Vol. 594: 107–117, 2018
https://doi.org/10.3354/meps12511

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published April 26

Genetic variation in anti-parasite behavior in oysters

Tal Ben-Horin[1,2], Standish K. Allen Jr.[3], Jessica M. Small[3], Dina A. Proestou[1,*]

[1]National Cold Water Marine Aquaculture Center, United States Department of Agriculture Agricultural Research Service, Kingston, RI 02881, USA

[2]Department of Fisheries, Animal and Veterinary Sciences, College of the Environment and Life Sciences, University of Rhode Island, Kingston, RI 02881, USA

[3]Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Sciences, College of William and Mary, Gloucester Point, VA 23062, USA

ABSTRACT: Behavioral avoidance of disease-causing parasites provides a first line of defense against the threat of infection. We report that the suspension-feeding oysters *Crassostrea virginica* respond to the presence of the water-borne parasite *Perkinsus marinus* by increasing the rate of shell closure and decreasing the clearance of suspended particles from the surrounding seawater. The extent of feeding behavior modification varied among selectively bred oyster families, indicating genetic variation in this trait. Oyster families susceptible to *P. marinus* infection were less likely to modify their feeding behavior when this parasite was present, if at all. Characterizing the role of anti-parasite behavior in minimizing *P. marinus* proliferation in oysters and the implications of variability among individuals will refine our understanding of mechanisms of disease resistance in suspension-feeding bivalves.

KEY WORDS: Anti-parasite behavior · Avoidance · Disease · Suspension feeding · Oysters

INTRODUCTION

The abundance and ubiquity of parasitic life forms necessitates the existence of anti-parasite defense mechanisms. Furthermore, genetic variation in defense traits is required to facilitate selection (by either natural or artificial means) of phenotypes with higher relative fitness in the presence of parasites. Numerous strategies involving behavioral, physiological, and molecular aspects of the immune system are employed by hosts to prevent and/or minimize infection. It has been shown that behavioral defenses against parasites can be one of the most effective mechanisms by which hosts protect themselves from disease (Hart 1990, Wisenden et al. 2009, de Roode & Lefèvre 2012). Because behaviors vary considerably (Wilson et al. 1994, Sih et al. 2004, Wolf et al. 2007, Biro & Stamps 2010) and consistently (Wolf & Weissing 2012) within species, it should follow that intraspecific variation in anti-parasite behaviors is the norm, particularly when the degree of parasite exposure and disease risk are unpredictable.

Behavioral defense against parasites requires that hosts detect parasites and decrease or neutralize their negative effects (Wisenden et al. 2009). Several parasite avoidance behaviors used by mammals have been summarized by Hart (1990, 1994), and include self and heterospecific grooming, herding, sexual selection, and selective foraging. Similar anti-parasite behaviors extend to aquatic species. For example, some stickleback species augment shoaling behaviors in the presence of brachyuran parasites, thereby reducing the risk of infection for individuals (i.e. safety in numbers; Poulin & FitzGerald 1989). Female sticklebacks also avoid consorting with males whose colors have been dulled by parasitic ciliates (Milinski

*Corresponding author: dina.proestou@ars.usda.gov

& Bakker 1990). Increased frequency of rapid body movements by chorus frog larvae significantly decreases attachment and migration of trematode cercariae, resulting in lower infection prevalence and intensity (Daly & Johnson 2011). Moreover, Daly & Johnson (2011) found that anti-trematode behaviors in chorus frogs were 25% more effective than innate immune responses in reducing infections.

Behavioral defense against parasites is not limited to vertebrates. Periwinkles detect ectoparasitic trematode cercariae in the mucus trails of conspecifics, and often refuse to graze near and cross mucus trails contaminated with these larval parasites (Davies & Knowles 2001). Despite their abundance and diversity, behavioral avoidance of microparasites (e.g. protists, haplosporidians, bacteria, and viruses) has scarcely been studied in marine invertebrates. Likely the best example involves the olfactory detection of virus-infected conspecific Caribbean spiny lobsters and subsequent change in sheltering behavior by this normally gregarious species. Healthy individuals actively avoid sheltering with the sick and choose no shelter (and increased predation risk) over risk of infection, even when alternative shelters are limited (Behringer et al. 2006, Behringer & Butler 2010). In the case of lobsters, predictive epidemiological models have shown that anti-parasite behaviors that reduce social transmission are the key to preventing disease outbreaks (Butler et al. 2015).

While evidence for the efficacy of behavioral immunity in aquatic species is accumulating, there have been few investigations targeting suspension-feeding, sessile, marine molluscs, which are highly prone to microparasitic infection. This is surprising considering the role host–parasite interactions play in shaping wild and cultured populations of ecologically and economically important shellfish (Coen & Bishop 2015). Since at least the early 1940s, populations of eastern oyster *Crassostrea virginica* along the US Atlantic and Gulf coasts have suffered extensive mortalities from Dermo disease, a chronic condition caused by the protozoan parasite *Perkinsus marinus* (Mackin et al. 1950). The parasite enters the oyster via pallial (i.e. mantle and gill) surfaces during suspension feeding (Perkins 1987). In addition to direct ingestion, recent evidence suggests that oysters preferentially reject *P. marinus*, and infection occurs along the mantle epithelium as rejected parasites contained in pseudofeces await discharge from the pallial cavity (Allam et al. 2013). These observations imply that oysters detect *P. marinus* as a non-nutritive particle and the parasite responds by utilizing an alternative route of infection to overcome this host defense. Behaviors that reduce feeding may therefore be important mechanisms to limit parasite encounter and disease.

Heavy losses from Dermo disease in wild and cultured populations have intensified interest in understanding mechanisms of parasite resistance and developing strategies to minimize the negative impacts of disease. Genetic improvement of cultured oysters over the last 3 decades has resulted in lines with varying levels of Dermo resistance, among other traits (Brown et al. 2005, Dégremont et al. 2015). Here we exploited oysters derived from a family-based selective breeding program to explore whether differences in feeding behavior contribute to variation in parasite defense in bivalve molluscs. Specifically, we asked whether variation in the rate of feeding was associated with variation in the capacity for oyster families to resist *P. marinus* infection. We employed a series of manipulative experiments to: (1) quantify variation in *P. marinus* resistance among families, and (2) examine variation in the effect of *P. marinus* exposure on feeding behavior in oysters.

