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Influence of Gonadal Stage and Ploidy on Human-Pathogenic Vibrio Levels in the Oyster Crassostrea virginica - Final Report

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FINAL REPORT

Influence of Gonadal Stage and Ploidy on Human-Pathogenic Vibrio Levels in the Oyster *Crassostrea virginica*

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OBJECTIVES

To determine whether oyster reproductive status and particularly ploidy influences levels of human-pathogenic *Vibrio* spp. in oyster tissues was the overall aim of this project.

Our project objectives were three-fold:

1) To determine the relationship between oyster gonadal stage and levels of human-pathogenic *Vibrio* spp. in two separate pairs of genetically related diploid and triploid oysters through analyses of individual oysters conducted during peak *Vibrio* season in the York River, Virginia;
2) To determine the relationship between oyster ploidy and levels of human-pathogenic *Vibrio* spp. in those same oysters; and
3) To determine the degree to which farm-level marine aquaculture practices change based on results of this project.

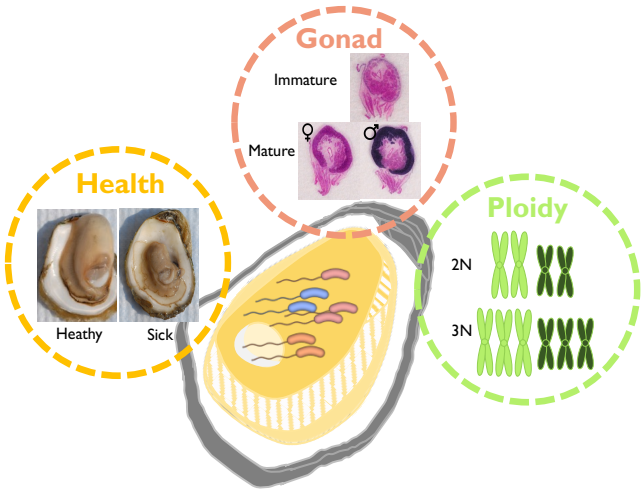

Fig. 1: Schematic presentation of the study focus.

MATERIALS AND METHODS

Sampling design

The oysters used for this project were produced by VIMS Aquaculture Genetics and Breeding Technology Center (ABC). A total of 4 lines were spawned in July 2017 and in July 2018, including 2 diploid lines and 2 triploid lines. More specifically, the following lines were chosen: 1/ a diploid line XN.LYN (2N XB) adapted to moderate salinity waters and bred for resistance to the two main oyster parasites along the East Coast of the United States, *Perkinsus marinus* and *Haplosporidium nelsoni*; 2/ a diploid line LOLA (2N LOLA) bred for *P. marinus* resistance as well as some *H. nelsoni* resistance but more tolerant of lower salinity waters; 3/ a triploid line representing a cross between the above diploid line XN.LYN and a tetraploid line, 4MGNL (3N XB); and 4/ a triploid line produced by crossing the above diploid LOLA line and the 4MGNL tetraploid line as above (3N LOLA).

After being reared at VIMS hatchery, oysters were held in the York River, the study site of this project. Early in May 2018 and May 2019, oysters produced the year before were distributed into plastic mesh oyster bags to initiate each year's experiment, with 3 bags per line, each containing 200 oysters, placed on racks in the lower intertidal of the York River at VIMS.

Samples were collected at each of 5 time points in 2018 and again in 2019. Sampling started in the Spring and ended in the Fall in order to capture the period during which *Vibrio* spp. are abundant in oysters, while also capturing different gonadal stages of oyster reproductive development. At each time point, 11 to 12 oysters per line were sampled and processed for histology and *Vibrio* levels (on 2 occasions, one of the oysters was a box, i.e. had died, and could not be processed). Water temperature and salinity were measured at time of collection.

Oyster gonadal stage and general health: histological analysis

Oysters were scrubbed of external fouling, measured using calipers (shell height, mm), and then shucked with a flame-sterilized oyster knife. Transverse sections including gill, mantle, gonad, digestive gland, stomach and intestine, and associated connective tissues were fixed in Davidson's fixative (Shaw and Battle 1957) and processed using standard paraffin histological methods. Six-micron sections were stained with hematoxylin and eosin and evaluated on an Olympus BX51 light microscope.

Sex of each oyster was noted and stage of gonadal development observed in this study was identified based on the categories described in Table 1.

Table 1: Categorical staging of oyster gonads.

