Vol. 574: 13–27, 2017
https://doi.org/10.3354/meps12207

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published July 4

Examining inducible defenses to novel predators across native and introduced populations

B. C. Turner*, C. E. de Rivera, M. E. Hepner

Department of Environmental Science and Management, Portland State University, Portland, Oregon 97201-0751, USA

ABSTRACT: An invading species should be more likely to establish if it can successfully identify and defend against predators in the recipient range, such as through the expression of inducible defenses. Inducible defenses are behavioral or physiological changes induced by the presence of the predator or related cues that reduce an organism's susceptibility to predation. The few studies that have examined inducible defenses in the context of biological invasions used introduced species that invaded many generations before rather than newly introduced prey naïve to predator cues. Therefore, we examined if inducible defenses may have benefited the purple varnish clam *Nuttallia obscurata* during the early stage of its introduction from Asia to the Northeast Pacific. Once we established that non-native *N. obscurata* increased burrowing depth in the presence of invaded-range predators, in particular Dungeness crabs *Metacarcinus magister*, we compared burrowing depth in aquaria of tethered *N. obscurata* collected from 2 introduced populations, viz. Oregon, USA, and British Columbia, Canada, versus those collected from a native population in Miyagi Prefecture, Japan. The physical presence of *M. magister* caused *N. obscurata* from the USA and Canada to increase their burrowing depth, while specimens from Japan did not. Whereas these findings suggest that inducible defenses may contribute to the continued success of *N. obscurata* in the Northeast Pacific, they do not support the idea that *N. obscurata* expressed inducible defenses in the early stage of invasion. Nonetheless, this mechanism may be important for the initial establishment of some species and population growth and expansion for other species once they learn the cues of local predators.

KEY WORDS: Depth refuge · Behavioral defense · Clan burrowing depth · *Nuttallia obscurata* · Non-native prey · Biological invasion · Predator avoidance · Risk cues

INTRODUCTION

A species invading a new location should be more likely to establish if it can successfully defend against novel predators in the recipient range, such as through the expression of inducible defenses. Inducible defenses are a form of phenotypic plasticity, which is the ability of a genotype to alter the expression of an organism's characteristics (e.g. morphology, behavior) in response to environmental cues (DeWitt et al. 1998, Dzialowski et al. 2003). Inducible defenses are triggered after exposure to risk cues, generally tactile interactions with predators, chemical signals from predators (kairomones), or chemical signals from injured conspecifics (alarm cues), and cause an organism to undergo morphological or behavioral changes that reduce susceptibility to predation (Trussell & Nicklin 2002, Bourdeau 2010). While it might be expected that organisms would be unable to identify and defend against novel predators with which they do not have a shared evolutionary history (akin to the naïve prey hypothesis; Sih et al. 2010, Carthey & Banks 2014, Berthon 2015), several studies have shown that some non-native organisms express inducible defenses in the presence of predators in their new

*Corresponding author: bcturner@pdx.edu

recipient range (Dzialowski et al. 2003, Flueck 2004, Grason & Miner 2012, Naddafi & Rudstam 2013, Cisterne et al. 2014, Castorani & Hovel 2016), which could have aided in their establishment.

Although non-native organisms will have no previous interactions with novel predators in their new recipient range, they may still be able to identify and defend against these predators. The non-native organism may respond to a novel predator if it is taxonomically similar to a known predator species. For example, the freshwater snail *Physella virgata* will express an inducible defense (development of rotund shell) in the presence of a range of sunfish species, even species that are non-molluscivorous (Langerhans & DeWitt 2002). Alternatively, non-native organisms may use general cues that happen to include the novel predator, such as the presence of any large novel organism or object (Dill 1974, Sih 1986).

Even when species do not immediately recognize and respond to novel predators, selective pressures from predators have resulted in the expression of anti-predator behaviors in prey with which they have coexisted for only a few generations (Thompson 1998, Yoshida et al. 2003, Nunes et al. 2014, Berthon 2015). An example of this phenomenon is the difference in the response of native blue mussels *Mytilus edulis* from habitats that had been invaded by the Asian shore crab *Hemigrapsus sanguineus* approximately 15 yr prior (7–15 generations of *M. edulis* prior) compared to populations where *H. sanguineus* was a newly arrived, novel predator. *M. edulis* that had coexisted with *H. sanguineus* increased their shell thickness in response to the crab's presence, whereas completely naïve mussels from other, uninvaded populations did not respond to its presence (Freeman & Byers 2006, Zagata et al. 2008). Although this example explored the response of native prey to a non-native predator, rather than the established predator and non-native prey dynamic of interest in the present study, it highlights the potential for relatively rapid change in the response of naïve prey to the presence of novel predators after a few generations.

Additionally, novel predators may not immediately recognize newly introduced non-native species as potential prey and there may be a delay in the initiation of predation (Sih et al. 2010). After non-native prey have coexisted with predators in the recipient range for multiple generations, examinations of inducible defenses in response to these predators may not be representative of what occurred during their initial introduction. Temporal separation between introduction and investigation complicates efforts to examine the relationship between inducible defenses and establishment success as the prey species used in inducible defense research involving non-native prey were introduced approximately 6 to 110 yr before each of these studies took place (see Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m574p013_supp.pdf).

Examining whether the expression of inducible defenses by an introduced species may increase its success during the difficult early stages of invasion would require either studying the invasion as it occurs or bringing specimens from the introduced species' native range in contact with recipient range predators. The lag time between introduction and detection of most unintentionally introduced species reduces the feasibility of the first approach (Crooks 2005). However, one could experimentally test for the potential immediate expression of inducible defenses given exposure to novel predators if naïve individuals were collected from the donor population. The expression of inducible defenses, or lack thereof, by specimens of an introduced species taken directly from their own native range when exposed to novel risk cues (e.g. kairomones from recipient range predators), should be similar to those from the species when it first invaded the recipient range.

We examined inducible defenses, including the potential for their expression by naïve prey newly introduced to a region, in the bivalve *Nuttallia obscurata* (Reeve, 1857), the purple varnish clam. The class Bivalvia contains many species with expansive non-native ranges, and a large number of bivalves are known to express behavioral and morphological inducible defenses (summarized by Castorani & Hovel 2016). Larval *N. obscurata* are thought to have been transported via ballast water to the Northeast Pacific from their native range, which includes Japan, China, and Korea (Forsyth 1993, Coan et al. 2000). This non-native bivalve was discovered in British Columbia, Canada, in 1991 (Forsyth 1993) and has since expanded its range south into Washington and Oregon, USA (Byers 2002). Crabs common to the Northeast Pacific are known to readily consume *N. obscurata* and play a strong role in limiting their intertidal distribution (Byers 2002, Dudas et al. 2005). Therefore, we hypothesized that established clam populations would recognize and respond to cues from Pacific Northeast crab predators. Although there was no investigation into the expression of inducible defenses by *N. obscurata* prior to this study, a similarly thin-shelled burrowing clam, *Mya arenaria*, increased its burrowing depth and consequently its survival in the presence of crab predators (Whitlow et

al. 2003, Whitlow 2009). Given the similarities between *N. obscurata* and *M. arenaria*, we hypothesized that *N. obscurata* would also increase its burrowing depth and correspondingly its chances of survival in the presence of predatory crabs.

