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Influence of oyster genetic background on levels of human-pathogenic *Vibrio* spp.

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ABSTRACT

Human-pathogenic *Vibrio* bacteria are common inhabitants of oyster tissues, but our understanding of factors driving the wide range of concentrations found in individual oysters is extremely limited. We examined the influence of oyster sex and parasitism in light of their profound effects on oyster tissues against a backdrop of eastern oysters, *Crassostrea virginica*, from two diploid and two triploid aquacultured lines. This allowed us to examine not only the effect of oyster ploidy but also of oyster genetics, a factor never investigated with regard to human-pathogenic *Vibrio* species. We measured levels of total *Vibrio vulnificus (vvhA)*, and of total (*tlh*) and pathogenic (*tdh*+, *trh*+) *V. parahaemolyticus*, in each oyster, and analyzed the data through generalized linear mixed-effects models. A key outcome of these analyses was the consistent inclusion of oyster line as a predictor variable across *Vibrio* targets. A potential effect of *Perkinsus marinus* infections and/or oyster sex was also suggested, although the combination of variables varied with *Vibrio* target. This study suggests that the influence of oyster genetic background should be further investigated, and that the dynamics of human-pathogenic *Vibrio* spp. in oysters is likely driven by multiple, interacting factors, some of which may be under oyster host genetic control.

1. Introduction

The natural occurrence of human-pathogenic *Vibrio* spp. in oysters, and the fact that consumption of oysters is the main route of transmission of these pathogens to humans, make the understanding of interactions between oysters and human-pathogenic *Vibrio* spp. central to protecting human consumers from seafood-borne *Vibrio*-related illnesses. This is particularly relevant in light of the highly variable concentrations of human-pathogenic *Vibrio* spp. in oysters cultured in close proximity and exposed to the same environmental conditions (DePaola et al., 2003; Kaufman et al., 2003; Klein and Lovell, 2017; Marcinkiewicz et al., 2017; Bienlien et al., 2022). These observations suggest that the dynamics of these bacteria at the scale of an oyster population are influenced by factors associated with the oyster itself, such as oyster health or physiology, rather than by environmental conditions alone (DePaola et al., 2003; FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization], 2011). Managing risks associated with these pathogens will benefit not only from a better understanding of factors controlling large-scale temporal and spatial dynamics of these bacteria (Takemura et al., 2014), but also from gaining a better understanding of the factors controlling these dynamics at the individual oyster scale. The few studies conducted in this context have failed to identify the factors driving the concentrations of *Vibrio* spp. in individual oysters, so our understanding of human-pathogenic *Vibrio* spp.-oyster interactions remains extremely limited.

Illnesses linked to the consumption of raw or improperly cooked oysters are mainly associated with two *Vibrio* species, *Vibrio vulnificus* and *V. parahaemolyticus* (Center for Disease Control and Prevention, 2019). *Vibrio parahaemolyticus* is mostly associated with mild illnesses, but in the U.S. it causes an estimated 45,000 cases per year (Center for Disease Control and Prevention, 2019). In comparison, *V. vulnificus* is associated with a small number of illness cases (~ 100/year) but remains a serious concern due to its high fatality rate (~30% of cases)–it is the leading cause of seafood-borne mortality in the U.S. (Mead et al., 1999; Jones and Oliver, 2009; Scallan et al., 2011; Oliver, 2015; Center for Disease Control and Prevention, 2019). For both species, not all strains are pathogenic, and unfortunately, identification of reliable pathogenic markers continues to be challenging. For *V. parahaemolyticus*, most studies estimate the occurrence or abundance of pathogenic strains through the detection of the thermostable direct

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hemolysin gene (*tdh*) and the *tdh*-related hemolysin gene (*trh*) (Honda and Iida, 1993), even though a small percentage of clinical isolates lack these genes (Jones et al., 2012; Miller et al., 2021). For *V. vulnificus*, polymorphisms within several genes have been proposed as markers to identify pathogenic strains, but there is not yet a consensus regarding the most appropriate marker (Dickerson et al., 2021), so most studies focus on total *V. vulnificus* measurements.

The rare studies centered on the influence of the oyster itself on levels of human-pathogenic *Vibrio* spp. have explored the effect of oyster parasitism (Sokolova et al., 2005; Bienlien, 2016; Bienlien et al., 2022). Parasitism can be particularly detrimental for the host by influencing overall health and impairing host physiology. Consequently, it has been hypothesized to have profound impact on the oyster-habitat in which human-pathogenic *Vibrio* spp. occur. These studies focused on the eastern oyster *Crassostrea virginica* and examined the influence of its two main parasites, the protozoans *Perkinsus marinus* and *Haplosporidium nelsoni*, on levels of *V. vulnificus* and *V. parahaemolyticus*. The studies of both Sokolova et al. (2005) and Bienlien et al. (2022) pointed to an absence of association between parasitism associated with *P. marinus* and levels of human-pathogenic *Vibrio* spp. The potential impact of *H. nelsoni* was more difficult to assess due to its low prevalence, suggesting that more studies would be needed to fully investigate the influence of these parasites (Bienlien et al., 2022).

In oysters, development of the gonad is associated with major physiological changes (Thompson et al., 1996) that could also have profound impacts on the intra-oyster habitat in which human-pathogenic *Vibrio* spp. occur. In *C. virginica*, gonadal development is seasonal, with gonad proliferation and sexual differentiation into females, males or hermaphrodites occurring in the spring, and spawning occurring from spring into fall (Kennedy and Battle, 1964). As documented by Kennedy and Battle (1964), histological examination of mature oysters indicated that gonadal tissues can almost represent 50% of the body mass, underscoring the changes in the tissue habitat associated with the reproductive cycle. The examination of the gonadal development or sex as a potential predictor variable of these bacteria is also supported by previous studies focusing on *Vibrio* bacteria. Williams et al. (2009) showed that *C. virginica* gonad harbored more *V. campbellii* per gram than other tissues, but also inhibited *V. campbellii* growth more effectively than other tissues. In addition, a study conducted on *Crassostrea gigas* showed the affinity of another *Vibrio* sp., *V. splendidus*, for gonadal tissues, and suggested that gonad may be both a portal of entry (via gonoducts) and a highly nutritive tissue for *Vibrio* growth (De Decker et al., 2011).