Ploidy	Code	Description
2N or 3N	*I*	*Indifferent*, no gonadal development
2N or 3N	*H*	Hermaphrodite
2N	*M*	*Mature* gonad with mature gametes
2N	*P*	Post-spawn gonad
3N	*b*	Gonad occupies *limited* area of tissues with *no or few* mature gametes
3N	*bM*	Gonad occupies *limited* area of tissues with *abundant* mature gametes
3N	*a*	Gonad occupies *extensive* area of tissues with *no or few* mature gametes
3N	*aM*	Gonad occupies *extensive* area of tissues with *abundant* mature gametes

The categories were different for diploid and triploid oysters reflecting the differences observed in terms of gonadal development associated with ploidy (Matt and Allen 2021). Oysters with no gonadal development observed were categorized as *indifferent* and hermaphrodites were observed in both diploid and triploid oysters.

Infection status with respect to the major pathogens *Perkinsus marinus* and *Haplosporidium nelsoni* was determined histologically, and intensity of infection was ranked based on the scale shown in Table 2. Intensity was rated as *rare*, *light*, *moderate* or *heavy* based on the Carnegie lab's standard rubric for each category (Carnegie and Burreson 2011, Mann et al. 2014), to provide additional perspective on the relationship between parasite infection and *Vibrio* spp. levels in individual oysters.

Table 2: Categorical staging of *Perkinsus marinus* and *Haplosporidium nelsoni* infections.

Parasite	Code	Description
Perkinsus marinus	*neg.*	No detection
	R-L	*Rare* to *Light* infection
	LM-M	*Light Moderate* to *Moderate* infection
	MH-H	*Moderate Heavy* to *Heavy* infection
Haplosporidium nelsoni	*neg.*	No detection
	R-L	*Rare* to *Light* infection
	L-M, MF	*Light* to *Moderate* infection, *Multi-Focal*
	M, S	*Moderate* infection, *Systemic*
	H, S	*Heavy* infection, *Systemic*

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

Vibrio spp. densities in samples was measured using a three-tube most-probable number (MPN) approach followed by quantitative PCR (qPCR) as previously described (Bienlien 2016). After sampling of the transverse section for histological analysis, 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) at ~55 rpm for 30 s (Bienlien 2016). In between homogenizations of different samples, the part of the Ultra-Turrax in contact with the tissues was washed in a bleach solution and rinsed three times in sterile water to prevent cross-contamination. Each oyster tissue homogenate was inoculated into an alkaline peptone water (APW) MPN series and incubated at 35°C for 18-24 h (U.S. FDA 2010, Kaysner & DePaola 2004). A 1-ml volume was removed from the top cm of each APW enrichment tube and boiled for 10 min to lyse cells (Jones et al. 2009), with this lysate subsequently used as the template DNA sample in each of the qPCR assays described below.

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. (2011) except that TaqMan® Fast Advanced Master Mix was used. Detection of total and pathogenic *V. parahaemolyticus* was performed using the primers and probes described by Nordstrom et al. (2007) with the 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 include an IAC. Results of the qPCRs were used to assess the MPN density values using approved MPN tables (US FDA 2010). Samples associated with an absence of detection of the gene targeted were given a value of 1.5 MPN/g (half the value of the detection limit of our method, i.e. 3MPN/g).

Statistical analysis

Data were analyzed using generalized linear mixed-effects models (GLMMs), 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. GLMMs are expressed as:

$$g\big(E(\mathbf{y}|\mathbf{b})\big) = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{b}$$
$$\mathbf{b} \sim \mathrm{N}(\mathbf{0}, \boldsymbol{\Sigma})$$

where $E(\mathbf{y}|\mathbf{b})$ is the expected value of the response vector $\mathbf{y}$ conditioned on the vector of random effects $\mathbf{b}$ that are independent and identically normally distributed, $\mathbf{X}\boldsymbol{\beta}$ is the fixed-effect linear parametric model component, $\mathbf{Z}\mathbf{b}$ is the random-effect model component, $\boldsymbol{\Sigma}$ is the variance-covariance matrix of the random effects, and g is the monotonic link function.

Statistical models were fitted to the generalized additive models for location, scale, and shape (GAMLSS) regression framework (Stasinopoulos et al. 2017) implemented through the *gamlss* package in R (R Core Team, 2020). GAMLSS models extend generalized linear and additive mixed effects models in two important ways: (i) a wider range of distributions for the response variable are allowed compared to those included in the exponential family, including distributions that are heavy- or light-tailed, and positively or negatively skewed; and (ii) all parameters of the distributions, namely location (mean or median), scale (variance), and shape (skewness and kurtosis), can be modeled as linear or smooth functions of predictor variables.