The goal of this research was to determine if inducible defenses could be expressed and decrease predation at the start of an invasion, rather than only after several generations. Specifically, we set out to determine if the expression of inducible defenses, in the form of increasing burrowing depth, could have contributed to the initial survival of the first generation of *N. obscurata* in the Northeast Pacific. We tested this question using specimens from the native range of *N. obscurata* as surrogates for the original colonizers of the Northeast Pacific. We examined the inducible defenses of *N. obscurata* in 4 stages. First, we examined if they did indeed demonstrate inducible defenses by increasing their burrowing depth. Second, we examined which cues induced the defense. Third, we compared the responses of clams collected from their native range (Japan) and from 2 non-native populations in the Northeast Pacific (USA and Canada). Finally, we examined whether deeper burrowing conferred a survival advantage.

By comparing the responses of clams from these native and introduced populations, we can determine if the expression of inducible defenses is reduced if *N. obscurata* is naïve to Northeast Pacific crab predator cues (USA vs. Japan) or varies across populations with previous exposure to the predators (USA vs. Canada). Given the many cases of bivalve inducible defense in multiple taxa and geographic regions (summarized by Castorani & Hovel 2016), we predicted all populations would burrow deeper given alarm (conspecific) or tactile stimulus cues. We expected that clams that had co-existed with the predators for generations (USA and Canada) might also respond to predator kairomones. Deeper burrowing by *N. obscurata* naïve to the predators (Japan) would support the idea that inducible defenses could have increased the survival of the species during the earliest stages of invasion in the Northeast Pacific, while deeper burrowing by recipient range clams would suggest such defenses contribute to the long-term success in the Northeast Pacific. Failure of *N. obscurata* from Japan to burrow deeper when exposed to risk cues would suggest that while inducible defenses may contribute to the species' long-term success in the Northeast Pacific, they likely did recognize and respond to risk cues during the earliest stages of invasion.

MATERIALS AND METHODS

Collection and care of research specimens

Specimens of *Nuttallia obscurata* (25–50 mm in length) were collected by hand from 3 locations: Sand Lake estuary, Oregon, USA (45° 16′ 26.7″ N, 123° 57′ 20.1″ W) from August 2013 to April 2016; Departure Bay, British Columbia, Canada (49° 12′ 20.0″ N, 123° 58′ 05.7″ W) in May 2015; and Hiroura Bay, Natori, Miyagi Prefecture, Japan (38° 09′ 58.1″ N, 140° 56′ 57.5″ E) in May 2015. Collected specimens were placed in small plastic containers (~500 ml) along with damp sediment collected on-site. They were transported on ice to Portland State University in Portland, Oregon, where all organisms were housed and experiments were conducted. Handling of all clams was standardized to approximate the expected collection and transport of specimens from Japan. Burrowing depth and body length were recorded in the field for clams collected at Sand Lake in August 2013 to examine any potential length–depth relationship in the field.

Three species of crabs were collected to act as potential predators of *N. obscurata* in the experiments: *Metacarcinus magister* (Dana, 1852), *Cancer productus* (Randall, 1839) and *Carcinus maenas* (Linnaeus, 1758). Both *M. magister* and *Cancer productus* are native to the Northeast Pacific and their habitat overlaps that of *N. obscurata* (Dudas et al. 2005). Although *Carcinus maenas* is native to Europe and Northern Africa, its introduced range in the Northeast Pacific occasionally overlaps with that of *N. obscurata* (Klassen & Locke 2007). All 3 crab species consume *N. obscurata* in laboratory settings, and predation by *Cancer productus* was observed in the wild (Byers 2002, Dudas et al. 2005, Curtis et al. 2012). Adult *M. magister* (95–125 mm carapace width [CW]; male and female) and adult *Cancer productus* (100–135 mm CW; male and female) were collected at Whiskey Creek in Netarts Bay, Oregon (45° 24′ 50.6″ N, 123° 56′ 05.3″ W), while adult *Carcinus maenas* (74–81 mm CW; male) were collected at Bodega Harbor, California (38° 19′ 25.00″ N, 123° 02′ 52.00″ W).

All collected specimens were housed in tanks filled with artificial seawater brought to a salinity of 35 psu with Instant Ocean® Sea Salt (Spectrum Brands) and kept at 13°C on recirculating water tables. *N. obscurata* were separated by origin and housed in 76 l tanks containing ~10 cm of fine to medium grain sand collected from Sand Lake, Ore-

gon. Before use in the lab or in our experiments, the sediment collected from Sand Lake was completely dried, thoroughly rinsed with fresh water, and allowed to dry again for 24 h. Shellfish Diet® 1800 (hereafter shellfish food; Reed Mariculture) was added to the table weekly to provide food for *N. obscurata*. Crabs were housed on a separate, independent water table, divided into 76 l tanks by species. Crabs were fed squid 1 or 2 times wk^{-1} and, as available, *N. obscurata* that had been used in experimental trials. For Expts 1 to 3 below, crabs selected for use in an upcoming experimental trial were placed in a separate tank and fed squid to excess 24 h prior to being added to experimental enclosures. Crab predators are often starved for 24 h to standardize hunger levels for experiments examining predation (Blundon & Kennedy 1982, Eggleston 1990, Dittel et al. 1995, Griffen & Williamson 2008, Curtis et al. 2012). However, since we aimed to standardize hunger while minimizing predation on tethered *N. obscurata*, we instead fed the crabs to satiation in the 24 h before the experiment. Water in each table was completely replaced between experiments.

Experimental enclosures

All experiments were conducted in 38 l tanks filled to 20 cm depth with sediment collected from Sand Lake, a depth cited as the species' most commonly observed maximum burrowing depth (Byers 2002) and one we did not find surpassed in the field. We added aerated artificial saltwater with salinity at 35 psu to each tank, which was maintained at 10°C using a 454 l h^{-1} pump (Ponicspumps®, Faster Harvest) and a chiller (Sea Line Platinum Series Chiller Model SL-150A, Sea Line). Salinity, temperature, and sediment composition reflected conditions where *N. obscurata* is found in the Northeast Pacific, specifically those at Sand Lake, the local collection site. Each tank was allowed to sit for 24 h prior to the addition of *N. obscurata* and was covered with a light diffuser grid with a plastic cage (16 cm length × 12.5 cm width × 6 cm depth) at its center. This cage allowed us to place crabs in the water column of a given enclosure, but prevented the crab from touching the clams or the sediment surface.

Between trials, the sediment was collected from each tank, mixed, rinsed with fresh water, and then air-dried for a minimum of 24 h. Tanks, chillers, and aerator stones were thoroughly rinsed with fresh water after each trial.

