with concentration up to 24 µM ($F(1, 18) = 10.2$, $P = 0.005$, $R^2 = 0.36$; Fig. 3B), which was close to the natural concentration maximum (22.1 µM) in blue crab urine from predator cue bioassays conducted in summer 2020 (SI Appendix, Fig. S1). However, trigonelline did not induce stronger oyster shells at the slightly higher concentration, 24.6 µM, tested in the 2020 predator cue bioassay (Fig. 1), nor at higher concentrations tested within the dose response experiment (SI Appendix, Table S3). In other words, juvenile oysters did not strengthen their shells when exposed to concentrations of trigonelline above natural concentrations in blue crab urine. In contrast, homarine potency did not significantly increase with concentration ($F(1, 16) = 4.01$, $P = 0.063$, $R^2 = 0.20$; Fig. 3A), however, it did induce oysters to make the strongest shells at 0.85 µM, almost twice as strong as oysters exposed only to seawater. Notably, this was the lowest non-zero concentration of homarine tested (ANOVA, $F(5, 12) = 4.00$, $P = 0.023$; Fisher's LSD, $P = 0.001$, Fig. 3A), suggesting that even lower concentrations of homarine might induce a stronger response. The potency of homarine in combination with trigonelline also increased with concentration, providing more support for the hypothesis that these molecules have the strongest effect on oyster shell strength when presented together ($F(1, 19) = 13.6$, $P = 0.002$, $R^2 = 0.42$; Fig. 3C).

Given that oyster response was not entirely explained by homarine and trigonelline concentrations (Fig. 2), an additional experiment was designed to test the dose dependency of blue crab urine as a natural mixture. Blue crab urine potency significantly increased with concentration ($F(1, 43) = 44.0$, $P < 0.0001$, $R^2 = 0.5785$, Fig. 4), providing further evidence for the dose-dependent nature of oyster shell strengthening and the role of other urinary metabolites in this interaction. The 6.6 million-fold dilution of blue crab urine (i.e., 1.50×10^{-7} within experimental aquaria) was the lowest dose that significantly induced oyster defenses (one-way ANOVA w. multiple comparisons, $P = 0.012$, Fig. 4), and was notably much lower than the two-thousand-fold dilution that was previously observed to induce oyster shell strengthening (Fig. 1).

Discussion

Juvenile eastern oysters made stronger shells in response to blue crab urine as well as urine constituents homarine and trigonelline, the same cues that were previously found to reduce mud crab foraging behavior (Poulin et al. 2018). These results confirm that prey from different marine taxa and trophic levels respond to the same molecules released by a shared predator, providing insights about induced defense mechanisms in response to a generalist predator. While there have been a wide array of studies focusing on how chemical cues from predators influence prey in terrestrial and aquatic systems (Godard et al. 1998; Leonard et al. 1999; Maerz et al. 2001; Smee and Weissburg 2006; Scherer et al. 2016, 2017; Weissburg et al. 2016; Poulin et al. 2018), previous efforts concentrate on how closely related prey respond to a common predator (Peckarsky 1980; Ferland-Raymond et al. 2010; Ferrero et al. 2011). Considerably fewer studies explored the interaction between a shared predator with diverse prey (Osada et al. 2014), and experiments with marine organisms remain rare (Poulin et al. 2018). Our findings reveal that taxonomically diverse prey from multiple trophic levels within this model system detect and respond to the same predation risk cues, supporting the idea that common fear molecules may be capable of influencing other ecological systems through various non-consumptive effects. Furthermore, ragworms may reduce foraging in response to these same cues (Fletcher et al. 2023), potentially providing further evidence for the existence of common fear molecules. The effects of a generalist predator may reduce those of an intermediate predator on basal prey, where the defense of the intermediate prey lowers its own efficacy or contact rate (e.g., by reducing activity) when exploiting the basal prey. The presence of a generalist predator may also amplify the effect of an intermediate predator by increasing efficacy or contact rate with basal prey, such as when the response of the intermediate prey is to move to refugia where basal prey are abundant. Furthermore, as is the case for multiple predator effects (Sih et al. 1998), pairwise interactions are not sufficient for explaining numerous prey responses to a multitude of cues from a single predator.