MATERIALS AND METHODS

Expt 1: genetic variation in *Perkinsus marinus* resistance

We assessed genetic variation in the response of 3 selectively bred oyster families to *P. marinus* exposure. The oyster families were derived from broodstock with varied putative resistance to Dermo disease (Dégremont et al. 2012, Proestou et al. 2016). Dams were sourced from 3 families (399, 450, and 383) found to be susceptible (399) or resistant (450 and 383) to *P. marinus* infection in a preliminary laboratory challenge (D. A. Proestou unpubl. data) and crossed with sires from selected oyster lines maintained at the Virginia Institute of Marine Science Aquaculture Genetics and Breeding Technology Center (ABC). The XB and hANA lines have respectively undergone 13 and 3 generations of selection for high performance in regions where *P. marinus* is endemic (Dégremont et al. 2012, Frank-Lawale et al. 2014, Proestou et al. 2016) while WT has been maintained as an unselected line. The crosses produced families WT399, XB450 and hANA383 which were maintained at ABC according to routine protocols (Frank-Lawale et al. 2014). Approximately 1 yr old seed (18 to 48 mm) were shipped to the US Department of Agriculture Agricultural Research Service

National Cold Water Marine Aquaculture Center (NCWMAC) on the University of Rhode Island campus, where the experiments were conducted in summer 2015. Upon arrival, we sacrificed 16 oysters from each family in order to (1) fit a linear regression of the relationship between oyster total weight and meat weight (required to calculate parasite dose; see Supplement S1 at www.int-res.com/articles/suppl/m594p107_supp.pdf) and (2) quantify baseline *P. marinus* densities in the experimental material. The remaining oysters were decontaminated with 0.1% bleach solution and freshwater dips (see Supplement S2) and maintained in recirculating aquaria containing 1 µm filtered UV-sterilized seawater at ambient temperature (~18°C) and 15 ppt. We gradually increased the temperature and salinity of the seawater to 25°C and 25 ppt over a period of 2 wk and fed all oysters a daily maintenance ration of Shellfish Diet 1800® instant algae (Reed Mariculture).

Following acclimation, we divided oysters from each of the families into 2 groups: control and exposed to *P. marinus* through feeding (n = 56 per group per family). Individual oysters were placed in static 1 l cups containing 500 ml 1 µm filtered UV-sterilized seawater and Shellfish Diet 1800® at an approximate concentration of 10^4 algal cells ml^{-1}. In the exposed group, we added cultured *P. marinus* cells (ATCC® strain 50509, 'DBNJ'; American Type Culture Collection) in artificial seawater. We harvested *P. marinus* cultures during the log phase of cell proliferation, and inoculated the cups containing oysters at a dose of 5×10^6 cells g^{-1} wet wt. We allowed oysters to feed on the algae/parasite mixture for 6 h prior to transferring them to 1 of 2 recirculating seawater systems consisting of eight 75 l tanks receiving 1 µm filtered UV-sterilized seawater from a 100 l head tank containing biofiltration (see Supplement S3). Control oysters were handled similarly, less the parasite exposure, and placed in the other recirculating system. We replaced the seawater in the head tanks and siphoned fecal material from the recirculating systems daily to minimize secondary *P. marinus* exposures in the exposed group.

The experiment lasted 42 d, during which we monitored survival and changes in parasite load over time. Oysters were checked daily and gaping, moribund oysters were recorded, removed, and shucked, and soft tissues preserved in 95% ethanol. In addition, 6 oysters per family per treatment were censored, shucked, and preserved at 4 different time points: 6 h, 36 h, 7 d, and 28 d post-exposure. We sacrificed all oysters surviving to the end of the experiment and preserved the soft tissues in 95% ethanol.

Measurements of parasite load for each dead, censored, and surviving oyster were obtained using the real-time quantitative PCR (qPCR) assay described by De Faveri et al. (2009). In brief, 5 to 10 mg of mantle and digestive tissue were dissected from the preserved oyster tissues and subjected to DNA extraction via a modified Chelex protocol (Aranishi & Okimoto 2006). DNA extracts were diluted to a standard concentration of 100 ng $µl^{-1}$ and 1 µl from each extract was included in the assay. All samples were run in duplicate on a Bio-Rad CFX96 Touch real-time PCR machine (Bio-Rad Laboratories) at the Aquatic Diagnostics Laboratory (Roger Williams University). We quantified parasite loads as the number of copies of *P. marinus* DNA per 100 ng total DNA averaged across the 2 replicates. We related these values to the Makin infection intensity ranking system (J. G. Mackin unpubl., cited in Ray 1954) using the fitted relationship described by De Faveri et al. (2009).

Expt 2: genetic variation in anti-parasite behavior

We characterized feeding behavior, both in the presence and absence of *P. marinus*, to assess variation in parasite avoidance among the 3 oyster families. Oysters from the same cohort used in Expt 1 were shipped to the NCWMAC in the fall of 2015. Upon arrival, they were decontaminated, transferred to recirculating aquaria, and gradually acclimated to 25°C and 25 ppt seawater over the course of 2 wk. Given the smaller sample size and shorter duration of this experiment, oysters were fed a mixed diet of live algae (*Isochrysis galbana* T-ISO strain, *Chaetoceros calcitrans*, and *Pavlova lutheri*).

One day prior to the experiment, we scrubbed all oysters to remove surface materials and biodeposits, and placed the animals back in the seawater system without food to ensure feeding during the challenge. The following day we allowed individual oysters (n = 20 oysters per family) to feed in static 1 l experimental cups containing 500 ml of 1 µm filtered UV-sterilized seawater mixed with the live algae at an initial density of approximately 10^4 algal cells ml^{-1} for 6 h. Half of the experimental cups were inoculated with the environmental *P. marinus* isolate at a dose of 10^6 cells per cup, and the remaining cups, serving as uninoculated controls, received 1 ml artificial seawater. We maintained 10 additional cups, 5 inoculated with *P. marinus* at a dose of 10^6 cells per cup, as no oyster controls to account for and quantify the particle settlement rate. We sampled 1 ml water from each cup immediately prior to placing oysters in the cups,

and again at the end of the 6 h experimental period, and quantified suspended particle concentrations for all samples in duplicate on a Coulter Counter particle sizing and counting analyzer (Beckman Coulter). We also noted whether shell valves were open or closed at 1 h intervals. At the end of the experiment, all oysters were removed from their cups using sterile forceps and shaken to dislodge bound materials, and the presence of accumulated biodeposits (i.e. feces and pseudofeces) noted. We then shucked all oysters and baked soft tissues at 80°C for 48 h to quantify dry tissue mass.