Clam tethering

For all experiments, *N. obscurata* were divided into similarly sized sets of 6 clams (1 set $tank^{-1}$). All clams were 25–50 mm in length (mean ± SE across all experiments; 37.0 ± 0.5 mm; see Table S3 in Supplement 1 for average sizes for each experiment). Each clam was attached to a garden staple by gluing a small loop (~1 cm in diameter) at the end of a 20 cm tether of 0.28 mm monofilament line to the clam's left valve using Super Glue Gel (Loctite®), allowing it to burrow to its natural maximum depth. Clams were placed in a 38 l tank umbo up with their garden staple planted beside them. Tethers can be used to determine the burrowing depth of clams while still submerged (as in Griffiths & Richardson 2006, Flynn & Smee 2010) and do not appear to impact the burrowing abilities of clams (Auffrey et al. 2004). Burrowing depth of each clam was determined by removing the garden staple, pulling the line gently until it met resistance, and then measuring the length of the exposed line and subtracting it from the pre-determined 20 cm of line length. By gently pulling on the line until taught, which allowed it to cut through the sand if needed, we removed the slack and ensured we were measuring directly above the clam, as there was occasionally horizontal movement by clams.

Expt 1: Do *N. obscurata* collected in Oregon express inducible defenses?

From August to December 2013, we tested if *N. obscurata* from Sand Lake would burrow deeper in the presence of cues from local crab predators and damaged conspecifics together. We used 4 treatments: (1) control (no risk cues); (2) 1 *Cancer productus* plus 4 crushed *N. obscurata*; (3) 1 *M. magister* plus 4 crushed *N. obscurata*; or (4) 1 *Carcinus maenas* plus 4 crushed *N. obscurata*. All crabs were allowed to move freely for the duration of their time in the enclosure. *N. obscurata* were crushed between thumb and forefinger above the enclosure into which they were to be added, and then placed on its sediment surface. Shellfish food was also added to each enclosure, including the control, to provide food for *N. obscurata* during the experiment. The experiment was run over 72 h time blocks (n = 8), with 1 replicate of each treatment per time. Prior to each time block, 6 tethered clams were placed in each tank and allowed 24 h to burrow and acclimatize (Dudas et al. 2005). At the end of each time block, all crabs and crushed *N. obscurata* were removed, water was drained to sand

level, and each clam's burrowing depth (nearest mm) and status (alive or eaten) was recorded. A clam was considered eaten if it was dead with extensive damage to its shell. Across all experiments, we found no evidence of clam mortality or shell damage that was not the result of a clam being eaten.

For all experiments, specimens (crabs and clams) were not reused. Surviving *N. obscurata* were fed to crabs in storage tanks, while crabs were either returned to their collection site (*M. magister* and *Cancer productus*) or euthanized via freezing (*Carcinus maenas*).

Expt 2: What risk cues trigger inducible defenses from Northeast Pacific *N. obscurata*?

While Expt 1 allowed us to examine whether *N. obscurata* would burrow deeper in the presence of a combination of risk cues, it did not allow us to determine the effect of specific cues (conspecific alarm cues, predator kairomones, and predator physical presence). From June to August 2014, we tested which of the different cues from Expt 1 caused *N. obscurata* from an introduced population at Sand Lake to burrow deeper than those in control treatments. We used the same general methods employed in Expt 1, but using 4 different cue treatments: (1) control (no risk cues); (2) crushed conspecifics only (4 crushed *N. obscurata*); (3) 1 caged *M. magister*; or (4) 1 roaming *M. magister*. Tanks containing a caged *M. magister* allowed *N. obscurata* to detect chemical signals from, but not physically interact with, the predator. In contrast, clams in tanks containing an uncaged roaming crab could detect chemical signals and physically interact with the predator. Shellfish food (for clams) and 25 mm^2 of squid meat (alternate food source for crabs) were added to each tank, including control tanks. The experiment was run over 72 h time blocks (n = 10), with 1 replicate of each treatment per time block.

Expt 3: Do individuals from the donor range burrow deeper in response to recipient-range predators?

From July to October 2015, we tested if naïve specimens of *N. obscurata* from their native range (Japan) responded to the presence of local, Northeast Pacific crab predators in a similar fashion to the established non-native populations (USA, Canada) of this species in the Northeast Pacific, and if the response of these non-native populations varied with geographic origin (USA vs. Canada). We used the same methods employed in Expts 1 and 2, with 1 major exception: cues were left in the tanks for 48 h rather than 72 h. The cue exposure time was reduced by 24 h for this experiment after observations suggested there was no consistent difference in clam depth after 48 or 72 h of exposure. Nine treatments were used in this experiment, representing all possible combinations of cue treatments (control, caged *M. magister*, and roaming *M. magister*) and geographic origin of *N. obscurata* (USA, Canada, and Japan). The experiment was run over 10 time blocks, with 1 replicate of each treatment per time block.

Expt 4: Do observed changes in burrowing depths reduce predation?

From August to September 2015, we conducted an experiment to determine if the observed differences in burrowing depth increased survival of *N. obscurata* in the presence of a Northeast Pacific crab predator. Sets of 6 clams from Oregon were tethered and placed into separate tanks. The clams were tethered in a manner similar to Expts 1–3, but this time the purpose of the garden staple was not to measure burrowing depth, but to keep the clams at a pre-determined depth while allowing them to re-orient themselves. To reduce the potential for *N. obscurata* to move vertically in the sediment, the tether length was reduced to 2 cm, the minimum length with which we were still able to attach them to the staple and provide them the ability to re-orient body angle as needed.

Each tank was randomly assigned 1 of 2 sediment depths representing the mean burrowing depth found from the control in Expt 1, viz. 10 cm deep (hereafter 'shallow'), and from the *M. magister* treatment in Expt 1, viz. 13 cm (hereafter 'deep'). Tanks either had a predator, *M. magister*, or served as a predator-free control treatment. The experiment was run across 7 time blocks, with 3, or once 4, tanks $block^{-1}$. One tank $block^{-1}$ was randomly assigned to be predator–deep and another to be predator–shallow sediment depth. The third, and once fourth, tank was randomly assigned to 1 of the treatments: predator–deep, predator–shallow, control–deep, or control–shallow. Across all time blocks, we used a total of 18 predator (9 deep, 9 shallow) and 4 control (no predator; 2 deep, 2 shallow) treatments.

A base layer of sediment (2 cm) was added to each tank to prevent clams from being flush against the

bottom of the tank. The 6 clams tank^{-1} were placed on the 2 cm of sediment with their garden staple laid down horizontally next to them. A wooden dowel was temporarily placed vertically next to the posterior of each clam to create a sand-free 'pocket' allowing for the extension of the siphons to the surface. The creation of a sand-free 'pocket' was implemented so the clams could access the surface after we added sand above them; otherwise, their access to the surface would be much more limited by our sand addition than if they were allowed to burrow naturally. Finally, sediment was added to bury the clams to the target depth for each replicate (10 cm of sediment added for shallow, 13 cm for deep), the dowels were removed, and the clams were allowed to acclimatize for 24 h.