Our study was designed to examine the effects of the above factors while also including factors particularly relevant to oyster aquaculture: oyster line (or genetics) and oyster ploidy. Triploid oysters are often grown in aquaculture due to their faster growth and better quality during spawning season compared to diploid oysters (Hudson, 2019). Higher performance of triploids over diploids is the result of reduced gonadogenesis, which allows the triploids to reallocate energy reserves to growth rather than to reproduction (Allen and Downing, 1986). Previous studies conducted in the Gulf Coast region showed no significant effect of oyster ploidy on levels of human-pathogenic *Vibrio* spp., however in two of these studies triploids tended to have a lower abundance of *V. parahaemolyticus* compared to diploids (Walton et al., 2013; Grodeska et al., 2019; Jones et al., 2020). In this study, we re-examine the potential effect of ploidy in the Mid-Atlantic region of the U.S. but we also examine the potential influence of oyster genetics. Oyster genetics has not been considered as a potential factor influencing intra-populational levels of human-pathogenic *Vibrio* spp., but this factor deserves investigation not only because it influences individual physiology and health, but also because of its connection with oyster microbiomes (Wegner et al., 2013; King et al., 2019; Dupont et al., 2020; Nguyen et al., 2020; Scanes et al., 2021). Interestingly, for both total *V. vulnificus* and total *V. parahaemolyticus* as well as for *tdh* and *trh* strains, we found that generalized linear mixed-effects models that included oyster line were consistently empirically supported among the various models that included combinations of measured oyster variables.

2. Materials and methods

2.1. Study design

Eastern oysters, *Crassostrea virginica*, used in this study originated from two diploid lines and two triploid lines produced by Virginia Institute of Marine Science Aquaculture Genetics and Breeding Technology Center (VIMS ABC) in July 2017 and again in July 2018. The two diploid lines were putatively bred to differ not only in terms of their resistance to protistan pathogens but also in tolerance of low salinities (J. Small, VIMS, pers. comm.). The XN.LYN diploid line (2 N XB) was bred for resistance to the two most significant oyster parasites along the East Coast of the United States, *P. marinus* and *H. nelsoni*, and for adaptation to moderate salinity waters (18–23 psu). The second diploid line, LOLA (2 N LOLA), was bred for *P. marinus* resistance but with only low to moderate resistance to *H. nelsoni*, and for adaptation to somewhat lower salinity waters (8–15 psu). The two triploid lines were produced by crossing the respective diploid lines above with a tetraploid line, 4MGNL, itself a cross between oysters originating from Virginia and Louisiana, following the method described by Guo et al. (1996). The resulting triploid lines were designated XN.LYN (3 N XB) and LOLA (3 N LOLA).

After being reared at the VIMS Gloucester Point hatchery, oysters from the four lines described above were held in the York River, the study site of this project. Early in May 2018 and May 2019, oysters produced the year prior were distributed into plastic mesh oyster bags to initiate each year's experiment, in which three bags per line each containing 200 oysters were placed on racks in the lower intertidal portion of the York River at VIMS.

2.2. Sample collection and oyster mortality measurements

Samples were collected at five time points in each study year, 2018 and 2019. Annually, sampling started in the spring and ended in the fall in order to capture the period during which *Vibrio* spp. are most abundant in oysters, while also capturing different reproductive stages and varying infection status with regards to *P. marinus* and *H. nelsoni*. Average oyster size measured on the first sampling time point was 41 mm in 2018 and 50 mm in 2019. At each time point, 11 to 12 oysters per line were sampled and processed for histopathological analyses and determination of *Vibrio* levels (on two occasions, one of the sampled oysters had died, with only an empty shell, or "box", remaining). Live and dead oysters were counted at each sampling to determine percent mortality. Water temperature and salinity were measured at time of collection using a hand-held thermometer and refractometer, respectively.

After collection, oysters were placed in a cooler with no direct contact with ice and transported to the laboratory at VIMS adjacent to the study site. In the laboratory, oysters were scrubbed with sterile brushes and were kept in a cold room at 10 °C to maximize survival and recovery of *Vibrio* spp. (Kaysner et al., 2004) until further processing for histopathological and microbiological analyses as described below. Processing of all oysters occurred within six hours of sample collection.

2.3. Oyster sex and general health: histopathological analysis

Oysters were shucked with a flame-sterilized oyster knife, and for each individual, a transverse section including gill, mantle, gonad, digestive gland, stomach and intestine, and associated connective tissues was preserved in Davidson's fixative (Shaw and Battle, 1957). After fixation, these sections were processed using standard paraffin histological methods and a six-micron section was stained with hematoxylin

and eosin. Each histological slide was evaluated on an Olympus BX51 light microscope to characterize oyster sex and general oyster health. Sex of each oyster was noted as *indistinct* when the individual was not identifiable as female, male or hermaphrodite. General oyster health was documented through the measurement of infection status with respect to the major pathogens *P. marinus* and *H. nelsoni*. Intensity of infection was rated categorically with infections rated as *negative* for no detection, *rare* (*R*), *light* (*L*), *moderate* (*M*) or *heavy* (*H*) (Carnegie and Burreson, 2011; Mann et al., 2014).