After oyster removal, all particulate material from each cup was collected on 47 mm diameter Nucleopore filters with a pore size of 3 µm, as described by Audemard et al. (2004), in order to enumerate *P. marinus* cells remaining in the water and biodeposits post-feeding. We performed Chelex extractions of DNA from each filter and diluted extracts 1:100. We included 1 µl of each diluted extract in duplicate qPCR assays on a Roche LightCycler® 480 real-time PCR machine (Roche Diagnostics) and quantified parasite densities as the number of copies of *P. marinus* DNA per qPCR reaction averaged across the 2 replicates. We then related the quantified densities of *P. marinus* DNA to the number of *P. marinus* cells per 500 ml water by spiking known concentrations of *P. marinus* cells into 500 ml of 1 µm filtered UV-sterilized seawater containing *I. galbana*, *C. calcitrans*, and *P. lutheri* (10^4 algal cells ml^{-1}), filtering and extracting DNA from particulate material, and qPCR quantification. Full details of the relationship between densities of *P. marinus* DNA quantified by the qPCR assay and the number of *P. marinus* cells per 500 ml water are presented in Supplement S4.

Data analysis

We tested for family differences in baseline parasite load in the oysters censored before the experiments using ANOVA. Because low parasite concentrations were detected in oyster tissue prior to the experiment start, we also characterized the change in parasite load over time in the control treatment using linear regression.

To quantify levels of *P. marinus* resistance and how they varied among the oyster families in Expt 1, we assessed patterns of oyster survival and parasite load. We estimated nonparametric, Kaplan-Meier survival functions for each family in the control and *P. marinus*-exposed treatments, and evaluated the effects of *P. marinus* exposure on the time to observed mortality for each family using non-parametric log-rank tests.

We used a linear mixed-effects model to test the effect of *P. marinus* exposure and oyster family on measured parasite loads, treating the time of sampling as a random factor. We assessed the significance of the fixed effects terms (*P. marinus* exposure, oyster family, and their interaction) using ANOVA. To characterize parasite load trajectories following *P. marinus* exposure, and determine whether these changes varied among the families, we used the 2-step function:

$$D_t = \begin{cases} \alpha e^{\beta e^{r_s t}} & \text{if } t \le 7\,\text{d} \\ D_7 e^{r_p (t-7)} & \text{if } t > 7\,\text{d} \end{cases} \quad (1)$$

where D_t is the density of *P. marinus* DNA in sampled oyster tissues at time t, α and β are, respectively, the asymptote and displacement of the Gompertz function describing the initial trajectory (<7 d) of *P. marinus* load, r_s is the rate parasites are initially shed from hosts following exposure, D_7 is the parasite load 7 d following exposure, and r_p is the proliferation rate of parasites once established within hosts. We fitted Eq. (1) to the parasite load data using nonlinear least squares regression. The term αe^{β} describes the parasite load immediately following parasite exposure (D_0), and we tested for differences in D_0 among the oyster families using pairwise z-tests of the estimated values for this term from the fitted regression coefficients.

We used the indirect clearance method (Riisgård 2001) to estimate dry weight standardized filtration rates from the measured suspended particle concentrations in Expt 2. Briefly, for each oyster used in the experiment, we estimated the filtration rate, or clearance rate (Cl), of suspended particles as:

$$\text{Cl} = \frac{V}{t w^b}\left(\log\frac{C_0}{C_t} - \bar{s}\right) \quad (2)$$

where V is the volume of water, w is the oyster dry weight, b is the exponent of the allometric relationship between feeding rate and oyster dry weight (see Supplement S5), C_0 is the concentration of suspended particles measured at time 0, C_t is the suspended particle concentration measured at time t, and $\bar{s}$ is the mean settling rate of suspended particles estimated from the no oyster controls. The clearance rate describes the total volume of water cleared of suspended particles over a specified period. We tested for differences in clearance rates among the oyster families and with *P. marinus* exposure using ANOVA. To further test for differences in feeding behavior among families, we analyzed valve-opening

data using a generalized linear mixed model (binomial variance and logit link) with the time of sampling as a random factor, and oyster family and *P. marinus* dose as fixed factors. In addition, we tested whether the concentrations of *P. marinus* DNA remaining in water and biodeposits varied across the oyster families using ANOVA. Statistical analyses were performed in MatLab version R2016a (The MathWorks).

RESULTS

Expt 1: genetic variation in *Perkinsus marinus* resistance

Very few mortalities (3/168 control and 12/167 exposed) were observed in Expt 1, and were primarily seen in family WT399 (Fig. 1). We did not detect a significant effect of *P. marinus* exposure on the survival of families XB450 (log-rank test: $z = 0.06$, p = 0.94) and hANA383 (log-rank test: $z = 0.00$, p = 1.00) over the 42 d experimental period. However, exposure to *P. marinus* markedly reduced the survival of family WT399 (log-rank test: $z = 3.34$, p < 0.001).

P. marinus infection levels were negligible in oysters censored prior to the start of Expt 1. One-third of the oysters tested negative for *P. marinus* while the remaining oysters had light infections (<116.23 DNA copies per 100 ng DNA; Mackin rating ≤ 0.5). No difference in baseline *P. marinus* was detected across families (ANOVA: $F_{2,47} = 0.40$, p = 0.67). Similarly, we detected only light infections in the unexposed control group, and in this group, parasite loads declined over the time course of the experiment (linear regression: $\beta_{time} = -0.008$, SE = 0.002, $t = -4.12$, p < 0.001). Thus, it is unlikely that the light, baseline infections we detected confounded the response to experimental exposure.

We observed significantly higher parasite loads in the *P. marinus*-exposed treatment compared to the control (linear mixed-effects model: $\beta_{exposure} = 0.36$, SE = 0.04, $t = 9.07$, p < 0.001). Parasite loads varied by family (mixed-effects ANOVA: $F_{2,318} = 4.46$, p = 0.01) and there was a strong interaction between the effects of oyster family and *P. marinus* exposure (mixed-effects ANOVA: $F_{2,318} = 6.49$, p = 0.002), suggesting variability in the capacity for the different oyster families to avoid *P. marinus* exposure or resist parasite proliferation once exposed, or both (Fig. 2a). Parasite loads immediately following exposure (D_0) were highest in family WT399 (Fig. 2b), which carried significantly higher initial parasite loads than family hANA383 (pairwise *z*-test; $z = 1.69$, p = 0.04). Immediate parasite loads in family XB450 were intermediate to families WT399 and hANA383. The trajectories of *P. marinus* densities were consistent with previously described *P. marinus* trajectories post-exposure (Bushek & Allen 1996, Chintala et al. 2002), increasing following a decrease to near detection limits in the week after exposure. The extent of the increase varied by family, and was particularly evident in family WT399 ($\hat{r_p} = 0.10$ d^{-1}, SE = 0.006). Family XB450 had the lowest rate of increase in measured parasite load ($\hat{r_p} = 0.04$ d^{-1}, SE = 0.009), and hANA383 was intermediate to families WT399 and XB450 ($\hat{r_p} = 0.07$ d^{-1}, SE = 0.009). Taken together, the survival and parasite density data confirmed that selectively bred oyster families differed in their resistance to *P. marinus* exposure. Family WT399 was more susceptible to the parasite than families XB450 and hANA383.