Unlike the previous experiments in which we attempted to completely satiate crabs prior to their addition to the enclosures in order to reduce predation, the *M. magister* used in this experiment were fed to satiation and then starved for 24 h prior to their addition to the predator treatments to standardize hunger levels and to increase the likelihood that *M. magister* would actively forage for the buried clams.

After the clam acclimatization and crab starvation periods, crabs were added to the predator treatments. Clam mortality was assessed 6 and 24 h after crabs were added. At 6 h, clams were recorded as consumed if the staple had been pulled up and a broken shell was visible. After 24 h, crabs and then clams were removed.

Testing for other possible influences on experimental outcomes

Multiple factors can influence the burrowing depth of marine bivalves. Some soft-sediment bivalves display a strong correlation between body size and burrowing depth (Zaklan & Ydenberg 1997). Factors associated with location also could affect burrow depths. All tanks were in a single location within each experiment; however, due to varying space availability, we used different locations for the different experiments. For Expts 1 and 3, tanks were located on shelves in the wet lab; tanks used during Expt 2 were housed on, but not hydrologically linked to, operational water tables. From March to April 2016, we ran an experiment to determine if the location of tanks affected burrowing depth of *N. obscurata* collected in Oregon. The same general methods as the controls of Expts 1–3 were used. Two tanks were housed at each location (shelf or water table) and clams were allowed 3–4 d to burrow, after which time burrowing depth was measured. The experiment was run over 9 time blocks for a total of 18 tanks at each location.

Statistical analyses

Using R statistical software version 3.1.0 (R Development Core Team 2014), we compared the burrowing depths of clams exposed to risk cue treatments to the depth of *N. obscurata* from the control treatment for Expts 1–3. After verifying that the data approximated the assumptions of a normal distribution and homoscedasticity of residuals, we created generalized linear mixed-effects models (GLMM) for each experiment using the package 'lme4' (Bates et al. 2015). We originally considered using analysis of variance (ANOVA) or analysis of covariance (ANCOVA) to analyze the experimental results but ultimately concluded a GLMM was more appropriate. For a breakdown of this rationale, see Supplement 2 at www.int-res.com/articles/suppl/m574p013_supp.pdf. A GLMM was used by Castorani & Hovel (2016) to examine the response of the introduced Asian nest mussel *Arcuatula senhousia* in the presence of kairomones and alarm cues. When using categorical predictor variables with this approach, each group within a category is compared against a selected reference group. For Expts 1 and 2, the reference group was the control treatment. For Expt 3, the reference group for all 1-way comparisons was the control treatment for US *N. obscurata*; the US control was also part of the comparison for the interaction term (see Table 3). The interaction term for Expt 3 tests for differences between treatments across geographic origin. For example, the model will test if the differences in *N. obscurata* burrowing depth for control versus roaming *M. magister* changes when using US *N. obscurata* compared to those from Japan (see Table 3). R code and complete model outputs for all GLMMs can be found in Supplement 2.

For Expts 1 and 2, final clam burrowing depth was the response variable and cue treatment was the independent variable. Time block (1–8 and 1–10, for Expts 1 and 2, respectively), Tank (1–4), and clam position (1–6; nested within tank) were included as random factors. For Expt 3, final burrowing depth was the response variable while cue treatment (control, caged *M. magister*, or roaming *M. magister*), geographic origin (USA, Canada, or Japan), and the cue treatment× geographic origin interaction term were fixed effects. Time block (1–10), Tank (1–9) and clam position (1–6;

nested within tank) were included as random factors. Clams housed within a given tank during a given time block cannot be considered independent. Therefore, we included them as a random factor nested within tank so the model would treat clam position (a proxy for the identity of the clam) as a random sample from within the tank. This approach has been used before to deal with the problem of pseudoreplication (Millar & Anderson 2004, Crawley 2012).

For Expt 3, we also performed a post hoc multiple comparison analysis using the R function 'glht()' from the package *multcomp*. This allowed us to compare the differences in burrowing depth across all cue treatment×geographic origin combinations. This allowed for the direct testing of differences in burrowing depth between controls and treatments with *M. magister* (caged or roaming) for Canadian and Japanese clams (these comparisons for US clams were performed as part of the original GLMM).

Clam body size was originally a covariate in the models for Expts 1–3 to determine if clam size influenced depth. However, we never found a significant or strong relationship between clam body size and burrowing depth in any of the models, and scatterplots of depth versus size for each experiment (1–3) using data collected from control treatments did not indicate strong trends (Fig. S1A–C in Supplement 1). The covariate was removed for the final models to improve statistical power, and the results of the models did not change qualitatively as a result of this removal.

While we considered the inclusion of a barrier to separate *N. obscurata* from crabs in the roaming crab treatment, we ultimately decided that the treatment would most closely mimic potential real-world interactions between predators and prey than would be possible using a barrier to separate them. However, this design came at the cost of rendering the clams vulnerable to predation. While alternate food sources were included to reduce predation on the clams, predation on tethered *N. obscurata* occasionally occurred. To eliminate the possibility of predation on any shallow clams making the data on remaining clam depths appear as if clams were burrowing deeper in predator treatments, we excluded from the analysis of Expts 1–3 any replicate in which more than 1 clam was consumed in a given tank. No more than 2 tanks were excluded per treatment per experiment (Table S2 in Supplement 1). Additionally, any clams that failed to burrow within a given tank were excluded from the analysis.

For Expt 4, we compared mortality of *N. obscurata* 6 and 24 h after burial at the 2 experimental depths, shallow and deep. The data from control tanks were also compared with the 2 experimental depths but were used to document the extent of clam mortality independent of crab predation.

Although the statistical analyses found no significant relationship between burrowing depth and size for *N. obscurata*, we also tested whether burrowing depth (mm) was correlated with size (nearest 5.0 mm) of clams *in situ* at Sand Lake using Pearson's correlation (R function 'cor.test'). We included all clams 25–50 mm in length, as this was the size range used in the experiments (Fig. S1D in Supplement 1).

To determine if the location of tanks during Expts 1–3 influenced clam burrowing depth, a GLMM similar to those in Expts 1 and 2 was constructed. Final clam burrowing depth was the response variable, and location in the lab (shelf vs. water table) was the independent variable. Time block (1–9), Tank (1–4), and clam position (1–6; nested within tank) were included as random factors.

RESULTS

Expt 1: Do *Nuttallia obscurata* collected in Oregon express inducible defenses?

N. obscurata collected from Sand Lake burrowed significantly deeper in the presence of *Metacarcinus magister* + crushed *N. obscurata* (Table 1, Fig. 1) and *Carcinus maenas* + crushed *N. obscurata* compared to the control treatments. There was no significant difference between burrowing depth of *N. obscurata* in controls and those exposed to *Cancer productus* + crushed *N. obscurata*.

