Gulf and Caribbean Research

Volume 34 | Issue 1

2023

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GULF AND CARIBBEAN

RESEARCH

Volume 34
2023
ISSN: 2572-1410

Published by

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

GULF COAST RESEARCH LABORATORY
Ocean Springs, Mississippi

Gulf and Caribbean Research Vol 34, SC30-SC34, 2023
DOI: 10.18785/gcr.3401.21
Manuscript received October 2, 2023; accepted December 11, 2023

SHORT COMMUNICATION

INTRASPECIFIC POTENCY OF PREDATION RISK CUES§

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Key Words: *Crassostrea virginica*, predator, phenotypic plasticity, chemical cue

Introduction

Predatory interactions are often mediated by reciprocal detection of predator and prey chemical cues that are released through physiological processes (Weissburg et al. 2002, Hay 2009). Avoiding detection is paramount to evade consumers and/or to successfully forage, but eventually animals need to release waste products, which can reveal their presence to enemies, competitors, or prey (Hay 2009). In turn, many prey species rely on detecting these chemical cues to notify them of nearby predators, and prey may subsequently evade predation by either developing predator resistant morphologies, chemical defenses, or avoidance behaviors in the presence of these cues (Cronin 2001, Preisser et al. 2005).

Such defenses of prey can be highly variable. The chemical exudates predators release can depend on a variety of variables ranging from the diet of an individual predator to variation in the metabolic processes of that predator, with sometimes subtle effects on the composition of molecules prey rely on to gauge predation risk and develop defensive traits (Poulin et al. 2018). Anti–predator defensive traits and the frequency and magnitude of prey response, in turn, are influenced by the quantity and quality of predator cues as well as environmental factors that may affect prey ability to detect and react to risk posed by predators (Weissburg et al. 2014, Scherer and Smee 2016, Scherer et al. 2016).

These defenses are also energetically costly and can reduce growth or fecundity (Harvell 1990, Cronin 2001, Relyea 2002, Miner et al. 2005). To reduce costs, some organisms can limit expression of defensive traits to situations where the risk posed by consumers is high and they have a reliable mechanism to evaluate risk (Harvell 1990, Relyea 2002, Preisser et al. 2005, Hay 2009), further adding to variation in response. As a result of this variation, the specific conditions under which organisms may make these trade–offs are poorly defined.

Understanding how much a species response is driven by internal processes (i.e., individual variation) versus external processes (i.e., environmental variation) is central for predicting the strength of species interactions. Oyster (*Crassostrea virginica*) spat react to blue crab (*Callinectes sapidus*) urine by growing heavier, stronger shells (Roney et al. 2023) and show highly variable growth and morphological change in response to this predation risk (Robinson et al. 2014, Scherer et al. 2016, Combs et al. 2019). Using this model system and controlling for predator urine (i.e., risk cue) concentration, we quantified the degree to which variation in oyster responses to predator risk cues was influenced by individual oyster growth versus differences among predators in the cues they released. We hypothesized that cue quality has large effects on oyster risk evaluation and response that can be observed despite high growth variability among individual oysters. Although some variation in oyster growth was observed, more than 32% of the variation was attributed to differences in cue mixtures from individual predators, suggesting that cue quality has large effects on oyster risk evaluation and response that can be observed despite high growth variability among individual oysters. By using predator urine as a risk cue, chemical analyses can be performed to identify the precise molecules responsible for this variation (sensu Poulin et al. 2018).

Materials and Methods

To investigate how natural variation in cue quality among individual predators influenced the development of prey defensive traits, oysters were exposed to blue crab urine as well as to a control of plain seawater and to water obtained from a tank of actively foraging blue crabs, which is known to stimulate oysters to develop stronger, heavier shells (Belgrad et al. 2021). By using predator water in conjunction with a seawater control without predator cues, we set the low and high thresholds for which to compare oyster morphological changes when exposed to only urine from predators.

Oyster larvae were reared and settled onto sun–bleached shell by the Auburn University Shellfish Laboratory on Dauphin Island, AL before being provided to the Dauphin Island Sea Lab (DISL) in mid–June 2019. We elected to maintain natural settlement densities on shells (10 – 40 individuals) to ensure enough individuals survived for the duration of the study. Oyster spat were <1 mm when the experiment began. Four spat–covered shells were placed in individual 4 L high–density polyethylene tanks to test oyster shell changes in response to our treatments. Shells with spat were distributed to obtain an approximately equal number of individuals per tank. Tanks were filled with 2 L of natural settled sand–filtered seawater