For each *Vibrio* spp. or strain measured in this study, two types of models were explored. In ***environmental models***, water temperature and salinity, which are known predictors of human-pathogenic *Vibrio* spp. abundance (Takemura et al. 2014), were included in association with various combinations of the variables associated with oysters (gonad stage, sex, oyster line, *P. marinus* infection intensity and *H. nelsoni* infection intensity). In this case, we asked whether oyster related variables may improve models based on water temperature and salinity, alone or in combination. In the second type of models, which we refer to as ***oyster models***, water temperature and salinity were not included as potential predictors, but month was used as a factor in addition to the oyster variables described above. We asked whether oyster variables can be a predictor of the levels of *Vibrio* spp. measured in this study. For each type of model, sampling time point was a random factor and a variety of model parameterizations were fitted to develop the most parsimonious description of the data. Ploidy being correlated to oyster lines, ploidy was not used as a predictor variable but oyster line was included. Hermaphrodites were excluded from the analysis due to the difficulties in attributing a gonadal developmental stage to these oysters.

Due to the high frequency of non-detection for both *tdh* and *trh* genes, a hurdle model approach was used. In this approach, the final models and associated predictions are the results of 2 steps: a first step in which models are built for binomial data (*0* for non-detect and *1* for detection of the target) using a binomial distribution, and a second step in which only the detectable concentrations are modeled using the most appropriate distribution. The final prediction is a product of the predictions associated with each of these steps.

Model selection and statistical inference was guided by Akaike's Information Criterion (AIC), the Bayesian Information Criterion (BIC), and standard model diagnostics such as QQ plots, detrended quantile-quantile plots also known as worm plots, and analyses of residuals. Marginal mean predictions (Searle et al. 1980) was generated from the selected model and uncertainty in the form of confidence intervals of predictions was estimated by applying the delta-method approximation (Dorfman 1938).

RESULTS

Environmental conditions

Water temperatures measured at each sampling time point ranged from 21˚C (6 June) to 26˚C (6 August and 4 September) in 2018, and from 14˚C (4 November) to 24˚C (8 July) in 2019. Salinity ranged from 13 psu (24 May) to 16 psu (4 September and 2 October) in 2018, and from 14 psu (8 July) to 22 psu (4 September and 4 November) in 2019.

Oyster sex and gonad

A total of 478 oysters were collected in this study. Sex ratios varied between time points and lines. The only consistent patterns across lines consisted of a predominance of males in May of 2019, and a predominance of oysters with no gonadal development (categorized as *indifferent*) in November 2019 (Fig. 2a). Hermaphrodites were only rarely observed.

Oyster gonadal development in diploids showed a predominance of mature individuals (*MS*) from May through August, followed by a predominance of the post-spawn (*P*) individuals in September, and an absence of development (again, *indifferent*) in October or early November (Fig. 2b). These observations aligned with the expected *C. virginica* reproductive cycle with spawning occurring in the summer and a cessation of spawning and resorption of gonad when temperature decreases in the Fall. For triploids, most observed gonadal development occupied a relatively small area of the tissues (development categorized as *b* or *bM*), with the exception of the May sampling time point in 2019 where most gonadal development occupied an extensive area of the tissues (categorized as *a* individuals). Mature gametes (*aM* or *bM*) were observed in triploids during this study, in particular in June, August and September in both studied years.

Oyster parasitism

Perkinsus marinus, the most significant oyster pathogen in our region and along the East and Gulf Coasts of the US, was detected in all oyster lines but prevalence and infection intensity of the parasite varied with the oyster line (Fig. 2c). 2N LOLAs were associated with higher prevalence (11/12 oysters positive for the parasite in October 2018 and in September and

October 2019) compared to the other lines. 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 higher in 2N LOLAs compared to the other lines. *Haplosporidium nelsoni* was only detected in 2018, and interestingly, its prevalence was highest in 2N LOLAs (Fig. 2d).