Table 1. Mean ± SE burrowing depth of *Nuttallia obscurata* and generalized linear mixed effect model estimated model coefficients ± SE, test statistic, and probability for risk cue treatments relative to control treatments in Expt 1 (see 'Materials and methods' for details). All specimens of *N. obscurata* were collected at Sand Lake, Oregon, USA. **Bold** p-values are significant ($p \leq 0.05$)

Variable	N	Depth (cm)	Coefficient	*t*	p
Control (no cues)	41	10.6 ± 0.8	–	–	–
Cancer productus + crushed *N. obscurata*	32	11.6 ± 0.7	−0.06 ± 0.90	−0.07	0.95
Carcinus maenas + crushed *N. obscurata*	26	12.9 ± 0.9	2.23 ± 0.99	2.26	**0.03**
Metacarcinus magister + crushed *N. obscurata*	32	12.7 ± 0.8	2.06 ± 0.92	2.25	**0.03**

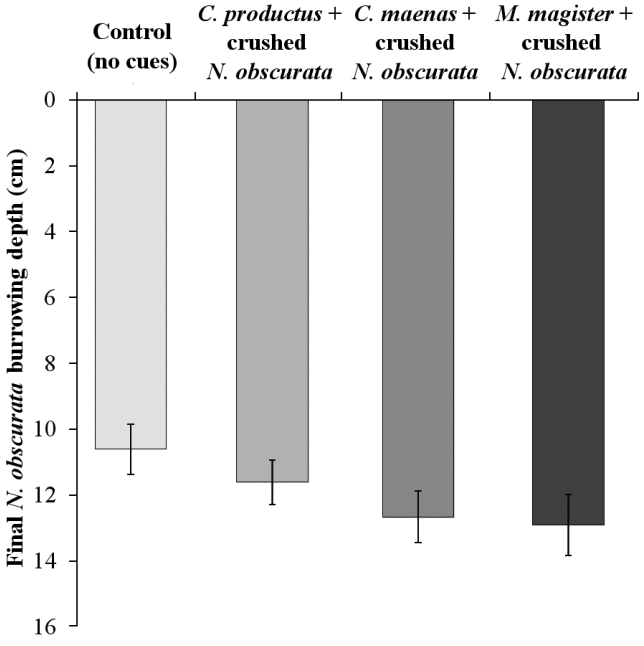

Fig. 1. Burrowing depth (mean ± 1 SE) of *Nuttallia obscurata* collected in Oregon (USA) and exposed to combinations of risk cues in Expt 1. Maximum possible burrowing depth is 20 cm. Clams burrowed significantly deeper in the presence of a free-roaming *Metacarcinus magister* + crushed *N. obscurata* (p = 0.03) and a free-roaming *Carcinus maenas* + crushed *N. obscurata* (p = 0.03) compared to control tanks. There was no difference in burrowing depth of clams in control treatment vs. clams in the presence of a free-roaming *Cancer productus* + crushed *N. obscurata* (p = 0.95)

Expt 2: What risk cues trigger inducible defenses from Northeast Pacific *N. obscurata*?

N. obscurata from Oregon burrowed deeper in the presence of a roaming *M. magister* than in control treatments, but not in the presence of crushed conspecifics or a caged *M. magister* (Table 2, Fig. 2). Burrowing depths of *N. obscurata* in the presence of a caged *M. magister* or crushed conspecifics were not significantly deeper than those in the control treatment.

Expt 3: Do individuals from the donor range burrow deeper in response to recipient-range predators?

N. obscurata from Oregon exposed to roaming *M. magister* burrowed marginally significantly deeper than clams in their control treatments, but clams from Miyagi Prefecture, Japan, did not (Table 3, Fig. 3). Specimens of *N. obscurata* collected in Canada in the presence of a roaming *M. magister* reacted similarly to clams from the USA, burrowing deeper than clams in the Canadian control treatment, although this difference was not statistically significant. In contrast, *N. obscurata* collected from their native range in Japan did not appear to respond to the presence of a roaming *M. magister,* burrowing slightly shallower compared to clams in their control treatment. It should be noted, however, that Japanese *N. obscurata* burrowed significantly deeper than their US counterparts in control treatments (Table 3).

Similar to Expt 2, none of the clams, regardless of source population, demonstrated significant increases in burrowing depth when *M. magister* was caged and clams were only exposed to effluent, suggesting chemical cues on their own did not induce significantly deeper burrowing in this experiment (Table 3).

No significant differences were found in the interactions between geographic origin and cue treatment when comparing the caged *M. magister* treatment with their control (Table 3). Similarly, there was no significant difference when comparing the interaction of the roaming *M. magister* treatment with their control for *N. obscurata* from Canada versus US clams. However, the difference in burrowing depth between control and roaming *M. magister* treatments was significantly greater for US *N. obscurata* compared to their Japanese counterparts.

Expt 4: Do observed changes in burrowing depths reduce predation?

The experiment testing if the response of *N. obscurata* to the presence of *M. magister* increased survival revealed that no *N. obscurata* buried at the deep depth (13 cm) were consumed after 6 or 24 h. In contrast, an average of 0.7 ± 0.4 (mean ± SE) *N. obs-*

Table 2. Mean ± SE burrowing depth of *Nuttallia obscurata* and generalized linear mixed effect model estimated model coefficients ± SE, test statistic, and probability for risk cue treatments involving *Metacarcinus magister* relative to control treatments in Expt 2 (see 'Materials and methods' for details). All specimens of *N. obscurata* were collected at Sand Lake, Oregon, USA. **Bold** p-values are significant (p ≤ 0.05)

Variable	N	Depth (cm)	Coefficient	*t*	p
Control (no cues)	46	8.8 ± 0.8	–	–	–
Crushed *N. obscurata*	50	9.4 ± 0.9	0.52 ± 1.00	0.52	0.61
Caged *M. magister*	57	10.1 ± 0.6	1.31 ± 0.97	1.35	0.18
Roaming *M. magister*	37	10.9 ± 0.7	2.18 ± 1.11	1.97	**0.05**

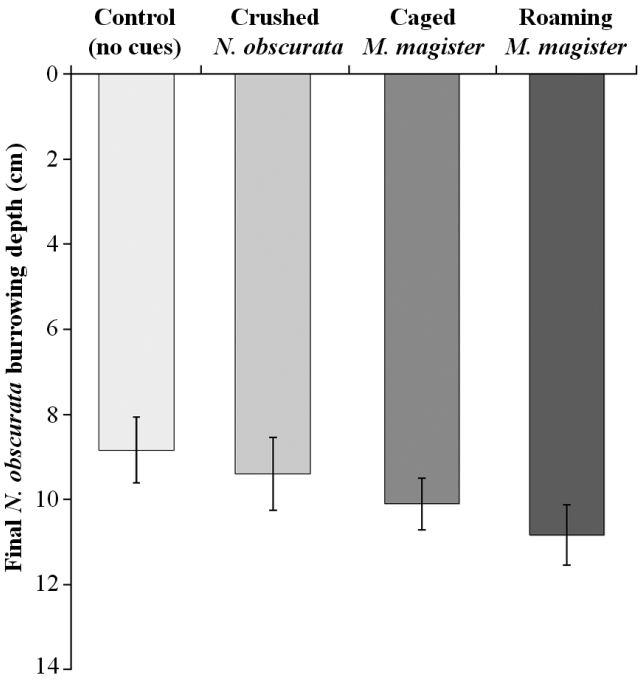

Fig. 2. Burrowing depth (mean ± 1 SE) of *Nuttallia obscurata* collected in Oregon (USA) exposed to individual risk cues in Expt 2. Maximum possible burrowing depth is 20 cm. Clams burrowed significantly deeper in the presence of a free-roaming *Metacarcinus magister* ($p = 0.05$) compared to control tanks. There was no difference in burrowing depth of clams in control treatment vs. clams in the presence of a caged *M. magister* ($p = 0.18$) or crushed *N. obscurata* ($p = 0.61$)