§*This research was conducted as part of the Dauphin Island Sea Lab's Research Experience for Undergraduates in the coastal and nearshore marine science program.*

(except for 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 a salinity of 20 (± 2). Each tank was aerated, covered with a lid to reduce evaporation, and stored indoors to regulate temperature. Tank aeration provided water circulation due to the small size of the system. Spat were fed to satiation using 1 mL of Shellfish Diet 1800 (Reed Mariculture) daily, which was gradually increased to 2 mL as spat grew. Regardless, prior study suggests food deprivation does not inhibit oyster shell morphological reactions to crab predators (Scherer and Smee 2017). Complete water changes and tank cleanings were conducted twice weekly, and immediately followed by 1 mL additions of predator urine mixtures, 1 mL of plain seawater, or 0.5 L of predator water depending on their treatment.

Predator water was acquired by placing 6 blue crabs in a 140 L mesocosm of recirculating settled natural seawater with 50% water changes performed every 1–2 weeks, and feeding each crab an adult oyster at least 3x weekly. Three to 5 h after feeding crabs, 0.5 L of this water was added to oyster tanks receiving predator water treatments. Crabs used for making predator water were not used for urine extraction.

Blue crab urine was collected following the procedures outlined in Roney et al. (2023). Briefly, adult blue crabs were caught locally using crab pots and housed in recirculating seawater systems at DISL. Crabs were adults at least 8.5 cm carapace width and included both male and female individuals. Crabs were kept in individual containers and tagged for identification. Each crab was maintained on a diet of a single adult oyster, fed 3x weekly for at least one week prior to use in the experiment to ensure all extracted metabolites were from the specified diets. Urine was collected from individual crabs twice each week. Crabs were cooled to quiescence, then a 23 gauge—needle was inserted about 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 because this indicated contamination with hemolymph. Urine was frozen at –80°C immediately after collection and kept separated by individual. Crabs would produce anywhere from 0 – 6 ml of urine during an extraction and extractions were generally only done 3 times before diminishing returns in urine collection necessitated crab release. Urine was later pooled into 8 unique mixtures using the fewest individuals possible (~36—38 ml urine per mixture). Mixtures 1—7 were made using 4—6 individuals and mixture 8 contained urine from 12 crabs. Thus, crab urine from a single individual would be present in only one mixture. These mixtures were then partitioned into 1 ml aliquots and stored at –80°C until use.

In this bioassay, each urine mixture had 2 replicate tanks while the negative seawater control and positive predator water control each had 10 replicate tanks. The bioassay was performed for 2 months from mid—June to mid—August 2019 with water changes and cue additions performed twice per week. At the conclusion of the experiment, 8 spat were haphazardly selected from each tank for assessment (n = 16 per urine mixture; n = 80 per seawater control and per predator water treatment), with 2 spat measured from each shell when available. Individual spat length was measured to 0.01 mm using a Vernier digital caliper, and the force necessary to crush the spat shell was quantified as a proxy of shell strength. Crushing force 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 length 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 by-product of their size (Robinson et al. 2014).

Separate generalized linear mixed models with a gamma distribution were performed on the entire dataset to determine the effect of chemical mixture on oyster shell size and standardized crushing force (R package: lme4, Bates et al. 2015). Cue mixture was treated as a fixed effect; the shell on which each spat was attached and tank were both treated as nested random effects. Tukey's multiple comparison tests were run on models to determine pairwise differences using the general linear hypotheses function in the R package multcomp (Hothorn et al. 2008). To quantify the influence of individual urine composition on oyster induced defenses, we calculated the proportion of variance associated with the effect of mixture, holding tank, and the interaction between the 2 variables (eta—squared, $\eta^2 = \frac{SS_{effect}}{SS_{total}}$) using just the data on urine mixtures (excluding control mixtures) (Norouzian and Plonsky 2018). We similarly calculated omega—squared (ω^2) as a measure of urine mixture effect size (conservatively calculated as $\omega^2 = \frac{SS_{effect} - (df_{effect} \times MS_{error})}{SS_{total} + MS_{error}}$; Dodd and Schultz 1973). All statistical analyses were conducted in R version 4.1.2.