2.4. Abundance of *V. vulnificus*, total and pathogenic *V. parahaemolyticus*: MPN-qPCR

Vibrio spp. densities in samples were measured using a three-tube, most-probable-number (MPN) approach followed by quantitative PCR (qPCR). After sampling of the transverse section for histology, remaining oyster tissues were weighed, an equal mass of cold phosphate-buffered saline (PBS) was added, and tissues were homogenized individually using a Janke & Kunkel Ultra-Turrax TP 18/10 S9 (IKA-Werk, Wilmington, NC, USA) at ~55 rpm for 30 s as described in Bienlien et al. (2022). Enrichments were set in 96-well plates with an APW volume of 1.5 mL dispensed in each well. Tissue homogenates were serially diluted in PBS to obtain concentrations ranging from 0.5 E-1 to 0.5 E-6 g/mL. For each dilution, 200 μL of the tissue dilutions were inoculated into each of three wells containing APW, leading to inoculate amounts ranging from 1.0 E-1 to 1.0 E-6 g. Plates were covered with a plastic film and were incubated at 35 °C for 18–24 h. After incubation, 50 μL of each enrichment was collected and boiled for 10 min to lyse cells (Jones et al., 2009). 1 μL of this lysate subsequently was used as the template DNA sample in each of the qPCR assays described below. Modifications to the enrichment volume compared to the 10 mL used in standard MPN protocol (U.S. Food and Drug Administration, 2010) were made to facilitate both the processing of a large number of oysters in a few hours and the subsequent analysis of the enrichments by qPCR.

Detection of total *V. vulnificus* was performed by targeting the hemolysin/cytolysin gene (*vvhA*) using the TaqMan assay designed by Campbell and Wright (2003) as described in Audemard et al. (2018), and as modified in Bienlien et al. (2022). Detection of total and pathogenic *V. parahaemolyticus* was performed using the primers and probes described by Nordstrom et al. (2007) with modifications recommended by the FDA Gulf Coast Seafood Laboratory (J. Jones, pers. comm.). Total *V. parahaemolyticus* was detected by targeting the thermolabile hemolysin gene (*tlh*) in a multiplex qPCR assay incorporating an internal amplification control (IAC) to ensure PCR integrity and eliminate false-negative reporting. Detection of *V. parahaemolyticus* pathogenic strains possessing the *tdh* and/or the *trh* gene was conducted in a second qPCR assay that also included an IAC. Results of the qPCRs were used to assess the MPN density values using approved MPN tables (U.S. Food and Drug Administration, 2010). Samples associated with an absence of detection of the targeted gene were given a value of 1.5 MPN/g (half the value of the detection limit of our method, i.e. 3MPN/g).

2.5. Statistical analysis

Data were analyzed using generalized linear mixed-effects models (GLMMs, Breslow and Clayton, 1993; Bolker et al., 2009; Zuur et al., 2009), which extend generalized linear models (GLMs) by including both fixed and random effects. These models can be useful for hierarchical data with autocorrelation due to repeated temporal sampling. The fixed-effect categorical predictor variables considered were sex (excluding hermaphrodites), oyster line, and *P. marinus* infection intensity. *Haplosporidium nelsoni* infection intensity was not included due to the low prevalence of the parasite in the collected oysters (see results below). A set of seven model parameterizations that ranged from the full model (sex, oyster line, *P. marinus* infection intensity) to reduced models (each predictor variable alone) were fitted and Akaike's Information Criterion (AIC; Akaike, 1973; Burnham and Anderson, 2002) was used for model selection. For *tdh* and *trh*, a two-component mixture distribution (zero-altered) that specified a probability of a zero value (non-detection) combined with the probability of a non-zero value was chosen. In this case, the seven model parameterizations were first used to model the probability of a zero-value (denoted as π) while keeping the full model parameterization for the mean of the non-zero values (denoted as μ). Once a form for π was chosen, the seven parameterizations were then used to model μ (see Supplementary Information for more details on model development and fitting).

For models with similar AIC values (typically ΔAIC values <5.0 where $\Delta AIC = AIC - AIC_{min}$), model validation was achieved through analysis of residuals (mean value of residuals and coefficients of skewness and kurtosis) and visual inspection of diagnostics (plots of residuals, QQ-norm plots, and detrended QQ-norm plots also known as worm plots). Since the study design involved two distinct cohorts of oysters where the three bags associated with each cohort were deployed during only one year, not all animals were exposed to experimental conditions during the full study duration. Therefore, to account for seasonal repeated measures of each set of oyster bags during each year, month was included as a random intercept with bag nested within month for all models. Note that in the mixture models for *tdh* and *trh*, the random effect was included for both π and μ model components. From the selected model for each *Vibrio* sp. or strain, marginal mean predictions (Searle et al., 1980) were generated and uncertainty in the form of 95% confidence intervals was based on standard errors approximated using the delta-method. These results differ from traditional statistical testing of effects associated with specific levels of the various categorical predictor variables included in each model which are based on the estimated uncertainty (standard error) associated with each parameter. In contrast, marginal mean predictions incorporate the uncertainty estimates of multiple parameters (though the delta-method variance approximation) and provide a more integrated characterization of the estimated central tendency (median, mean) of the four response variables. And in the cases of *tdh* + and *trh*+, the marginal mean predictions also combine the model component describing absence of strains (probability of a zero observation) with the conditional model (mean concentration given presence). All statistical analyses were conducted using the R software program (R Core Team, 2020).

3. Results

3.1. Environmental conditions

Water temperatures measured during this study were warmest at August and September time points in 2018 (26 °C on both 6 August and 4 September) and at July and September time points in 2019 (24 °C on 8 July and 23 °C on 4 September). As expected, water temperatures were lower at earlier time points, in May and June (20–25 °C), or later in October and November (13–22 °C).

Salinity was abnormally low in 2018 due to heavy precipitation in the region, ranging from 13 psu (24 May) to 16 psu (4 September and 2 October). The salinities recorded in 2019 were more typical for this study site, especially during the later sampling time points, ranging from 14 psu (8 July) to 22 psu (4 September and 4 November).