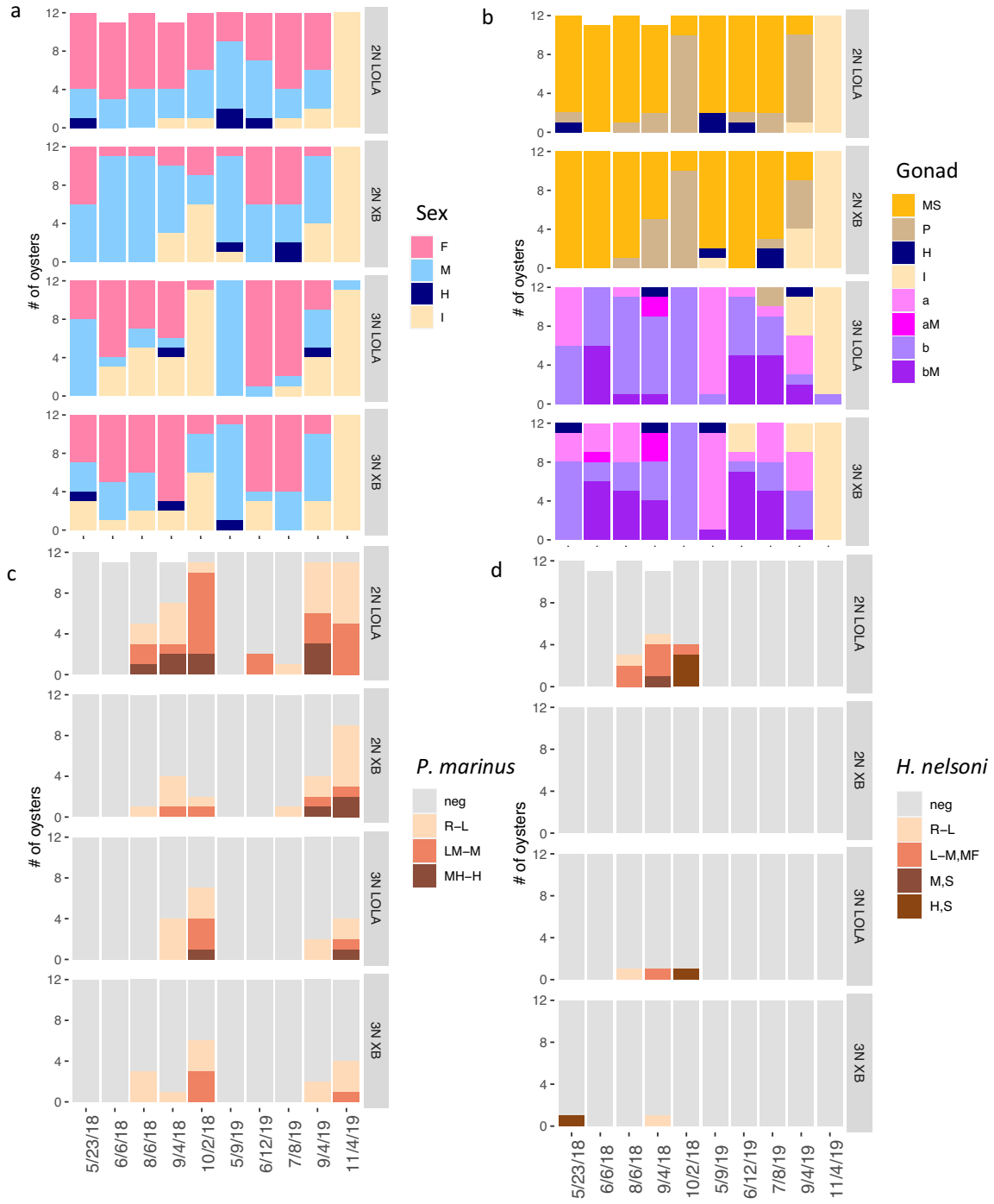

Fig. 2: Characterization of oyster sex (**a**), oyster gonadal development (**b**), *Perkinsus marinus* infection intensity (**c**) and *Haplosporidium nelsoni* infection intensity (**d**) in each of the oysters collected during the study. Details regarding the listed categories can be found in Table 1 and Table 2.

Vibrio spp. prevalence and abundance in individual oysters

Total *V. vulnificus* (*vvhA* gene) was detected in all oysters except two that were collected in November of 2019 when temperature was its lowest during the study (14˚C). Total *V. parahaemolyticus* (*tlh gene*) was detected in all collected oysters. *Vibrio parahaemolyticus tdh+* strains were detected in 48% (238 out of 478) of the collected oysters and *V. parahaemolyticus trh+* strains were detected in 64% (308 out of 478) (Fig. 3).

In 2018, the highest levels of strains carrying *tdh* were detected in May and June across all lines. In 2019, the highest levels of strains carrying this gene were observed in May, June and July (Fig. 3a). The patterns observed for *tdh* strains were also observed for strains carrying the *trh* (Fig. 3b) gene although in 2018, *trh* detectability was also very high in August in addition to May and June, and as noted above strains with this gene were detected more frequently overall than those with the *tdh* gene in both studied years.