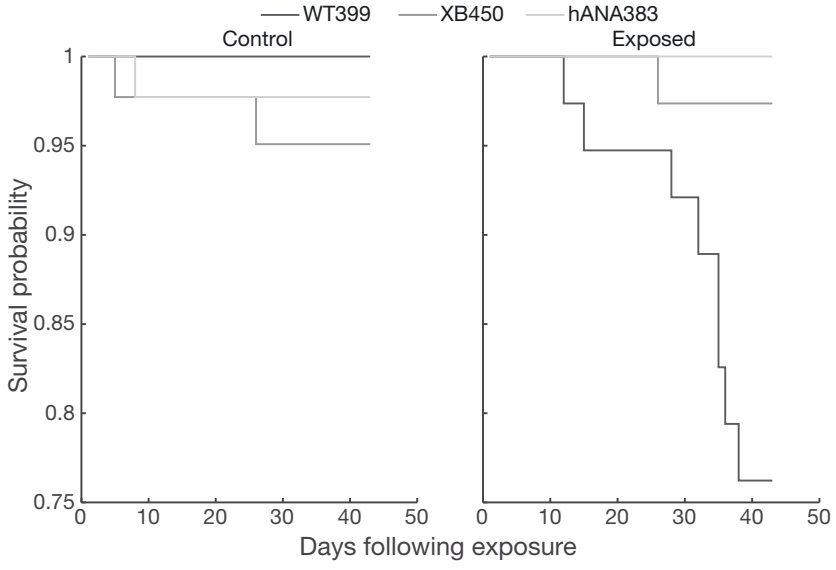

Fig. 1. Kaplan-Meier survival functions for the 3 oyster *Crassostrea virginica* families in the control and *Perkinsus marinus*-exposed groups in Expt 1

Expt 2: genetic variation in anti-parasite behavior

Feeding behavior did not vary among the families in the absence of *P. marinus* exposure, when measured as either particle clearance rate or the proportion of oysters with open