3.2. Oyster sex

A total of 478 oysters were collected during this study. While sex ratios varied between time points and lines, consistent patterns across lines included a predominance of males in May 2019, and a predominance of oysters with no gonadal development (categorized as *indistinct*) in November 2019 (Fig. 1A). During the warmer months, both triploid lines and the 2 N LOLA line had a higher proportion of females than males, however, a different trend was observed for 2 N XBs, with males tending to be more abundant than females. Hermaphrodites were

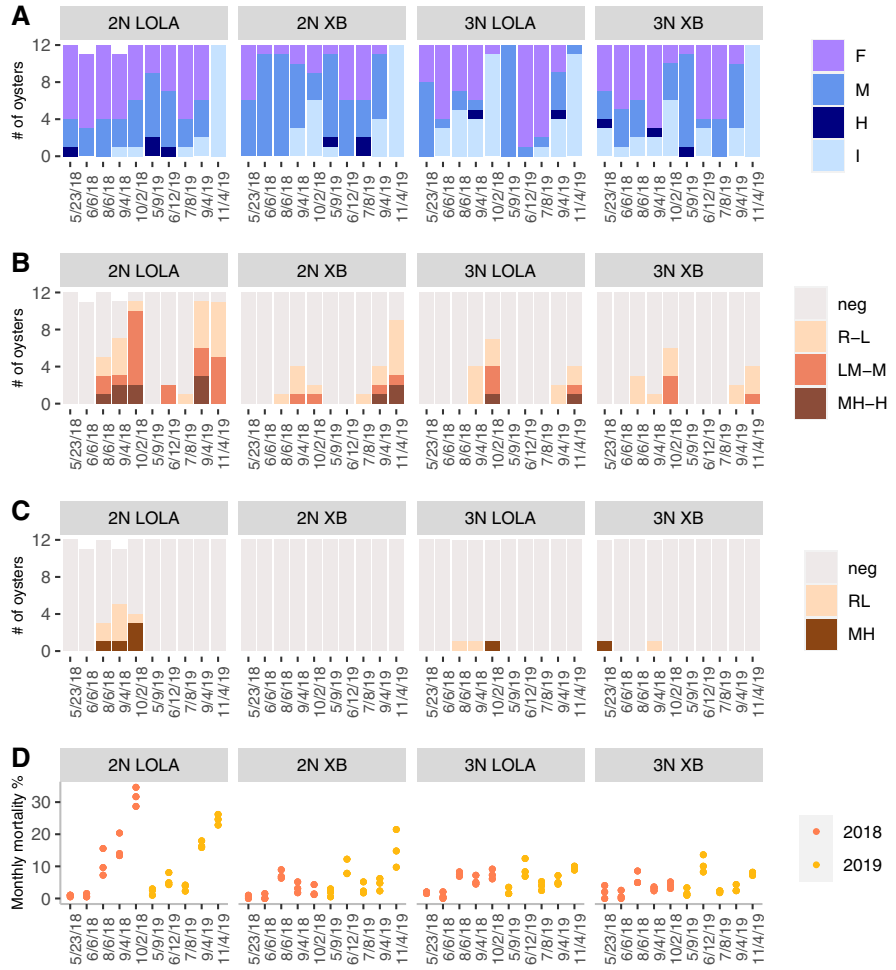

Fig. 1. Characterization of oyster sex (A), *Perkinsus marinus* infection intensity (B), *Haplosporidium nelsoni* infection intensity (C), and percent monthly mortality (D) in each of the oysters collected during the study. Categories for sex included: female (*F*), male (*M*), hermaphrodite (*H*), and indistinct (*I*). For each parasite, absence of detection was indicated by *neg*, and when infection was detected intensity was noted as *rare* (*R*), *light* (*L*), *moderate* (*M*) or *heavy* (*H*).

occasionally observed but only in <3% of all collected oysters (12 out of 478).

3.3. Oyster parasitism and mortality

Overall prevalence of *P. marinus* in oysters collected in this study was 21% (102/478). The parasite was detected in all oyster lines but prevalence and infection intensity of the parasite varied with oyster line (Fig. 1B). The highest prevalence was detected in the 2 N LOLA line. As expected, increased prevalence and increased infection intensity of *P. marinus* was observed in the fall compared to the earlier sampling time points. Infection intensity ranged from rare to heavy and again the prevalence of light to moderate (*LM-M*) and moderate-heavy to heavy infections (*MH-H*) was highest in 2 N LOLAs. *Haplosporidium nelsoni* was detected in <4% (17/478) of the oysters collected in the study with most of these infections (12/17) observed in 2 N LOLAs (Fig. 1C).

Mortality measured at each sampling time point was generally <21% (the value measured in 2 N XB on 4 November 2019), except in the 2 N LOLAs, which experienced higher mortality than the other lines in both years (Fig. 1D). Monthly mortality in the 2 N LOLAs peaked in the fall of both years and reached a maximum value of 35% on 2 October 2018.

3.4. Vibrio spp. prevalence and abundance in individual oysters

Both total *V. vulnificus* and total *V. parahaemolyticus* were highly prevalent in the oysters collected in this study. Total *V. vulnificus* was detected in 99.6% of oysters (476 of 478) and total *V. parahaemolyticus* was detected in all the collected oysters. During the study, concentrations of total *V. vulnificus* in individual oysters ranged from no detection to concentrations as high as 394,000 MPN/g (Fig. 2A). Concentrations of total *V. parahaemolyticus* ranged from 9.2 to 731,000 MPN/g (Fig. 2B). For both species, concentrations in oysters tended to be lower when water temperature was also lower, i.e., in May, October and November, compared to the other time points.

Vibrio parahaemolyticus tdh + strains were detected in 48% (238 of 478) of the collected oysters and *V. parahaemolyticus trh* + strains were detected in 64% (308 of 478). Concentrations of *V. parahaemolyticus tdh* + strains ranged from no detection to 1470 MPN/g observed on 6/12/19 in one 2 N LOLA oyster (Fig. 2C). For *trh*+, concentrations ranged from no detection to 1500 MPN/g observed in one 2 N LOLA and one 2 N XB oyster collected on 5/23/2018 (Fig. 2D). One additional high *trh* + value of 1200 MPN/g was observed on the same date in one 2 N LOLA individual. The dynamics of concentrations of these strains differed from total *V. parahaemolyticus* dynamics since these strains were more abundant in early summer (May and June) compared mid or late summer. While this trend was observed in both years of the study, the tendency for these pathogenic strains to be less abundant in 2019 compared to 2018 made this pattern less discernable in 2019.