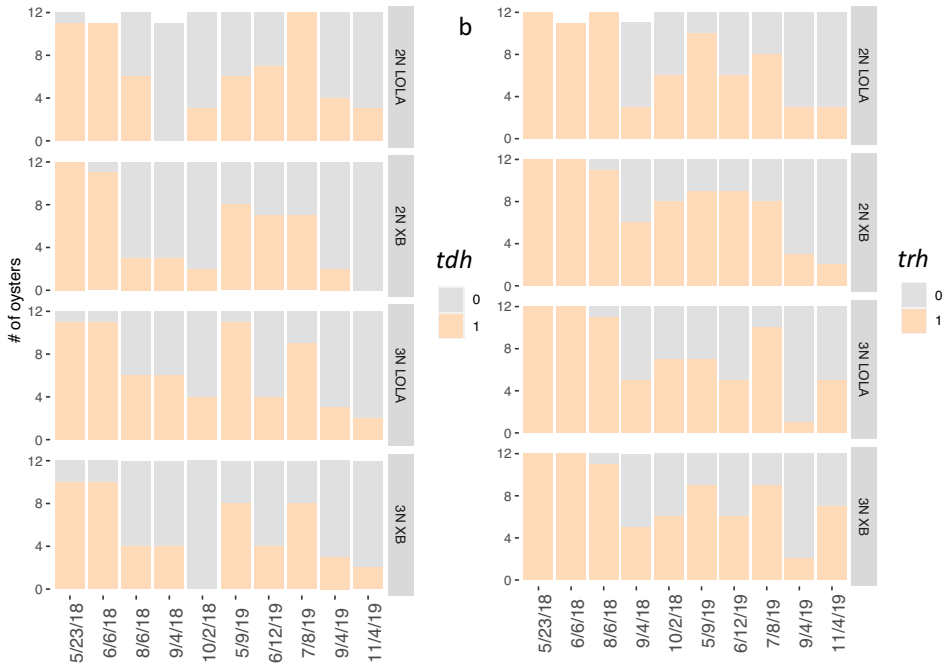

Fig. 3: Presence (*1*) or absence (*0*) of detection of *V. parahaemolyticus* strains carrying the *tdh* (**a**) or the *trh* gene (**b**) in each of the oysters collected during the study.

Concentrations of total *V. vulnificus* (*vvhA*) in individual oysters ranged from no detection as mentioned above in two oysters to concentrations as high as 394,000 MPN/g (Fig. 4). Although this study did not encompass the colder months of the year, hindering our ability to fully examine seasonal variations, the time points associated with the lowest temperatures (May, October and November) showed a tendency for lower total *V. vulnificus* concentrations compared to other time points. Concentrations of total *V. parahaemolyticus* (*tlh*) ranged from 9.2 to 731,000 MPN/g, and similar to total *V. vulnificus*, concentrations of this species were lower overall in May, October and November compared to the other time points. Concentrations of *V. parahaemolyticus* strains associated with either *tdh* or *trh* gene detection ranged from no detection to 1,470 MPN/g, and from no detection to 1,500 MPN/g, for *tdh* and *trh*, respectively. The dynamics of concentrations of these strains differed from total *V. parahaemolyticus* dynamics. In 2018, both *tdh* and *trh* concentrations were more elevated in general in May and June 2018 compared to other time points. A similar pattern was suggested by the 2019 data,

however, concentrations of these strains were lower than in 2018 making these patterns less discernable.