Results and Discussion

Oyster shell sizes were not significantly different when exposed to predator water (estimate = 0.01, t = 0.66, p = 0.51) or urine mixtures (estimate = 0.01, t = 1.03, p = 0.30), with no significant pairwise size differences across mixtures (p > 0.80). Oysters grew stronger shells when exposed to predator exudates, both from water from a tank with foraging blue crabs (estimate = 0.33, t = 2.68, p = 0.0073) and from blue crab urine (estimate = 0.23, t = 2.10, p = 0.035; Figure 1). Urine mixture quality had a large effect on oyster shell hardness (ω^2 = 0.288), where individual mixture accounted for 32.2% of the observed variation in individual oyster shell hardness (η^2 = 0.322). Here, the most potent urine mixture produced a mean shell hardness that was 90% stronger than the least potent urine mixture (Figure 1). In contrast, holding tank had a negligible main effect on shell hardness (ω^2 = 0.00), accounting for only 0.02% of the variation (η^2 = 0.0002). However, the interaction between holding tank and mixture was modest (ω^2 = 0.112) and accounted

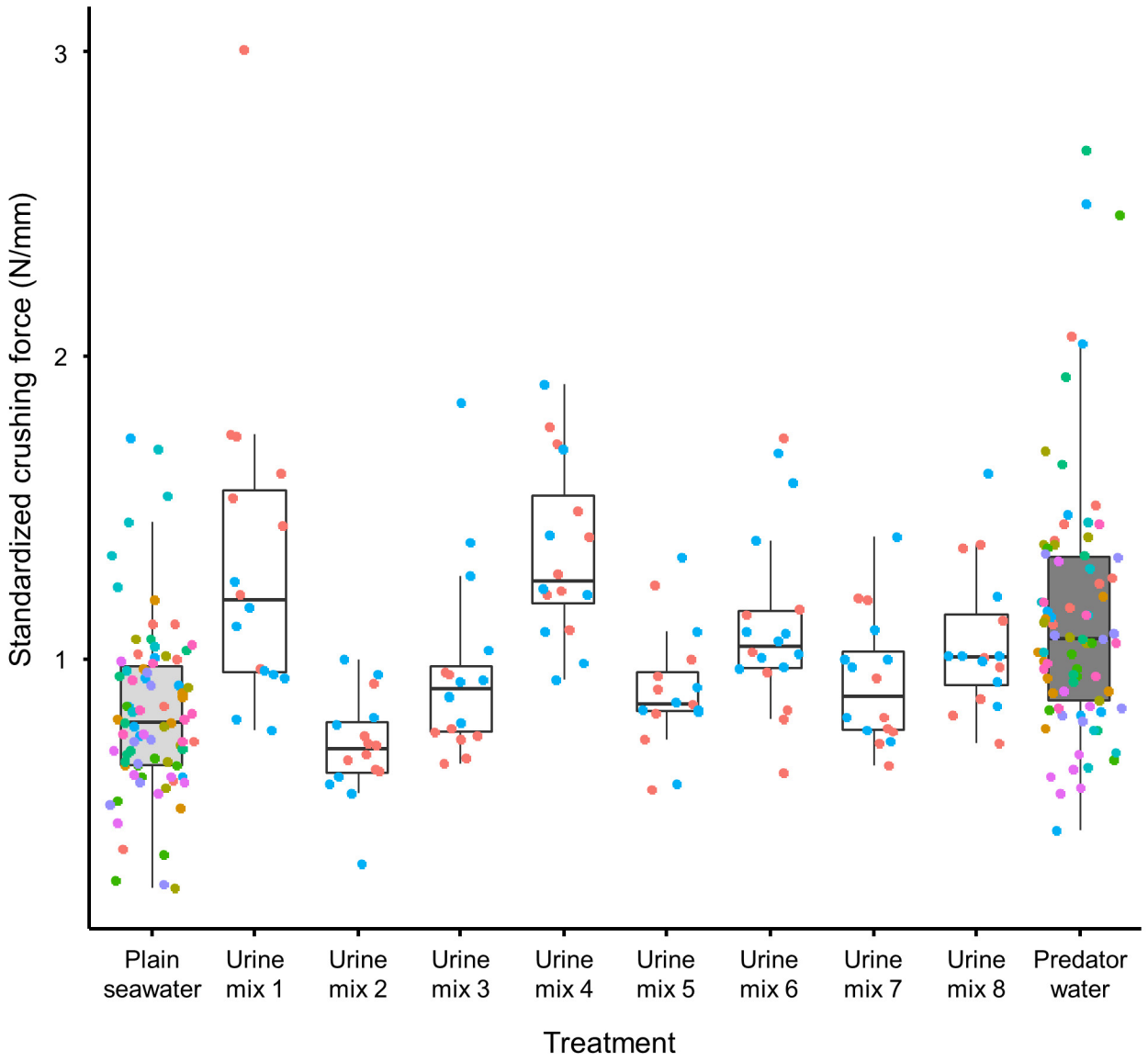

FIGURE 1. *Variation in individual oyster standardized shell strength (N/mm) as result of predator cue treatment. Oysters were either exposed to a control of plain seawater, a positive control of water collected from a tank with actively feeding blue crab predators, or one of 8 different mixtures of blue crab urine. Boxes represent the median and interquartile range (IQR) while whiskers denote 1.5x the IQR. Dots depict individual oyster shell strengths (n = 16 for each urine mixture, n = 80 each for seawater control and predator water treatments). Dot color represents different holding tanks within a treatment (2 tanks for urine treatments, 16 tanks total; 10 tanks each for control and blue crab predator water treatments, 20 tanks total). Jittering of dots within treatments was performed to avoid overlap.*