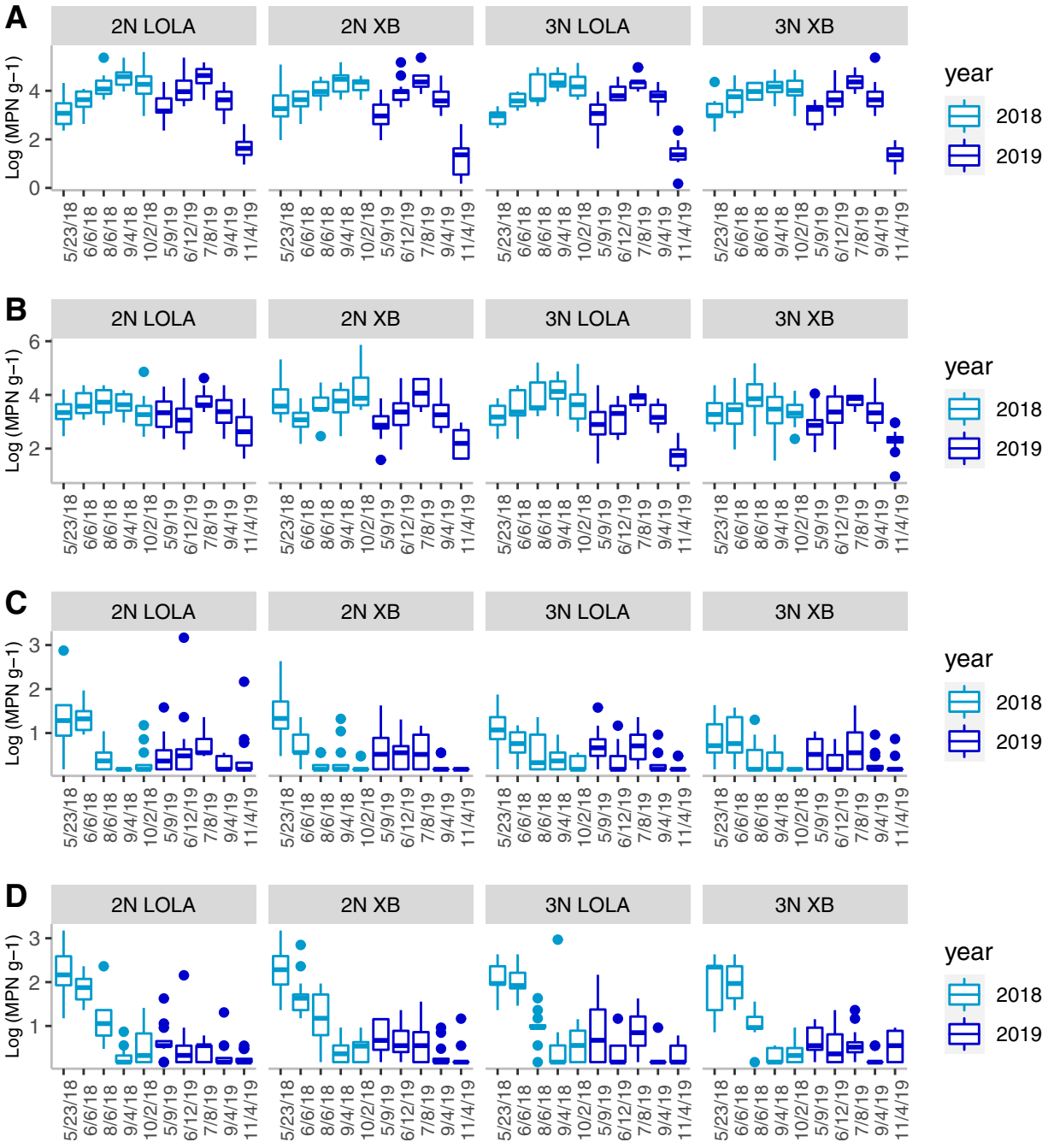

Fig. 2. Boxplots showing the concentrations (log_{10} MPN/g of tissue) of each *Vibrio* species or strain measured in each oyster line and at each time point during the study; total *V. vulnificus* (*vvhA*) (A), total *V. parahaemolyticus* (*tlh*) (B), and pathogenic *V. parahaemolyticus* strains possessing the *tdh* gene (C) or the *trh* gene (D).

3.5. Statistical analysis

The combination of variables included in the highest-ranking models differed across the *Vibrio* targets, however, each of these models consistently included oyster line among the predictor variables, highlighting the importance of genetic lineage with respect to *Vibrio* (Table S2). The model identified for *vvhA* was among the most simplistic, with oyster line included as the sole predictor. The model identified for *tlh* was the most complex and included oyster line, sex and *P. marinus* infection intensity. For *vvhA*, parameter estimates showed that the highest median concentrations occurred in 2 N LOLAs, and that median concentrations in the other lines relative to 2 N LOLA were all lower, with statistical significance detected for 3 N XB (Table S3). For *tlh*, estimated effects across the categories of the other predictor variables were relative to 2 N LOLA females with light to medium *P. marinus* infection intensity (*LM-M*). Within this comparative framework, the estimated median concentrations in oysters of indistinct sex and in males were both lower, with the latter being statistically significant. Estimated effects for all other oyster lines were lower and statistical significance was associated with the two triploid lines (Table S3). Parameter estimates across levels of *P. marinus* infection intensity indicated statistically significantly higher median concentrations in oysters with lighter infection levels (*R-L* and *negative*).

In the case of *tdh*+, the model identified for π included sex as a predictor of the absence of strains possessing this gene (Table S2), such that results showed the probability of absence in both *indistinct* and males was higher than in females, with the effect for males being statistically significant (Table S3). The model identified for μ included sex

and oyster line as predictors where female 2 N LOLA line was again the reference. Parameter estimates suggested that when *tdh* + was present, mean concentrations were statistically significantly lower in oysters of indistinct sex, and also lower, though not rising to statistical significance, in males of the 2 N LOLA line (Table S3). Across the various oyster lines, mean concentrations of *tdh* + were all lower than that of 2 N LOLA, with the negative effect associated with 3 N LOLA being statistically significant.