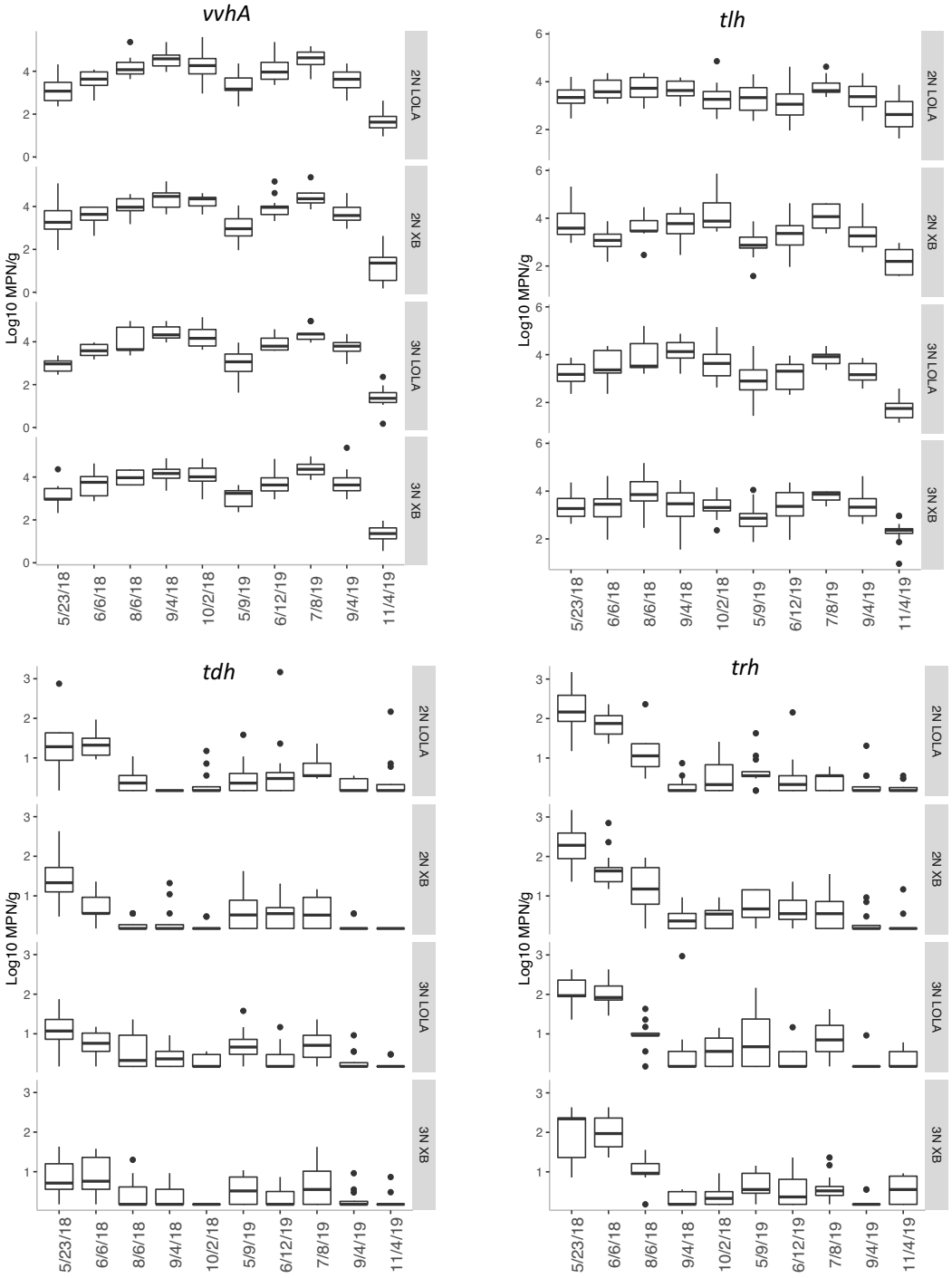

Fig. 4: Boxplots showing the concentrations (log_{10} MPN/g of tissue) of each *Vibrio* species or strain measured in oyster line and at each time point during the study; total *V. vulnificus* (*vvhA*), total *V. parahaemolyticus* (*tlh*), and pathogenic *V. parahaemolyticus* strains possessing the *tdh* or the *trh* gene.

Statistical analysis

Environmental *models*

Using this approach, predictor variables included in the models were water temperature and/or salinity in combination with variables associated with oyster gonad, sex or parasitism. Models including only temperature (Fig. 5), and in some instances models including only salinity, ranked the highest using AIC and BIC compared to more complex models including additional measures associated with oysters.