In the current study, oyster defenses were especially noteworthy when both homarine and trigonelline were present simultaneously (Fig. 1), indicating that chemical blends induce the greatest prey response. The potency of homarine and trigonelline in combination increased with dose within the natural concentration ranges of these molecules found in blue crab urine (Fig. 3), and as hypothesized, the potency of blue crab urine as a whole significantly increased with dose (Fig. 4). These data suggest that oysters use urine concentration as a proxy for danger, whereby high risk could correspond to several large crabs or higher numbers of smaller blue crabs present nearby. An oysters' ability to successfully interpret the risk of predator encounter and respond to chemical blends can increase the survival of individual spat in nature (Belgrad et al. 2023b, a), incentivizing the selection of accurate responses. Dose dependent responses to chemical cues are not unusual, whereby the magnitude of the response increases with cue concentration (Tollrian 1993; Fraker 2008), and it is not uncommon for this dose dependency to rely on a blend of multiple cues, including conspecific cues (Laforsch et al. 2006). Our findings suggest homarine and trigonelline are utilized by oysters for detecting blue crabs and it is likely that other bioactive molecules are also involved in this interaction. This is supported by the observation that there was no correlation between oyster shell strengthening and concentrations of homarine and trigonelline quantified from blue crab urine samples (Fig. 2; SI Appendix, Fig. S5), a relationship expected to be significant if these compounds alone were responsible for oysters responding to predation risk. Although this does not negate the importance of homarine and trigonelline in mediating the interaction between oysters, blue crabs, and mud crabs, our findings suggest that these are only two of perhaps several molecules comprising a cue blend and that more work is needed to identify all relevant chemical cues in this interaction.

Generalist prey such as oysters may use a chemical blend as a proxy for danger, whereby the presence of multiple chemical cues at the right ratios indicates a greater risk, prompting a stronger response at higher concentrations (Tollrian 1993). The use of chemical blends, and concentration-dependent responses, occurs across many systems in a variety of predator–prey interactions (Fraker 2008; Osada et al. 2014; Selander et al. 2015; Poulin et al. 2018; Weiss et al. 2018; Hahn et al. 2019). Blends, such as metabolic waste products, may be more reliable than individual cues because they reduce the possibility of falsely interpreting danger; therefore, selective pressure for this type of perception by prey is strong. The possibility of these cues originating from a relevant predator is higher if prey detect multiple molecules in the environment, especially if cues are primary metabolites shared by many predators, as certain combinations of chemical cues may act as an indicator of species type. Homarine and trigonelline may serve as these generalizable basal cues indicative of many marine predators, while the addition of unidentified chemical cues allows for marine prey to identify predator species; this explains the lack of trend between homarine and trigonelline concentration quantified in blue crab urine samples and standardized crushing force. This is also true for cue concentration, whereby prey may assess the concentration of a cue to determine relevant information for risk, including nearness, size,

or species of a predator, time since last fed, or other clues to interpret risk of consumption (Tollrian 1993; Laforsch et al. 2006; Fraker 2008).

As an important basal resource to numerous consumers in coastal communities, eastern oysters experience considerable selection pressure to recognize the risk of predation, suggesting that the most reliable cues could be those common to many predators. Utilizing molecules involved in life-sustaining pathways shared by many taxa, as fear cues, is advantageous to oysters and mud crabs; these cues are produced and emitted by relevant predators that cannot prevent their release, making detection of these organisms by prey more certain. Furthermore, metabolites like homarine and trigonelline are enriched in the waste of some organisms in a diet-dependent manner (Poulin et al. 2018), and it is likely that many fear cues come from a predator's diet or result from other metabolic processes. Sensing these urine molecules directly associates the predator with feeding on relevant prey because many marine invertebrates contain homarine and trigonelline within their tissues (Carr et al. 1996). This hypothesis is reinforced by evidence that homarine and trigonelline are more abundant in mud crab tissues compared to other blue crab prey, and that both cues were enriched in blue crab urine when blue crabs fed exclusively on mud crabs (Poulin et al. 2018). Since danger is associated with metabolic activity of the predator, primary metabolites are more definitive cues than those with a specialized function or which are non-diet derived. This notion may apply to many ecosystems, in which primary metabolites serve as common fear molecules for broadly consumed prey species, which in response have evolved to detect predation risk through metabolic waste products that are common to many predators. Therefore, primary metabolites in many taxa are likely to serve as common fear molecules within multiple ecosystems, although the distinction between primary and secondary is inexact as more and more metabolites are discovered to be multifunctional (Erb and Kliebenstein 2020).