For *trh*+, the model for π did not include any fixed-effect predictor variables (intercept-only), and the model for μ included oyster line and *P. marinus* infection intensity (Table S2). In contrast to the output derived for the other gene targets, parameter estimates indicated that when present, mean *trh* + concentrations were statistically significantly higher in all other lines when compared to 2 N LOLAs with light to moderate *P. marinus* infection intensity (*LM-M*), and highest in 3 N LOLAs (Table S3). When comparing the effects of different *P. marinus* intensity levels within 2 N LOLAs, results showed lower mean concentrations in oysters with all other infection intensities when compared to *LM-M*. Thus, the pattern of mean *trh* + was non-monotonic across levels of increasing *P. marinus* infection intensity, but statistical significance was detected only with oysters with no infection (*negative*).

Uncertainty in the marginal mean predictions, as expressed through 95% confidence intervals associated with the predictor variables identified in the selected model for each target, are shown in Fig. 3. The results associated with each target/predictor variable combination highlight overlapping error bars in a majority of cases. Overall, similar trends as observed through the analysis of the parameter estimates were observed. 2 N LOLAs tended to be associated with increased predicted median of total *V. vulnificus* and total *V. parahaemolyticus* compared to other lines and in particular with 3 N XB (Fig. 3A, B). For *tdh* + and *trh*+, the trend differed between the two strains with the *tdh* + predicted mean tending to be higher in 2 N LOLAs and lower in 3 N LOLAs whereas *trh* + showed an opposite trend (Fig. 3C, D). With regard to the effect of *P. marinus*, predicted medians for total *V. parahaemolyticus* (Fig. 3E) showed the most discernable trend with higher predicted medians in oysters with no *P. marinus* detection (*negative*) to *light* infections (*R-L*) compared to oysters with *light-moderate* (*LM-M*) to *heavy* (*MH-H*) infections. For *trh*+, a somewhat opposite trend was observed since predicted means tended to be lower in *negative* oysters compared to infected individuals, especially compared to light to moderately (*LM-M*) infected animals, so as stated above this represents a non-monotonic pattern (Fig. 3F). Finally, predictions suggested lower abundance of total *V. parahaemolyticus* in males compared to females and oysters of indistinct sex (Fig. 3G), or in males and indistinct oysters compared to

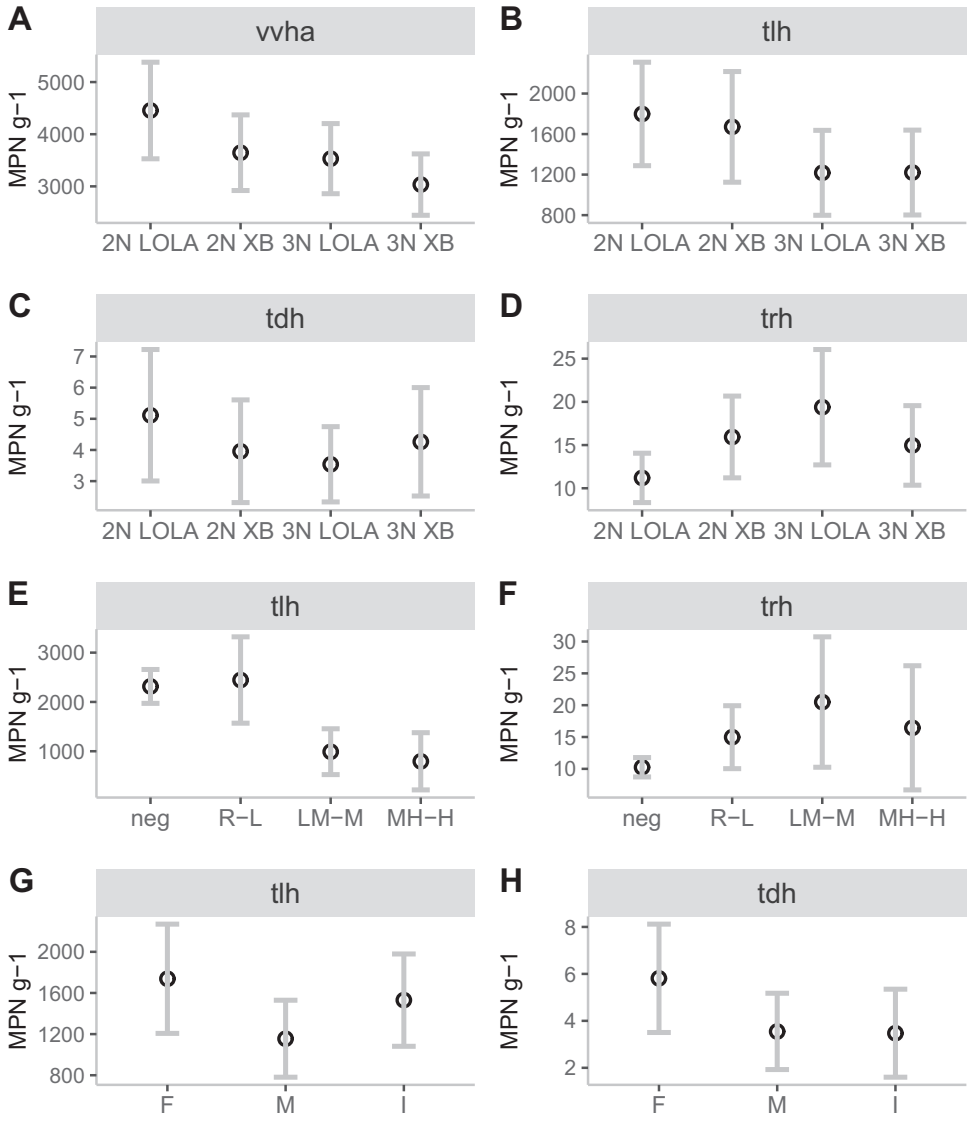

Fig. 3. Model predictions of median total *V. vulnificus* (*vvhA*) and total *V. parahaemolyticus* (*tlh*), or mean pathogenic *V. parahaemolyticus tdh* + and *trh* + over the observed domain of the identified predictor variables i.e., oyster line (A, B, C and D), *P. marinus* (E and F) or sex (G and H). Error bars are 95% confidence intervals. Categories for sex included: female (*F*), male (*M*), and indistinct (*I*). For *P. marinus*, absence of detection was indicated by *neg*, and categories for infection intensity were noted as *rare* (*R*), *light* (*L*), *moderate* (*M*) or *heavy* (*H*).

females for *tdh* + (Fig. 3H).