curata (11%) were consumed by *M. magister* per tank after 6 h, while 1.7 ± 0.7 *N. obscurata* (28%) were consumed after 24 h when buried at the shallow depth (10 cm). No mortality occurred in the control treatments. We saw no evidence of vertical migration (up or down) of clams from their original depths. All clams that were consumed were brought to the sediment surface by *M. magister.*

Testing for other possible influences on experimental outcomes

There was no significant positive relationship between body size and burrowing depth for *N. obscurata* collected at Sand Lake for clams with body sizes consistent with those used in the experiments (25–50 cm; $N = 49$, $r = 0.09$, $t = 0.64$, $p = 0.26$; Fig. S1D in Supplement 1).

N. obscurata tended to burrow deeper when tanks were placed on the wet lab shelves, as was done during Expts 1 and 3, compared with tanks placed on the water tables, as was done during Expt 2 (Fig. S2 in Supplement 1). However, the GLMM results showed that this difference was not statistically significant.

DISCUSSION

Nuttallia obscurata from Oregon increased their burrowing depth in the presence of *Metacarcinus magister*, consistent with the use of inducible defenses in the presence of known predators. However, not all populations responded to this recipient range predator similarly and not all potential risk cues used in these experiments elicited this defensive response. Clams collected from introduced populations in the USA and Canada both responded by burrowing deeper in the physical presence of *M. magister*, although the response was not significant among Canadian clams. Specimens from Japan, however, did not burrow deeper as a response to chemical cues or the physical presence of *M. magister.* These findings suggest that while inducible defenses may play a role in the perpetuation and spread of *N. obscurata* in its introduced range, naïve clams, such as individuals newly arrived from their native range, would not recognize recipient range predators and express inducible defenses.

Differences in the response of *N. obscurata* to cues from *M. magister* appeared to be related to the overlap between the location where the clams where collected (USA, Canada, or Japan) and the native range of *M. magister*. Clams from the USA and Canada have coexisted with and have been preyed upon by *M. magister* in the Northeast Pacific for >20 yr, providing an opportunity for these populations to recognize and respond to *M. magister* as a threat. In contrast, clams from Japan have had no previous exposure to *M. magister*, and their lack of response may be due to prey naiveté (akin to the naïve prey hypothesis; Sih et al. 2010, Berthon 2015). It should be noted, however, that responses to predators may vary between populations or even genotypes within populations (Fawcett 1984, Lively et al. 2000, Smee & Weissburg 2008, Large et al. 2011, Bourdeau 2012). Given that we sampled from only 1 population in each geographic range (USA, Canada, Japan), it is possible that the responses we observed in our experiments may vary across other populations within each region.

Contrary to our initial predictions, Japanese clams showed no evidence of inducible defenses, even when exposed to a tactile stimulus from a free-roaming crab. A previous study by Flynn & Smee (2010)

Table 3. Mean ± SE burrowing depth of *Nuttallia obscurata*, generalized linear mixed-effect model (GLMM) estimated model coefficients ± SE and statistical output for risk cue treatments involving *Metacarcinus magister* relative to the US *N. obscurata*, control treatment in Expt 3 (see 'Materials and methods' for details). **Bold** p-values are significant ($p \leq 0.05$)

Variable	N	Depth (cm)	Comparison in GLMM	Coefficient	*t*	p
US *N. obscurata*, control	55	10.5 ± 0.8	–	–	–	–
US *N. obscurata*, caged *M. magister*	53	10.1 ± 0.8	US *N. obscurata*, control	–0.24 ± 0.94	–0.26	0.80
US *N. obscurata*, roaming *M. magister*	41	12.1 ± 0.7	US *N. obscurata*, control	1.92 ± 1.01	1.90	0.06
Canadian *N. obscurata*, control	53	10.5 ± 0.7	US *N. obscurata*, control	0.29 ± 0.93	0.31	0.76
Canadian *N. obscurata*, caged *M. magister*	53	11.3 ± 0.7	Canadian *N. obscurata*, control[a]	0.74 ± 0.94	0.78	0.43
Canadian *N. obscurata*, roaming *M. magister*	49	12.0 ± 0.7	Canadian *N. obscurata*, control[a]	1.40 ± 0.96	1.45	0.15
Japanese *N. obscurata*, control	54	12.5 ± 0.7	US *N. obscurata*, control	2.16 ± 0.93	2.33	**0.02**
Japanese *N. obscurata*, caged *M. magister*	49	11.9 ± 0.7	Japanese *N. obscurata*, control[a]	–0.46 ± 0.96	–0.48	0.63
Japanese *N. obscurata*, roaming *M. magister*	48	11.2 ± 0.6	Japanese *N. obscurata*, control[a]	–1.55 ± 0.97	–1.60	0.11
Interaction term (Canadian *N. obscurata*, control vs. caged *M. magister*)	–	–	US *N. obscurata*, control vs. caged *M. magister*	0.98 ± 1.33	0.74	0.46
Interaction term (Canadian *N. obscurata*, control vs. roaming *M. magister*)	–	–	US *N. obscurata*, control vs. roaming *M. magister*	–0.52 ± 1.39	–0.37	0.71
Interaction term (Japanese *N. obscurata*, control vs. caged *M. magister*)	–	–	US *N. obscurata*, control vs. caged *M. magister*	–0.22 ± 1.34	–0.16	0.87
Interaction term (Japanese *N. obscurata*, control vs. roaming *M. magister*)	–	–	US *N. obscurata*, control vs. *M. magister*	–3.46 ± 1.39	–2.48	**0.01**

[a]Coefficients ± SE and statistical output were determined via post hoc multiple comparison analysis

found that tactile cues in the absence of predator kairomones caused the soft-shell clam *Mya arenaria* to increase its burrowing depth. Hence, tactile cues from any predator, familiar or novel, could elicit a response from prey. However, the stimulus applied by Flynn & Smee (2010) to *M. arenaria* (directly prodding each clam's siphon once daily) likely was more intense than that experienced by clams in our study.