for 14.6% of the variation in shell strength ($\eta^2 = 0.146$), as some mixtures showed greater variation from being held in separate tanks (e.g., urine mixes 1 and 3) than in others (e.g., urine mixes 5 and 8).

Oysters exhibit substantial variation in growth among individuals (Singh and Zouros 1978, Varney et al. 2009) and are known to react to blue crab urine by developing heavier, stronger shells (Roney et al. 2023). When presented with equal volumes of blue crab urine from different groups of blue crab predators, we saw markedly more variation among oysters exposed to different urine mixtures than among oysters within the same mixture. Although 52.2% of the variation was unexplained, much of this can be attributed to differences in growth among individual oysters. However, variation among urine mixtures indicates that the cue quality (composition of cue) was important in modulating oyster responses to predation risk, consistent with prior studies (Okuyama 2008, Belgrad and Griffen 2016). This finding also indicates that variability in similar assays may be explained by variation in the cues released by individual predators (Scherer et al. 2016, Scherer and Smee 2017). Furthermore, individual prey organisms may respond differently to the same stimuli (Belgrad and Griffen 2016). Finally, variation among individuals of the same species can also be caused by many factors such as prey condition, competition intensity, and resource quality (Harvell 1990, Croy and Hughes 1991, Relyea 2002).

Despite decades of research on phenotypic plasticity and predatory interactions in general, experiments on cue quality are curtailed because the chemical composition of exudates modulating these interactions remains largely unknown, particularly in aquatic environments (Poulin et al. 2018). Once signals are released, they dilute quickly and are impossible to distinguish from ambient organic molecules. Lack of chemical identification limits understanding of both risk evaluation and response but also the evolution of fear responses in prey. Extracting urine for bioassays as performed here allows for chemical analyses to determine the cue components to which oysters or other prey react (Poulin et al. 2018, Roney et al. 2023).

Oysters provide many economic benefits and are a key component of the culture of many coastal communities. Ecologically, oysters provide a plethora of benefits including water filtration, shoreline protection, serving as a carbon sink, and creating habitat for many other species that ultimately improves fishing. Unfortunately, oyster reefs are one of the most degraded habitats in the world (Beck et al. 2011). Numerous restoration efforts have attempted to rebuild oyster populations, and these efforts most often involve planting cultch (oyster shells or other hard substrates) to provide settlement surfaces for oysters to attach and subsequently grow into a new reef (La Peyre et al. 2014).

In some areas, oyster populations are so low that larval supply is limiting, and cultch planting is not effective. Instead, using remote setting is employed where oyster larvae are settled onto oyster shells in a nursery and grown for 2—4 weeks before being transported into the field. Although this technique has been successful in some areas, in the Gulf of Mexico, it is plagued by predation.

Our findings, along with recent research, suggests that oyster phenotypic plasticity can be manipulated in the nursery to encourage oysters to develop stronger shells, providing protection from predators, which increases their survival in the field (Belgrad et al. 2021, 2023). Phenotypic plasticity significantly improves survival, and greater understanding of the mechanisms driving this plasticity will open new avenues for manipulating species.

By adding chemical cues from oyster predators such as blue crabs or oyster drills into the nursery where juvenile oysters are growing, shells can be induced to toughen them against predation. We have shown that predator urine alone induces oyster shell changes, and it is possible that the specific chemical constituents of the urine that cause oysters to grow thicker shells can be identified. Once these molecules are known, artificial fear cues may be created and added to oyster nurseries to stimulate oyster shell growth and improve restoration outcomes. Our results, along with Roney et al. (2023), suggest this achievement is within reach and could provide a logistically feasible strategy to improve remote setting and help rebuild oyster populations by "scaring them strong."

Acknowledgements

Funding for this project was provided by the Dauphin Island REU program NSF Award #1838618. Members of the Smee Lab provided essential help for animal collection and care as well as support and encouragement during the experiments. Emily Combs, a DISL REU student, was especially helpful with animal care that made these experiments possible.

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