4. Discussion

From this study, a novel hypothesis emerges that oyster genetics may be important in influencing levels of total *V. vulnificus* and total and pathogenic *V. parahaemolyticus* in oysters. This hypothesis is supported by the observation that for each of the *Vibrio* targets analyzed, the highest ranked model included oyster line. This variable was identified as the sole predictor in the model associated with total *V. vulnificus*, and was a predictor in combination with other factors i.e., oyster sex or infection by *P. marinus*, in the case of the other targets. Oyster genetic background, here evaluated through the deployment of oyster selected lines, may influence not only general microbial communities (Wegner et al., 2013; de Lorgeril et al., 2018; King et al., 2019; Dupont et al., 2020; Nguyen et al., 2020; Scanes et al., 2021) but also levels of human-pathogenic *Vibrio* spp. in oyster tissues. This study also suggests that the concentration of human-pathogenic *Vibrio* spp. in oysters is likely not driven by a single oyster intrinsic factor, but rather by a combination of factors, some of which may be under the influence of oyster genetics.

Oysters from the 2 N LOLA line tended to be associated with higher abundance of total *V. vulnificus*, total *V. parahaemolyticus* and, although not as clearly, of *tdh* + compared to the other oyster lines. Interestingly, 2 N LOLAs tended to be associated with higher mortality levels and higher parasite infection levels compared to the other lines, so this line appeared to be less well adapted than the other oyster lines to conditions associated with our study site. This line was bred specifically for lower salinity waters (< 15 psu), so part of the stress experienced by these oysters may have arisen from exposure to moderate salinities or to conditions associated with moderate salinities at our study site. For example, parasite infection levels suggest that these animals were more acutely challenged by *P. marinus* and *H. nelsoni* compared to the other lines. With these measures of health in mind, we ask whether oyster immune status could influence not only the abundance of shellfish-pathogenic *Vibrio* spp. (Petton et al., 2015; Travers et al., 2015; Le Roux et al., 2016; de Lorgeril et al., 2018; Destoumieux-Garzón et al., 2020), but also the abundance of human-pathogenic *Vibrio* spp. This holistic approach considering the overall health of the oyster as a key parameter influencing its safety for human consumption with regard to human-pathogenic *Vibrio* spp. has been hypothesized earlier, but was never tested (Lacoste et al., 2001; Clarke, 2001). While oyster health may influence human-pathogenic *Vibrio* spp. dynamics within oyster tissues through altered immune functions, it can also be hypothesized that oyster health may have an indirect effect on *Vibrio* spp. through a potential influence on oyster physiology or behavior. Health impacts on filtration rate in particular could affect the uptake but also potentially the depuration or purging of these bacteria from oyster tissues. To our knowledge, the influence of oyster health on oyster behavior, specifically on oyster feeding or filtration, has not been investigated. In light of our study, further investigations would be warranted considering the potential impact on *Vibrio* spp. levels.

Our study suggests that the intersection between oyster health and levels of human-pathogenic *Vibrio* spp. should be further investigated but also raises the question of the impact of human-pathogenic *Vibrio* spp. on oyster health. The few studies evaluating the association of *Vibrio* spp. with *C. virginica* disease have been conducted in hatchery settings (Tubiash et al., 1965; Richards et al., 2015), so more research would be needed to assess the potential contribution of *Vibrio* spp. including strains of *V. parahaemolyticus* and *V. vulnificus* to mortalities of this oyster species. The results associated with *trh*+, however, suggest that the response of *Vibrio* spp. to oyster stress may depend on the *Vibrio* species or strain and that caution should be used before drawing general conclusions regarding the influence of oyster genetics and health on these pathogens. Indeed, for *trh* + predictions suggested a lower concentration in 2 N LOLAs compared to the other lines, thus an opposite trend to that observed for the other species or strains. As shown by Matz et al. (2011) during a study focusing on *V. parahaemolyticus* strains, genetic differences among strains can be associated with differential environmental fitness. Therefore, it is conceivable that *tdh* + and *trh* + strains may be associated with a different response to the 'state' of the oyster habitat.