The response of *N. obscurata* from US and Canadian populations to the presence of *M. magister* appears to confer increased protection from predation. All specimens buried at 13 cm (deep treatment) survived the predation trials, while some clams were consumed by *M. magister* when buried at 10 cm (shallow treatment). While the differences in burrowing depth resulting from the presence of *M. magister* may seem insufficient to provide increased protection for *N. obscurata*, it not only increased survival in our lab experiment but is in line with the findings of similar studies. Flynn & Smee (2010) observed that a 1.5 cm (15%) increase in burrowing depth by *M. arenaria* exposed to the crab *Carcinus maenas* resulted in a 38% increase in clam survival. Additionally, Byers (2002) found a significant reduction in the mortality of *N. obscurata* with a ~3 cm increase in burrowing depth, although these were for clams at shallower depths than in our study or what we observed in the field. Given that *M. magister* has been observed digging as deep as 30 cm to collect bivalve prey, even deep-burrowing *N. obscurata* would still be vulnerable to some predation, albeit to a lesser degree (Auster & Crockett 1984).

Although Japanese clams did not burrow deeper in the presence of *M. magister*, they did burrow significantly deeper in controls than clams from the USA or Canada. One possible explanation for the relatively consistent, deep burrowing of Japanese specimens is that they may have been subject to higher predator encounter

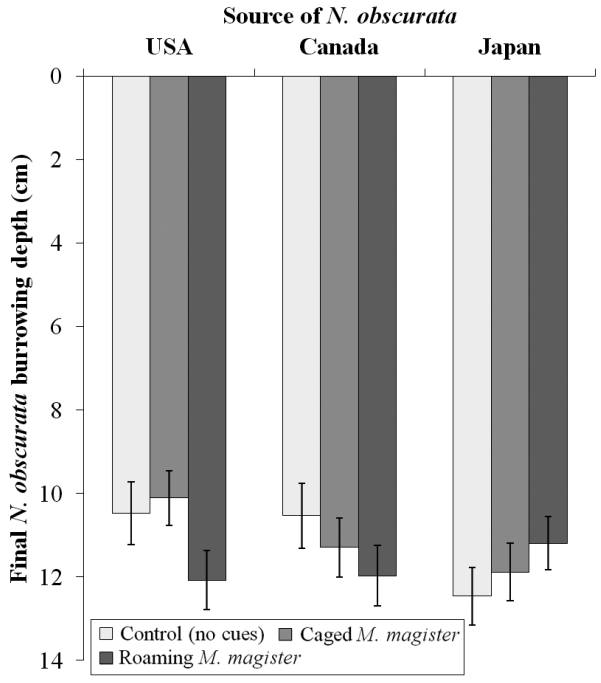

Fig. 3. Burrowing depth (mean ± 1 SE) of *Nuttallia obscurata* from 2 regions it has invaded (USA and Canada) and 1 native region (Japan) exposed to individual risk cues in Expt 3. Maximum possible burrowing depth is 20 cm. There was no significant difference in burrowing depth for clams in control tanks from Canada (p = 0.76) relative to those collected in the USA, although Japanese clams burrowed significantly deeper (p = 0.02). USA clams burrowed marginally deeper in the presence of a roaming *Metacarcinus magister* (p = 0.06) than those in control treatments but not in the presence of a caged crab (p = 0.80). No significant differences were found in the interaction between geographic origin and cue treatment when comparing the caged *M. magister* treatment with the control (USA vs. Canada, p = 0.46; USA vs. Japan, p = 0.71) There was also no significant interaction for *N. obscurata* from the USA and Canada when comparing the roaming *M. magister* treatment with the control (p = 0.87). However, the difference in burrowing depth between roaming *M. magister* treatments and the control was significantly greater for US *N. obscurata* compared to their Japanese counterparts (p = 0.01)

rates in Japan compared to clams from the Northeast Pacific. Habitats where predator encounter rates are high and consistent are thought to promote the development of ever-present constitutive defenses (Tollrian & Harvell 1998), and habitats with variable encounter rates promote plastic inducible defenses (Lively 1986, Harvell 1990), although this is not always the case (Bourdeau 2011). Either plastic or general constitutive defenses (e.g. depth refuge) may aid invasion into areas with generalist predators such as *M. magister* because both defense strategies should reduce susceptibility to predation (Blundon & Kennedy 1982, Stevens et al. 1982, Zwarts & Wanink 1989, Jensen & Asplen 1998). Alternatively, it may be that *N. obscurata* are not commonly consumed by crabs in the sampled Japanese population, and thus do not burrow deeper after exposure to physical or chemical cues from crabs. However, if predation risk is low, one would expect clams to burrow more shallowly, as growth and feeding rates are higher at shallower burrowing depths (Zaklan & Ydenberg 1997, De Goeij & Luttikhuizen 1998, Edelaar et al. 2003). While information on predation pressure of *N. obscurata* in Japan would aid in the interpretation of our observed responses, we were unable to locate any records of predation on *N. obscurata* by Japanese crab species. Whatever the cause, if the observed burrowing depth of Japanese clams is reflective of the first clams to be introduced to the Northeastern Pacific, then they may have experienced reduced susceptibility to predation without expressing an inducible response.

The intensity and nature of an organism's inducible defenses can vary with predator identity (Kishida & Nishimura 2005, Bourdeau 2009, Freeman et al. 2009, Kishida et al. 2009, Garner & Litvaitis 2013, Naddafi & Rudstam 2013, Castorani & Hovel 2016). Non-native species may respond to novel predators if the response of a non-native species is a generalized response to a broad class of predators (e.g. predatory crustaceans) that includes the novel predator or if the novel predator is evolutionarily similar to predators present in the home range of the non-native species. In our study, clams responded to *M. magister* (Cancridae) and *C. maenas* (Portunidae) but not to *Cancer productus* (Cancridae), which suggests that *N. obscurata* is responding to cues that vary among species rather than solely to cues common to all brachyuran (Infraorder) or cancrid crabs. A similar trend was observed for introduced populations of the Atlantic oyster drill *Urosalpinx cinerea* along the Northeast Pacific coast; work by Grason & Miner (2012) found that *U. cinerea* reduced their feeding rates in the presence of *C. productus*, whereas Kimbro et al. (2009) did not observe a similar response to the closely related *Romaleon antennarium* (formerly *Cancer antennarius*). However, *Carcinus maenas* is in the same family (Portunidae) as a potential (though unconfirmed) crab predator of *N. obscurata*. The ishigani *Charybdis japonica* is a predator of bivalves and other benthic organisms and has been found near Hiroura Bay, though generally in lower tidal zones than *N. obscurata* is found (Jiang et al. 1997, Urabe et al. 2013). It is possible that *N. obscurata* from Oregon responded to

Carcinus maenas primarily because of its similarity to *Charybdis japonica*, although this idea is somewhat contradicted by the fact the clams also responded to the more distantly related *M. magister.* In addition, *Carcinus* sp. has invaded parts of Japan and could have interacted with ancestors of the *N. obscurata* that arrived in Oregon (Geller et al. 1997).