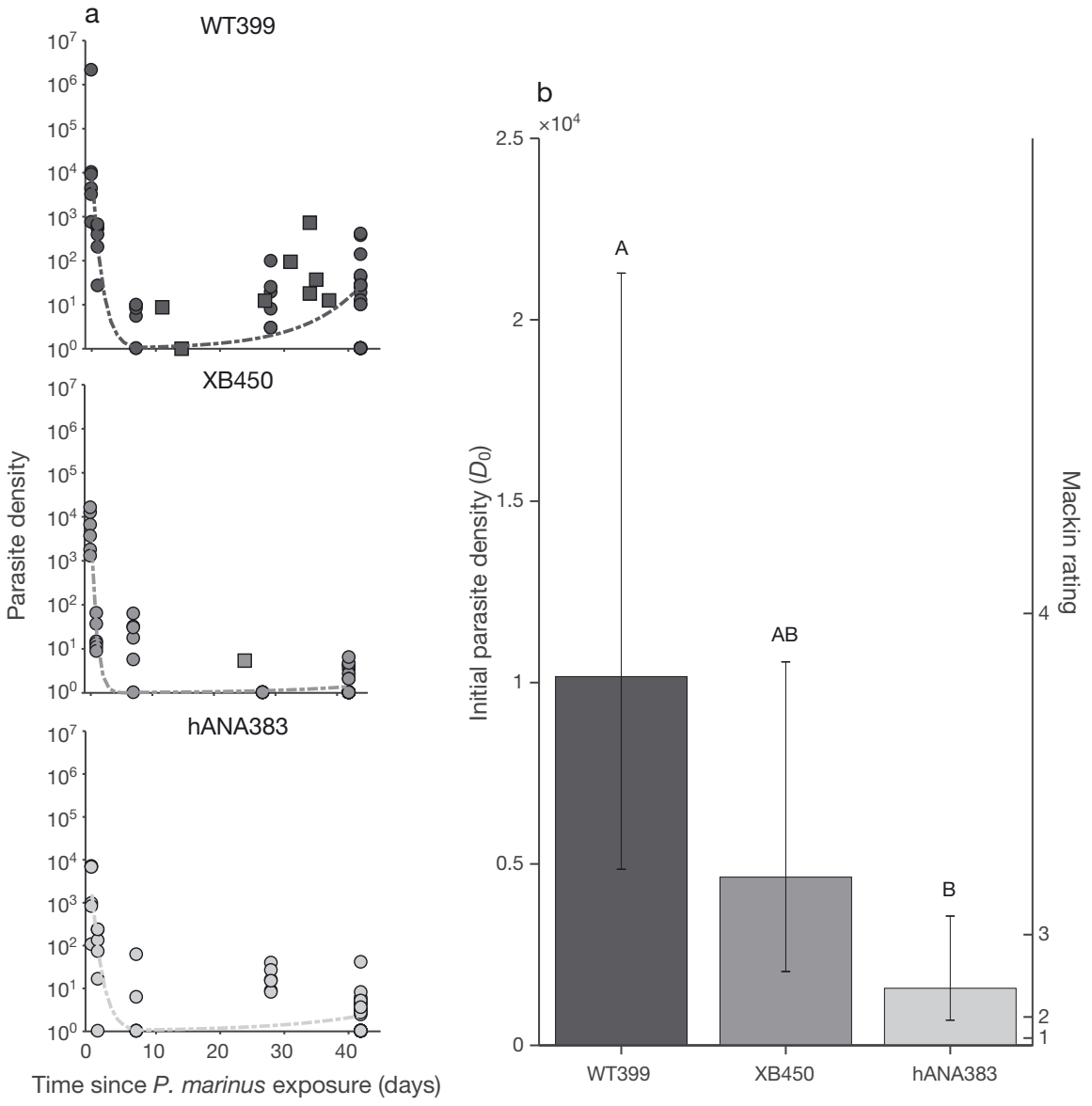

Fig. 2. (a) Densities of *Perkinsus marinus* DNA, expressed as plasmid DNA copies per 100 ng total DNA, sampled from oysters *Crassostrea virginica* in the *P. marinus*-exposed groups in Expt 1. (Circles) Censored samples, (squares) observed mortalities. (Dashed lines) Fitted values of Eq. (1) for the 3 oyster families. (b) The initial parasite density (D_0) estimated from the fitted values of Eq. (1) for the 3 oyster families. The letters above each bar show groups based on significant ($\alpha = 0.05$) pairwise differences from *z*-tests of the estimated values of D_0 from the fitted regression coefficients. The right *y*-axis reports the densities of *P. marinus* measured as plasmid DNA copies per 100 ng total DNA, while the left *y*-axis reflects parasite densities according to the Mackin infection intensity ranking system. The Mackin ratings were calculated based on the linear relationship derived from quantifying parasite densities for a set of samples using both qPCR and Ray's fluid thioglycollate media (RFTM) enumeration methods

valves (Fig. 3a,b). In the latter case, the more resistant families XB450 (mixed effects logistic regression: odds ratio = 1.07, SE = 0.41, $z = 0.18$, p = 0.86) and hANA383 (mixed effects logistic regression: odds ratio = 0.52, SE = 0.20, $z = -1.71$, p = 0.09) were as likely as WT399 to be observed with open valves (Fig. 3b). Exposure to *P. marinus* led to a marked reduction in measured clearance rates (Fig. 3a), but only in families XB450 and hANA383 (ANOVA: $F_{2,54} = 3.28$, p = 0.04). In addition, when exposed to *P. marinus*, individuals from families XB450 and hANA383 were only 0.20 times (mixed effects logistic regression: odds ratio = 0.20, SE = 0.11, $z = -2.96$, p = 0.003) and 0.16 times (mixed effects logistic regression: odds ratio = 0.16, SE = 0.09, $z = -3.05$, p = 0.002) as likely as family WT399 to be observed with open valves (Fig. 3b). The majority of individuals from all 3 families (90% of WT399 and XB450, and 80% of

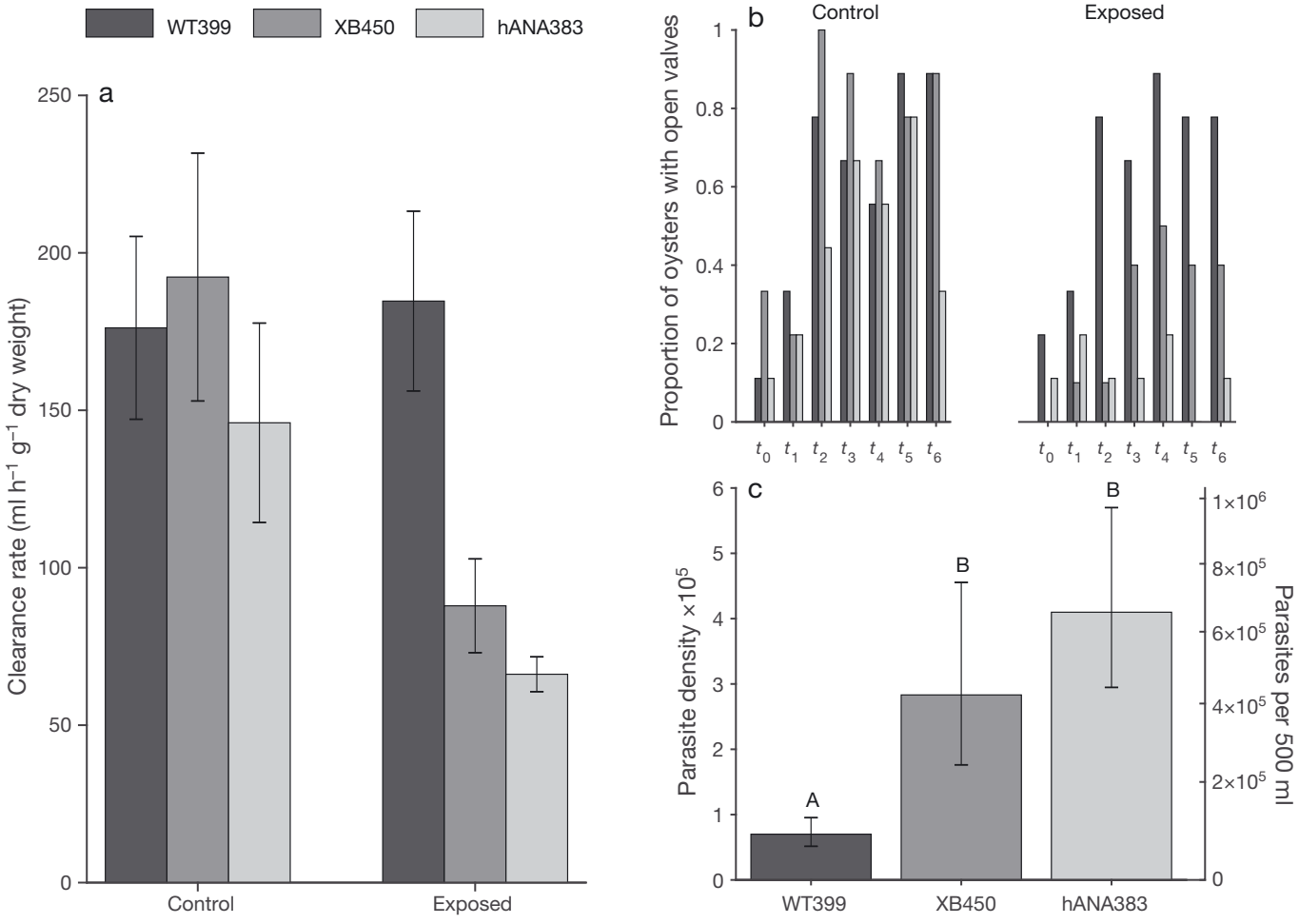

Fig. 3. (a) Mean (±SE) suspended particle clearance rates of the 3 oyster *Crassostrea virginica* families in the control and *Perkinsus marinus*-exposed groups in Expt 2. (b) The proportion of oysters in each family observed with open valves each hour over the 6 h experimental period in Expt 2. (c) Estimated concentrations of *P. marinus* remaining in the seawater and produced biodeposits in the exposed group at the end of Expt 2. The letters above each bar show groups based on significant ($\alpha = 0.05$) pairwise differences estimated from a post hoc Tukey's HSD test

hANA383) produced biodeposits when *P. marinus* was absent. However, when *P. marinus* was present in the seawater, the percentage of individuals producing evident biodeposits declined to 70% in XB450 and 30% in hANA383, while remaining at 90% in WT399. The concentration of *P. marinus* DNA remaining in the water at the end of the 6 h experimental period was correlated with the concentration of total suspended particles remaining (Pearson's $\rho = 0.51$, $p = 0.006$). We detected an order of magnitude greater density of *P. marinus* DNA in seawater collected from cups containing oysters from families XB450 and hANA383 than from those containing individuals from WT399 (Fig. 3c; ANOVA: $F_{2, 28} = 5.97$, $p = 0.007$). Therefore, the families that best modified their feeding behavior in Expt 2 left the highest concentrations of *P. marinus* in the water.