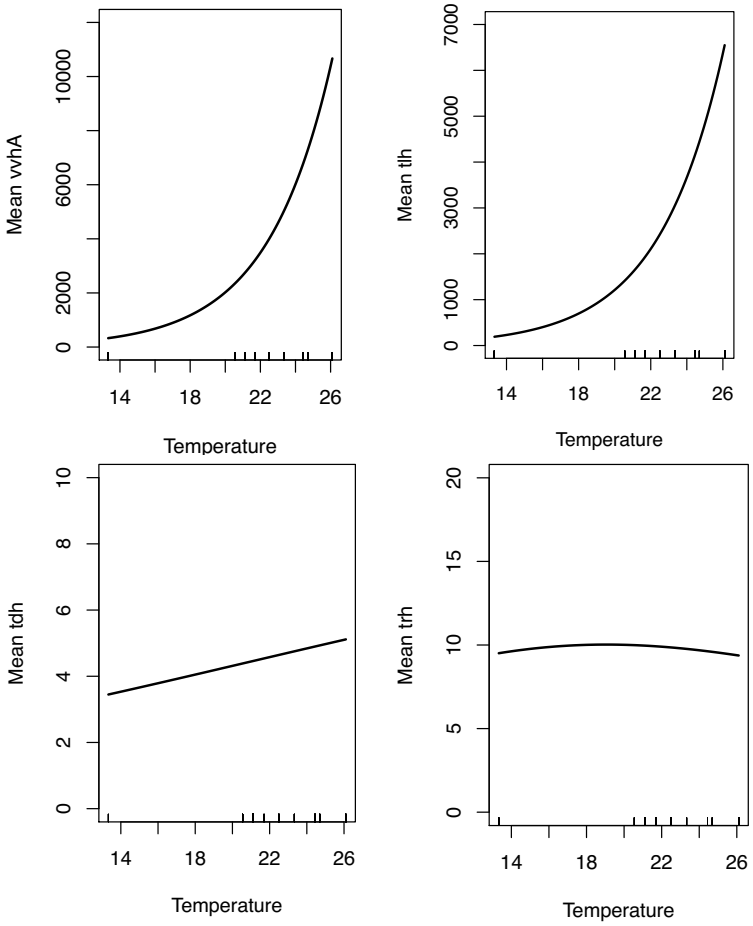

Fig. 5: Model predictions of mean *Vibrio* species and strains in oyster over the observed domain of the predictor variable i.e. temperature. Rug on x-axes show observed values of the predictor.

Distributions chosen in GAMLSS models as the most appropriately fitting the concentrations of total *V. vulnificus* and total *V. parahaemolyticus* were Box-Cox-t-orig (BCTo). Models including water temperature as a single predictor variable compared to other parameterizations ranked consistently higher than models including variables associated with oyster gonad, sex or parasitism using AIC and BIC. Models including salinity as a single predictor variable also ranked high for total *V. vulnificus* (data not shown) and suggested a negative association; however, under the range of salinities included in this study, the shape of the prediction suggested a very small effect of salinity, at least in comparison to the effect of temperature. For these two gene targets, standard diagnostic plots suggested that models including temperature as a predictor were well adapted to the data. Predictions associated with

these models showed a positive association of total *V. vulnificus* and total *V. parahaemolyticus* with temperature (Fig. 5).

The hurdle model approach used for concentrations of *tdh* and *trh* also identified BCTo as the most appropriate distribution in the second step of this approach. As with total *V. vulnificus* and total *V. parahaemolyticus*, concentrations of *tdh* and *trh* were best described by models including temperature as the single predictor variable, with a slight positive association of *tdh* and *trh* with water temperature (Fig. 5). For *tdh*, the model including salinity as the single predictor ranked high and suggested a negative association. For the *tdh*+ and *trh*+ strains, however, the diagnostic plots suggested that models may not be fully adapted to the observed data and caution should be used in the interpretation of these results.

Oyster *models*

Removing water temperature and salinity and using month as a factor in these oyster models enabled the identification of oyster related variables as potential predictors of *Vibrio* spp. concentrations in oysters. Specifically, models including only oyster line or only oyster sex consistently ranked the highest based on AIC and BIC across all *Vibrio* species or strains analyzed (Fig. 6).

When examining the predictions associated with oyster sex, no general trend emerged across the different gene targets. In some instances, predictions suggested that females are associated with higher concentrations of total *V. parahaemolyticus* (*tlh*) and of *tdh*+ strains. Predictions associated with *vvha* showed a tendency for concentrations in males to be more elevated than in females or indifferent. Finally, predictions suggested a minimal effect of sex on the concentration of *trh*+ strains.

Predictions associated with models including oyster line suggested a stronger effect of oyster line on *Vibrio* spp. concentrations in oysters compared to sex. Interestingly, predictions also suggested that concentrations of *vvha*, *tlh* and *tdh*+ strains were more elevated in 2N LOLAs compared to 3N XB. The observed pattern differed for *trh*+ since predictions suggested a more minimal effect of oyster line as illustrated by the relatively low difference in predicted mean concentrations among oyster lines.