It is also possible that differences in the response of *N. obscurata* across predators may have less to do with their phylogenetic similarity to the predators in the clam's native range, but rather the result of predator foraging behavior. *Cancer productus* has powerful claws and has been observed to select shallower-burrowing but thicker-shelled clams even when thinner-shelled but deeper-burrowing clams were available (Yamada & Boulding 1998, Dudas et al. 2005). In contrast, *M. magister* and *Carcinus maenas* have weaker claws and will preferentially choose thinner-shelled clams, even when this requires more active foraging (Hunt & Yamada 2003, Dudas et al. 2005, Curtis et al. 2012). The more active foraging of *M. magister* and *C. maenas* may be more likely to disturb siphons of *N. obscurata* compared to *Cancer productus*, causing the clams to burrow deeper in response.

Specimens of *N. obscurata* collected in the USA and Canada increased their burrowing depth when *M. magister* was free to roam throughout the tank. In contrast, clams exposed solely to kairomones from caged *M. magister* or alarm cues from crushed conspecifics appeared to have no measured effect. Alarm cues are considered a general risk cue because they would be present regardless of the predator species involved and therefore response to alarm cues should be more likely to facilitate invasion than cues from specific predator species (Payne et al. 2004, Sih et al. 2010). Previous studies have observed that alarm cues alone can trigger inducible defenses for non-native prey, although they are generally weaker responses than those triggered by predator kairomones (Grason & Miner 2012, Garner & Litvaitis 2013, Castorani & Hovel 2016). Our data presented here suggest that alarm cues alone did not contribute to the inducible burrowing behavior we observed for *N. obscurata*.

Another factor that could have influenced the burrowing depth of clams from Japan is differences in sediment characteristics. Byers (2002) found that the burrowing depth of *N. obscurata* increased in looser, sandier sediment compared with more densely packed, muddier sediment. The sediment used in all of our experiments was collected from the same location within Sand Lake and was handled in the same way to reduce variation in sediment type or compaction. In contrast, conditions between *N. obscurata* collection sites were not identical. Specimens were collected from small areas (<100 m^2) at the same tidal height at each embayment (Sand Lake, Departure Bay, Hiroura Bay) to minimize the impact of habitat variation on the behavior of our specimens. Sediment type was similar but not identical across embayments, with sediment being somewhat finer and denser at Hiroura Bay compared with Sand Lake (Fig. S3 in Supplement 1). However, the burrowing depths of clams during collection were similar between embayments.

The burrowing depths of some species of soft-sediment bivalves have a strong correlation with body size, which could influence the expression of a behavioral inducible defense like those displayed by *N. obscurata* (Zaklan & Ydenberg 1997). However, this size–depth pattern does not hold for all species. For example, *Mercenaria mercenaria* did not display this size–depth relationship (Roberts et al. 1989). There did not appear to be a clear relationship between body size and burrowing depth for *N. obscurata*, described as having a 'shotgun distribution' (Gillespie et al. 1999). Data from the control treatments in our experiments and from the Sand Lake field site found no significant relationship between body size and burrowing depth for the size range used in the 4 experiments.

Other factors aside from sediment characteristics and predator presence have been observed to influence the burrowing depth of soft-sediment bivalves. Environmental stresses will influence burrowing depth, with hypoxia driving bivalves closer to the surface while extreme temperatures and risk of being washed away will drive them deeper (Sutherland 1982, Tallqvist 2001). Burrowing depths will also vary with the tide, with clams generally burrowing shallowest at high tide and deepest at low tide (Roberts et al. 1989). For species like *N. obscurata* that are both suspension and deposit feeders, the feeding method employed should also influence burrowing depth (Zwarts & Wanink 1989). However, given that every effort was taken to ensure conditions (e.g. sediment type, food availability, temperature, salinity) were identical across specimen storage tanks and experimental tanks, these factors likely did not cause any of the observed differences in burrowing depth of *N. obscurata* in our experiments.

Due to logistical constraints, not all conditions were identical across the experiments (although all were within experiments). Location within the laboratory setting did not significantly influence burrowing depth, although there was a weak but consistent pat-

tern based on location. Specimens of *N. obscurata* from Oregon burrowed an average of 2.2 cm and 1.1 cm deeper in Expts 1 & 3, respectively, than clams in Expt 2, which were housed on water tables. The follow-up experiment similarly found that clams housed on shelves burrowed an average of 2 cm deeper than those housed on water tables. This potential location-based variation in burrowing depth across experiments may be due to lighting differences. An inverse relationship between day length and burrowing depth was found for the clam *Macoma balthica* (Reading & McGrorty 1978). Although all the experiments had the same day–night durations, the lighting is brighter at the water table than at the shelves. Each experiment took place in only 1 location (shelf or table), so lighting or other location-specific factors should not have influenced the findings of each given experiment. Moreover, patterns in the response of *N. obscurata* to risk cues were consistent across all experiments. Therefore, we remain confident that the response of clams to risk cues strongly influenced burrowing depth within each experiment.

Similar to tank location, trial duration varied across experiments in our study. Clams used in these experiments had between 72 (Expt 3) and 96 h (Expts 1 & 2) (acclimatization period and experiment duration together) to reach their equilibrium depth. It is possible that the clams did not have sufficient time to burrow in the shorter timeframe. However, burrowing depths achieved under both experimental durations are within ranges we observed in the field and those observed by other researchers (Gillespie et al. 1999, Byers 2002).

For species brought to a new area with suitable abiotic conditions, overcoming biotic resistance from local predators and competitors will facilitate establishment and spread. While we did not find evidence to suggest that the first-generation invaders may have expressed an inducible defense, as mimicked by exposing individuals just arrived from the native range to the recipient predators in tanks, this mechanism may still be important for the initial establishment of some species and for delayed success in other species once they learn the cues of local predators. Many non-native species experience a lag time between introduction and population growth resulting from small population sizes (i.e. Allee effect), and effectively responding to local predators may increase non-native survival and decrease the duration of this lag. Expanding the scope of research to include additional non-native species and offspring of previously naïve prey would allow for a clearer picture of the role of inducible defenses in the invasion process.

Acknowledgements. For their generous hospitality and invaluable help during collection efforts in Japan and Canada, we thank T. Sazuki and R. Harbo. For assistance collecting specimens along the Oregon coast, we thank L. Bliss-Ketchum, M. Bradley, M. Essig, S. Gonsalvez, A. Harwood, E. Kincaid, L. Lehman, G. Lehman, S. Lehman, A. McCandless, W. McClees, J. Miller, B. Mohammad, V. Robertson-Rojas, A. Truitt, A. Turner, C. Turner, and G. Turner. For invaluable help in the lab and the field, we thank R. Mallorson and S. Harger. Financial support was provided by NSF GK12 (0948041) in support of B.C.T., the American Malacological Society, the Bushby Travel and Graduate scholarships, and generous donors to our SciFund Challenge.

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Editorial responsibility: Martin Solan, Southampton, UK

Submitted: November 9, 2016; Accepted: May 23, 2017
Proofs received from author(s): June 24, 2017