DISCUSSION

Anti-parasite behaviors have repeatedly evolved in host populations that are highly vulnerable to parasitism, and differences in relative fitness provide the fodder for selection. Here we have shown that the presence of *Perkinsus marinus* can induce changes in oyster feeding behavior and that those changes are associated with measured levels of Dermo resistance across eastern oyster families. The family most susceptible to the parasite, WT399, was also the family that showed the least change in its feeding behavior to limit the number of invading *P. marinus* cells. Therefore, our findings provide evidence for variation in anti-parasite behavior, and behavioral immunity, in a sessile, suspension-feeding, bivalve mollusc.

Although we performed our experiments with only 3 oyster families and a single parasite strain, we were able to demonstrate genetic variation specifically for Dermo resistance by quantifying survival and changes in parasite load under laboratory-controlled conditions. Currently, eastern oyster breeding programs select for disease resistance indirectly, using survival in field trials, where disease pressure is inconsistent from year to year and site to site, as a measure of resistance (Brown et al. 2005, Dégremont et al. 2015). By conducting laboratory disease challenges, we not only confirmed that oysters selected for disease resistance (families XB450 and hANA383) were less susceptible to *P. marinus* than those that were not (family WT399), we also provide evidence that varied ability to modify feeding behavior in the presence of *P. marinus* contributes to differences in resistance.

Parasite avoidance behaviors require that hosts can detect potential danger. Sessile species such as oysters, which cannot move and interact with their environment largely by filtering the surrounding water, must rely on chemical and tactile cues from filtered particles. They have evolved to discriminate and select nutritive particles from a complex mixture containing minerals, detritus, and live algae suspended in the water column. Factors such as particle size, nutritional quality, and the biochemical and physical surface properties of particles all have demonstrated effects on feeding rates (Milke & Ward 2003, Ward & Shumway 2004, Pales Espinosa et al. 2009, Rosa et al. 2013). In addition, recent work has indicated that particle retention is mediated by interactions between carbohydrate-binding proteins (i.e. lectins) present in the mucus layer of bivalve-feeding organs and the surface carbohydrates of filtered particles (Pales Espinosa et al. 2009, 2010, 2016).

Potentially harmful particles can elicit responses from suspension-feeding bivalves ranging from the reduction of water movement through the shell (i.e. shell closure) to limiting particle retention and/or rejecting them as pseudofeces before ingestion (Ward & Shumway 2004). For example, many bivalve species, including clams, mussels, and eastern oysters, exhibit reduced valve gape and lower clearance rates when exposed to toxic algae such as *Alexandrium monilatum* (May et al. 2010). Only 1 study has demonstrated the ability of bivalves to recognize protozoan parasites. While working with oysters produced at a commercial hatchery, Allam et al. (2013) observed the preferential rejection of *P. marinus* with pseudofeces prior to ingestion, indicating that these oysters could discriminate *P. marinus* from microalgae. Allam et al. (2013) also showed that the parasite utilizes an alternative route of infection (entering along the mantle epithelium before pseudofeces are discharged from the pallial cavity) to overcome preingestive rejection. The fact that oysters can detect and reject parasites is consistent with what we observed with respect to increased shell closure and reduced clearance rates in some families. Our recovery of a significantly higher percentage of the nominal starting *P. marinus* dose (10^6 cells) in the cups containing oysters from more resistant families (40% and 60% for XB450 and hANA383, compared to 10% for WT399) lends further support for modified feeding in response to parasites. Since we processed water and biodeposits together, we can only speculate on whether reduced filtration or enhanced particle selection is the primary mechanism of parasite avoidance; however, as with toxic algae (e.g. May et al. 2010), reducing valve gape and feeding can effectively limit parasite encounter when particle rejection alone is insufficient for eliminating the risk of parasite transmission.

While effective anti-parasite behaviors have been shown to reduce direct parasitism and the risk of disease, there is growing recognition that these behaviors are energetically and/or ecologically costly (Raffel et al. 2008, Buck & Ripple 2017). For example, per capita growth in sticklebacks is reduced by parasite-avoiding shoaling behavior (Herczeg et al. 2009), and periwinkles that forgo mutual shell grazing in favor of avoiding the parasite-laden mucus trails of their conspecifics are commonly found with heavily fouled shells (Mouritsen 2017). However, since anti-parasite behaviors often represent a composite of many behaviors over various contexts, with each behavior accomplishing numerous and potentially interacting functions, it is often difficult to determine the fitness level consequences of these behavioral traits (Lind & Cresswell 2005). Anti-predator defense strategies, such as energy allocations to defensive structures and tactics that minimize prey visibility, have been found to carry fitness level consequences that often equal and can even exceed the effects of direct consumption (Preisser et al. 2005). It is reasonable to expect significant energetic costs to reduced feeding in oysters avoiding *P. marinus*. However, in our experiments, we did not observe differences in feeding behavior among the families in the unexposed treatment, suggesting flexibility in feeding behavior in response to parasite exposure. How flexibility in oyster feeding behavior influences fitness across a gradient of parasite exposure and disease risk will

provide insight for the stability of anti-parasite behavior in wild and cultured oysters.

Ours is not the first study to highlight how flexibility in feeding behavior affects performance traits in oysters. Bayne (2000) related genetic variation for growth in Sydney rock and Pacific oysters to differences in energy acquisition (i.e. filtration), energy allocation toward growth, and metabolic efficiency in response to ephemeral food environments. Oysters selected for rapid growth have faster feeding rates, select particles more efficiently, experience lower costs to growth, and allocate more of their energy intake to growth, particularly protein deposition, compared to unselected oysters (Bayne 2004, Pernet et al. 2008). Importantly, faster feeding rates do not impair flexibility in feeding behavior (Bayne 2004), which compensates for changes in the food environment. Since water column concentrations of *P. marinus* vary considerably in space and time (Audemard et al. 2006), it is reasonable to expect that flexibility in feeding behavior can compensate for changes in parasite exposure with little sacrifice to the rate of energy acquisition.