When a predator urinates, cues are released into the surrounding water as a concentrated plume, transported over space and time via diffusion or advection (Webster and Weissburg 2009). Nearby prey may then be exposed to these cues at a range of concentrations depending on their spatial proximity to an excretion or secretion event, transport of the cue in the water column, or molecular diffusion from particulate organic carbon. While homarine and trigonelline have yet to be quantified in seawater from relevant oyster reef habitats (i.e., along the eastern coast of the United States through the Gulf of Mexico), several studies have detected these compounds in marine particles (Heal et al. 2021; Boysen et al. 2021), sea ice (Dawson et al. 2020), and environmental seawater samples (Muslin 2017; Rasyid 2021; Sacks et al. 2022) at concentrations ranging from pM to µM. These ambient concentrations are largely below the effective doses for trigonelline and homarine in the current study (Fig. 3c), although at the medium-to-high end, they overlap with concentrations at which these urinary metabolites can impact species interactions. Collectively these studies suggest that these molecules are prevalent in the marine environment, although the concentration range is wide, further supporting our conclusion that specific concentrations are required for inducing prey defenses and that these concentrations must be above an ambient threshold. Additionally, reported concentrations of homarine and trigonelline in seawater field samples (Muslin 2017; Rasyid 2021) support the hypothesis that they are present at lower levels in offshore waters than in coastal habitats where the presence of predators and associated fear cues are higher.

Identifying waterborne fear cues from seawater is challenging (Berlinck et al. 2021; Bayona et al. 2022), so it is unsurprising that such molecules have rarely been identified. Homarine and trigonelline are widespread metabolites (Carr et al. 1996), but their principal ecological role in marine environments remains elusive. Both molecules have been described as osmolytes (Dickson and Kirst 1986; Tikunov et al. 2010; Gebser and Pohnert 2013), and their prominence as primary metabolites is further supported by their ubiquity in the tissues of marine invertebrates (Mathias et al. 1960; Dickson and Kirst 1986; Carr et al. 1996; Ashihara 2008; Ashihara et al. 2015) and some vertebrates, such as elasmobranchs (Dove et al. 2012), as well as by their presence in the urine of diverse species (Lapan 1975; Poulin et al. 2018; Gibson et al. 2020). These metabolites are also proposed to fulfill a variety of functions in other marine organisms and are perhaps more specialized in purpose than expected. Homarine and trigonelline are necessary for proper polyp development and larval metamorphosis in hydroids (Berking 1987), altering the morphology of these individuals, and both molecules were also identified as antifouling agents utilized by different species of soft corals (Targett et al. 1983; Kawamata et al. 1994). Additionally, some Antarctic soft corals use homarine as an antimicrobial defense (Slattery et al. 1997), while it serves as a predator feeding deterrent in Antarctic gastropods (McClintock et al. 1994). The pervasive roles of both cues in diverse ecological systems supports their utility as a general cue and implies that primary metabolites may be more important in ecological function than previously considered.

Discovery of new functions for known metabolites allows for study of ecological and evolutionary insights of predatory interactions, such as the plasticity of prey responses, subsequent evolution of sensory systems, and epigenetic consequences to morphological induced defenses. Currently, many studies rely on simply adding live predators to induce prey responses which places limits on researchers' ability to standardize experiments due to the individual nature of live organisms. However, the cue concentrations

described here provide targets for researchers to begin providing known threat exposure treatments. As advancements are made towards discovery of additional molecules that mediate predator–prey interactions, accurate identification and verification of biological activity will be paramount for understanding and managing the impact of chemical cues on ecosystems. Future research should focus on identifying chemical cues from additional marine predators, analogous to studies of terrestrial systems to further understand the breadth of homarine and trigonelline as universal fear cues. Strong collaborations between ecologists and chemists continue to result in important breakthroughs for the field, notably where efforts are directed towards the development of chemometric tools for metabolomics studies. Without these partnerships, identification of the molecules mediating these important interactions would be improbable.

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Availability for data and materials All data have been submitted to the Biological and Chemical Oceanography Data Management Office repository (https://www.bco-dmo.org).

Code availability All statistical analysis was performed in GraphPad Prism 9.3.0 for Windows and no original code was written for this study.

Declarations

Conflict of interest The authors of this manuscript declare they have no competing interests at time of publication.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

Consent to participate Not applicable.

Consent for publication Not applicable.

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