For three of our *Vibrio* targets, the model chosen included either *P. marinus* infection, sex, or both variables in addition to oyster line suggesting that multiple factors could be influencing human-pathogenic *Vibrio* spp. levels in oysters. A potential influence of parasitism by *P. marinus* was observed for total *V. parahaemolyticus* and *trh*+, but not for *tdh* + or total *V. vulnificus*. The known pathological impact of *P. marinus* on its host (Carnegie and Burreson, 2012) would reinforce the above hypothesis of oyster health as a parameter driving *Vibrio* spp. abundance in oysters, however our results as well as previous studies suggest a more complex relationship. Based on the results observed for total *V. parahaemolyticus* and *trh*+, the type of association between *P. marinus* infections and human-pathogenic *Vibrio* spp. may be species or strain specific. Results for these two *Vibrio* targets not only diverge but also seem to contradict results obtained by Bienlien et al. (2022), who found no association between infection by this parasite and any of these bacteria. The study of Bienlien et al. (2022) focused on a single oyster line, 2 N XB, while our study included four lines, but also included more animals and a more thorough investigation of the variables analyzed using generalized linear mixed-effects models. As mentioned above, such differences in response or, in the case of total *V. vulnificus* and *tdh*+, a lack of response, may be explained by species and strain specific differences. Alternatively, the response to *P. marinus* may be indirect and may be modulated by other factors including oyster genetics and sex as seen in this study, but also by factors that were not measured. Such complex interactions with this parasite may partly explain the contrasting results obtained in this study and in previous studies which showed either an absence of association between *P. marinus* and both *V. vulnificus* and *V. parahaemolyticus* (Sokolova et al., 2005; Bienlien et al., 2022), a negative relationship between *P. marinus* and both *V. vulnificus* and *V. parahaemolyticus* (Carnegie et al., 2013), or a positive relationship between *P. marinus* and *V. vulnificus* (Tall et al., 1999). It has to be acknowledged that differences in terms of experimental design, oyster genetics, as well as methods of detection of the parasite and of the *Vibrio* spp. may also explain discrepancies among results. While suggesting that parasitism by *P. marinus* alone is not sufficient to explain oyster intrapopulation differences in *Vibrio* levels, our study also suggests that this factor cannot be discounted, especially in the case of total *V. parahaemolyticus*.

Oyster sex was identified as a predictor variable for total *V. parahaemolyticus* and for *tdh*+. Model parameter estimates suggested that females and *indistinct* individuals were associated with higher total *V. parahaemolyticus* but lower *tdh* + concentrations compared to males. Besides the fact that gonadal tissues of a male or female oyster are different and may be represent different 'habitats' for these bacteria, differential immune capacity among males and females has been reported in previous studies on other bivalve species (Duchemin et al., 2007; Dang et al., 2012; Hurtado-Oliva et al., 2015; Lu et al., 2021) and also may explain such observations. Oxidative metabolism (Dang et al., 2012, Hurtado-Oliva et al., 2015) and phagocytosis (Duchemin et al., 2007) were shown to be influenced by sex, however, the response was found to vary not only depending on the species analyzed but also on the gonadal maturation stage. To our knowledge the influence of sex on the immune response in *C. virginica* has not been investigated, so more work is needed to assess whether there is an association between sex and immune response, and if there is an effect on levels of human-pathogenic *Vibrio* spp. With this in mind, our analysis focusing on sex and *indistinct* individuals appears to be limited, and as suggested by Bienlien (2016), further studies will be needed to fully document the influence of gonadal maturation. Finally, as observed previously (Guo et al., 1998), our study suggests that an influence of oyster genetics on sex ratios, in particular with in light of the sex ratios observed in our diploid lines. This again

suggests that oyster genetics, due to its effects on overall health and physiology, may be a key factor to consider as we design future studies aimed at better understanding the dynamics of human-pathogenic *Vibrio* spp. in oysters.

Our analysis did not reveal a clear influence of ploidy on levels of human-pathogenic *Vibrio* spp.; rather, as stated above, it suggested that the genetic background of the oyster line should be considered as a potential predictor. In our study, crossing either 2 N LOLA and 2 N XB with the tetraploid increased the hardiness in the triploid progeny as gauged by mortality rates and parasitism. However, the differences seen among the two diploid lines in terms of *Vibrio* spp. responses suggest that ploidy is not the primary factor driving these differences. Previous studies reporting a tendency for triploids to be associated with lower *Vibrio* spp. levels than diploids (Walton et al., 2013; Grodeska et al., 2019) may also have revealed a trend that was not associated with the ploidy per se but with the specific lines studied. However, because only one diploid and one triploid line were studied, the potential influence of genetics (or lines) could not be evaluated in these studies.

As seen in previous studies (DePaola et al., 2003; Kaufman et al., 2003; Klein and Lovell, 2017; Marcinkiewicz et al., 2017; Bienlien et al., 2022), within each time point, individual levels of total *V. vulnificus*, total *V. parahaemolyticus*, *tdh* + and *trh* + varied over several orders of magnitude. Across times points, the influence of temperature on levels of total *V. vulnificus* and *V. parahaemolyticus* was observed despite the absence of time points within the colder months. The total mean levels of these species tended to be lower in the spring and fall when temperatures were lower compared to the summer. A different dynamic was observed for *V. parahaemolyticus tdh* + and *trh* + strains, which tended to occur at higher mean abundance in the spring and early summer compared to late summer. Differences in the dynamics of total *V. parahaemolyticus* and pathogenic strains has been observed in other locations (DePaola et al., 2003; Zimmerman et al., 2007; Johnson et al., 2012; Williams et al., 2017; Flynn et al., 2019), and this study continues to emphasize the need to measure pathogenic strains rather than only total *V. parahaemolyticus* in the context of public health but also to gain a better understanding of the ecology of these particular strains.

5. Conclusion

Our results suggest that oyster genetics influence levels of human-pathogenic *Vibrio* spp. in oysters. Factors at least partly controlled by genetics such as resistance or tolerance to parasites and sex were included as potential predictors suggesting that their effect should be further investigated. Interestingly, because of the observed link between oyster line and oyster health, our study raised the question whether stressful conditions for an oyster, either due to poor adaptation of the oyster to an environment (as seen in this study), or lack of resilience to a changing environment, may favor increased levels of human-pathogenic *Vibrio* spp. in oyster tissues. This study also continues to underscore that intrinsic levels of these human pathogens in oysters are likely driven by a combination of factors rather than by a single one.

CRediT authorship contribution statement

Corinne Audemard: Conceptualization, Methodology, Formal analysis, Funding acquisition, Investigation, Visualization, Writing – original draft. **Kimberly S. Reece:** Conceptualization, Methodology, Funding acquisition, Investigation, Writing – review & editing. **Robert J. Latour:** Methodology, Formal analysis, Visualization, Writing – review & editing. **Lydia M. Bienlien:** Methodology, Writing – review & editing. **Ryan B. Carnegie:** Conceptualization, Methodology, Funding acquisition, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests of personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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