The present focus on behavioral avoidance is not intended to detract from the role of innate immunity in bivalve defense against parasites. Numerous studies have underscored the importance of innate immunity by noting that gene families involved in immune and stress responses are highly expanded in bivalve genomes compared to other species (Zhang et al. 2012, Zhang et al. 2014, Guo et al. 2015). In addition, there is a growing body of knowledge describing cellular and molecular mediators of infection processes in bivalve molluscs (Allam & Raftos 2015), including interactions occurring at the mucosal surfaces of the feeding organs, where initial encounter between host and parasite occurs (Allam et al. 2013, Allam & Pales Espinosa 2016). By identifying the specific behavioral, humoral, and immunological phenotypes that define disease resistance, and characterizing how they vary and interact, we are better positioned to define mechanisms underlying Dermo resistance in oysters. This is of applied interest, as the accurate measurement and targeted selection of key host defense traits will expedite the genetic improvement of cultured oyster stocks to benefit the aquaculture industry (Kube et al. 2012). In addition, as our recognition of the distribution and impact of marine diseases continues to expand, and the potential for disease outbreaks intensifies with changing environmental conditions (Ford & Smolowitz 2007, Burge et al. 2014), the varied ability of hosts to defend themselves against parasites will buffer the impact of disease in wild and cultured host populations.

Acknowledgements. We thank R. Corbett, C. O'Brien, K. Lundgren, and Z. ElShaer for assistance in the laboratory. Special thanks to J. Alix at the NOAA Milford Laboratory for providing algae isolates. This work was funded through USDA ARS CRIS Project No. 8030-31000-004-00D and a standard cooperative agreement between USDA ARS and the University of Rhode Island. T.B.-H. was further supported by a USDA-NIFA Agriculture and Food Research Initiative Postdoctoral Fellowship (RI000912). Access to the URI Marine Science Research Facility and URI Genomics and Sequencing Center, which are supported in part by the National Science Foundation under EPSCoR Grant Nos. 0554548 and EPS-1004057, were also instrumental for completion of this work. T.B.-H. and D.A.P. conceived the study. S.K.A. and J.M.S. produced and maintained the oyster families prior to experiments. T.B.-H. and D.A.P. performed the experiments. T.B.-H. and D.A.P. analyzed the data and wrote the manuscript. T.B.-H., D.A.P., S.K.A., and J.M.S. contributed to manuscript revisions.

LITERATURE CITED

Allam B, Pales Espinosa E (2016) Bivalve immunity and response to infections: are we looking at the right place? Fish Shellfish Immunol 53:4–12

Allam B, Raftos D (2015) Immune responses to infectious diseases in bivalves. J Invertebr Pathol 131:121–136

Allam B, Carden WE, Ward JE, Ralph G, Winnicki S, Pales Espinosa E (2013) Early host–pathogen interactions in marine bivalves: evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces. J Invertebr Pathol 113: 26–34

Aranishi F, Okimoto T (2006) A simple and reliable method for DNA extraction from bivalve mantle. J Appl Genet 47:251–254

Audemard C, Reece KS, Burreson EM (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. Appl Environ Microbiol 70:6611–6618

Audemard C, Ragone Calvo LM, Paynter KT, Reece KS, Burreson EM (2006) Real-time PCR investigation of parasite ecology: *in situ* determination of oyster parasite *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Parasitology 132:827–842

Bayne BL (2000) Relations between variable rates of growth, metabolic costs and growth efficiencies in individual Sydney rock oysters (*Saccostrea commercialis*). J Exp Mar Biol Ecol 251:185–203

Bayne BL (2004) Phenotypic flexibility and physiological tradeoffs in the feeding and growth of marine bivalve molluscs. Integr Comp Biol 44:425–432

Behringer DC, Butler MJ (2010) Disease avoidance influences shelter use and predation in Caribbean spiny lobster. Behav Ecol Sociobiol 64:747–755

Behringer DC, Butler MJ, Shields JD (2006) Ecology: avoidance of disease by social lobsters. Nature 441:421

Biro PA, Stamps JA (2010) Do consistent individual differences in metabolic rate promote consistent differences in behavior? Trends Ecol Evol 25:653–659

Brown BL, Butt AJ, Meritt D, Paynter KT (2005) Evaluation of resistance to Dermo in eastern oyster strains tested in Chesapeake Bay. Aquacult Res 36:1544–1554

Buck JC, Ripple WJ (2017) Infectious agents trigger trophic cascades. Trends Ecol Evol 32:681–694

Burge CA, Mark Eakin C, Friedman CS, Froelich B and others (2014) Climate change influences on marine infectious diseases: implications for management and society. Annu Rev Mar Sci 6:249–277

Bushek D, Allen SK Jr (1996) Host–parasite interactions among broadly distributed populations of the eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus.* Mar Ecol Prog Ser 139:127–141

Butler MJ IV, Behringer DC Jr, Dolan TW III, Moss J, Shields JD (2015) Behavioral immunity suppresses an epizootic in Caribbean spiny lobsters. PLOS ONE 10:e0126374

Chintala MM, Bushek D, Ford SE (2002) Comparison of *in vitro*-cultured and wild-type *Perkinsus marinus.* II. Dosing methods and host response. Dis Aquat Org 51: 203–216

Coen LD, Bishop MJ (2015) The ecology, evolution, impacts and management of host–parasite interactions of marine molluscs. J Invertebr Pathol 131:177–211

Daly EW, Johnson PTJ (2011) Beyond immunity: quantifying the effects of host anti-parasite behavior on parasite transmission. Oecologia 165:1043–1050

Davies MS, Knowles AJ (2001) Effects of trematode parasitism on the behaviour and ecology of a common marine snail (*Littorina littorea* (L.)). J Exp Mar Biol Ecol 260: 155–167

De Faveri J, Smolowitz RM, Roberts SB (2009) Development and validation of a real-time quantitative PCR assay for the detection and quantification of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica.* J Shellfish Res 28:459–464

de Roode JC, Lefèvre T (2012) Behavioral immunity in insects. Insects 3:789–820

Dégremont L, Garcia C, Frank-Lawale A, Allen SK (2012) Triploid oysters in the Chesapeake Bay: comparison of diploid and triploid *Crassostrea virginica.* J Shellfish Res 31:21–31

Dégremont L, Garcia C, Allen SK Jr (2015) Genetic improvement for disease resistance in oysters: a review. J Invertebr Pathol 131:226–241

Ford SE, Smolowitz R (2007) Infection dynamics of an oyster parasite in its newly expanded range. Mar Biol 151: 119–133

Frank-Lawale A, Allen SK, Dégremont L (2014) Breeding and domestication of eastern oyster (*Crassostrea virginica*) lines for culture in the mid-Atlantic, USA: line development and mass selection for disease resistance. J Shellfish Res 33:153–165

Guo X, He Y, Zhang L, Lelong C, Jouaux A (2015) Immune and stress responses in oysters with insights on adaptation. Fish Shellfish Immunol 46:107–119

Hart BL (1990) Behavioral adaptations to pathogens and parasites: five strategies. Neurosci Biobehav Rev 14: 273–294

Hart BL (1994) Behavioural defense against parasites: interaction with parasite invasiveness. Parasitology 109: S139–S151

Herczeg G, Gonda A, Merilä J (2009) The social cost of shoaling covaries with predation risk in nine-spined stickleback, *Pungitius pungitius*, populations. Anim Behav 77:575–580

Kube PD, Taylor RS, Elliott NG (2012) Genetic variation in parasite resistance of Atlantic salmon to amoebic gill disease over multiple infections. Aquaculture 364-365: 165–172

Lind J, Cresswell W (2005) Determining the fitness consequences of anti-predation behavior. Behav Ecol 16: 945–956

Mackin JG, Owen HM, Collier A (1950) Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* sp. in *Crassostrea virginica* (Gmelin). Science 111:328–329

May SP, Burkholder JM, Shumway SE, Hégaret H, Wikfors GH, Frank D (2010) Effects of the toxic dinoflagellate *Alexandrium monilatum* on survival, grazing and behavioral response of three ecologically important bivalve molluscs. Harmful Algae 9:281–293

Milinski M, Bakker TCM (1990) Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. Nature 344:330–333

Milke LM, Ward JE (2003) Influence of diet on pre-ingestive particle processing in bivalves. J Exp Mar Biol Ecol 293: 151–172

Mouritsen KN (2017) Periwinkle regulation: parasitism and epibiosis are linked. Mar Ecol Prog Ser 579:227–231

Pales Espinosa E, Perrigault M, Ward JE, Sumway SE, Allam B (2009) Lectins associated with the feeding organs of the oyster *Crassostrea virginica* can mediate particle selection. Biol Bull 217:130–141

Pales Espinosa E, Perrigault M, Ward JE, Shumway SE, Allam B (2010) Microalgal cell surface carbohydrates as recognition sites for particle sorting in suspension-feeding bivalves. Biol Bull 218:75–86

Pales Espinosa E, Cerrato RM, Wikfors GH, Allam B (2016) Modeling food choice in the two suspension-feeding bivalves, *Crassostrea virginica* and *Mytilus edulis.* Mar Biol 163:40

Perkins FO (1987) Protistan parasites of commercially significant marine bivalve molluscs — life cycles, ultrastructure, and phylogeny. Aquaculture 67:240–243

Pernet F, Tremblay R, Redjah I, Sevigny JM, Gionet C (2008) Physiological and biochemical traits correlate with differences in growth rate and temperature adaptation among groups of the eastern oyster *Crassostrea viriginica.* J Exp Biol 211:969–977

Poulin R, FitzGerald GJ (1989) Shoaling as an anti-ectoparasite mechanism in juvenile sticklebacks (*Gasterosteus* spp.). Behav Ecol Sociobiol 24:251–255

Preisser EL, Bolnick DI, Benard MF (2005) Scared to death? The effects of intimidation and consumption in predator–prey interactions. Ecology 86:501–509

Proestou DA, Vinyard BT, Corbett RJ, Piesz J and others (2016) Performance of selectively-bred lines of eastern oyster, *Crassostrea virginica*, across eastern US estuaries. Aquaculture 464:17–27

Raffel TR, Martin LB, Rohr JR (2008) Parasites as predators: unifying natural enemy ecology. Trends Ecol Evol 23: 610–618

Ray SM (1954) Studies on the occurrence of *Dermocystidium marinum* in young oysters. Proc Natl Shellfish Assoc 1953:80–92

Riisgård HU (2001) On measurement of filtration rate in bivalves — the stony road to reliable data: review and interpretation. Mar Ecol Prog Ser 211:275–291

Rosa M, Ward JE, Shumway SE, Wikfors GH, Pales-Espinosa E, Allam B (2013) Effects of particle surface

properties on feeding selectivity in the eastern oyster *Crassostrea virginica* and the blue mussel *Mytilus edulis*. J Exp Mar Biol Ecol 446:320–327

Sih A, Bell AM, Johnson JC, Ziemba RE (2004) Behavioral syndromes: an integrative review. Q Rev Biol 79: 241–277

Ward JE, Shumway SE (2004) Separating the grain from the chaff: particle selection in suspension- and deposit-feeding bivalves. J Exp Mar Biol Ecol 300:83–130

Wilson DS, Clark AB, Coleman K, Dearstyne T (1994) Shyness and boldness in humans and other animals. Trends Ecol Evol 9:442–446

Wisenden B, Goater C, James C (2009) Behavioral defenses against parasites and pathogens. In: Zaccone G, Perrière C, Mathis A, Kapoor BG (eds) Fish defenses, Vol 2. Pathogens, parasites and predators. Science Publishers, Enfield, p 151–168

Wolf M, Weissing FJ (2012) Animal personalities: consequences for ecology and evolution. Trends Ecol Evol 27: 452–461

Wolf M, van Doorn GS, Leimar O, Weissing FJ (2007) Life history trade-offs favour the evolution of animal personalities. Nature 447:581–584

Zhang G, Fang X, Guo X, Li L and others (2012) The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490:49–54

Zhang L, Li L, Zhu Y, Zhang G, Guo X (2014) Transcriptome analysis reveals a rich gene set related to innate immunity in the eastern oyster (*Crassostrea virginica*). Mar Biotechnol (NY) 16:17–33

Editorial responsibility: Karen Miller, Hobart, Tasmania, Australia

Submitted: September 22, 2017; Accepted: January 30, 2018
Proofs received from author(s): April 16, 2018