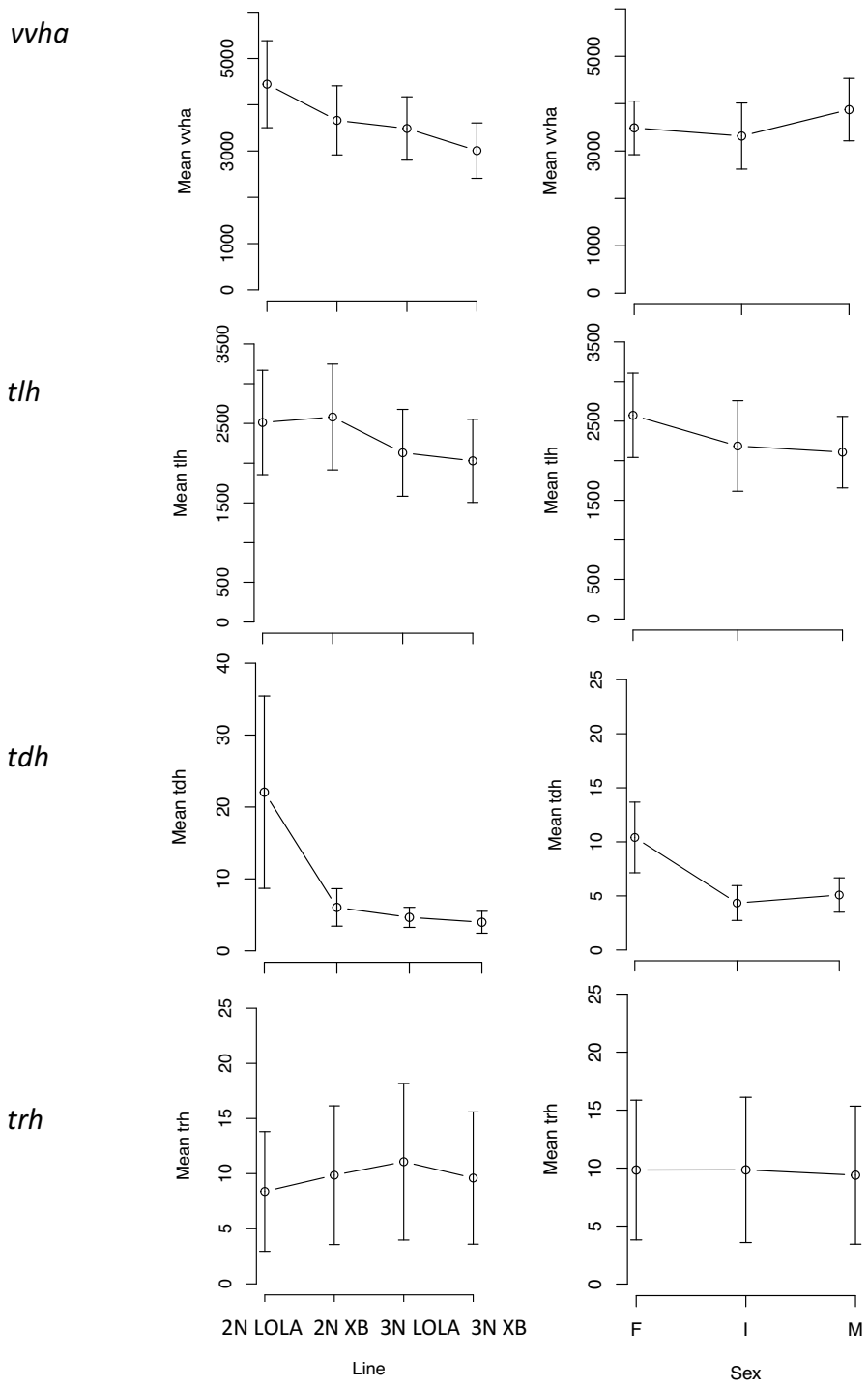

Fig. 6: Model predictions of mean *Vibrio* species and strains in oyster over the observed domain of the predictor variable i.e. oyster line or sex. Rug on x-axes show observed values of the predictor. Error bars are 95% confidence intervals.

DISCUSSION

Previous studies conducted on total *V. vulnificus* and on total and pathogenic *V. parahaemolyticus* (Bienlien 2016), or on other *Vibrio* spp. such as *V. campbelli* (Williams et al. 2009) and the oyster pathogen *V. splendidus* (De Decker et al. 2011), have suggested an affinity of these bacteria for oyster gonadal tissue. One of our objectives was to further inv estigate the potential influence of oyster gonadal stage on concentrations of human-pathogenic *Vibrio* species in the eastern oyster *C. virginica*. Our study included data associated with 478 individual oysters, which were analyzed using generalized linear mixed-effects models to identify predictor variable(s) of total *V. vulnificus* and total and pathogenic *V. parahaemolyticus* concentrations in oysters. Using this approach, **our results suggest that gonadal stage does not influence levels of these bacteria in oysters**. While this result seems to contradict the one from Bienlien (2016), who suggested a higher prevalence of pathogenic *V. parahaemolyticus* strains in oysters of peak gametogenic development compared to the prevalence across all oysters, one of the limits of that study was the overall low number of oysters associated with detection of these strains. In our study, strains associated with *tdh* gene detection or with *trh* gene detection were detected among 238 and 308 oysters respectively, allowing better evaluation of potential associations of these strains with gonadal stages or other variables. Based on this more thorough analysis, we suggest that the wide range of concentrations of either *V. vulnificus* or of total and pathogenic *V. parahaemolyticus* observed in oysters collected from the same location at the same time is not driven by differential gonadal development among oysters.

A second objective was to evaluate the influence of oyster ploidy on concentrations of these bacteria in oysters. This objective was tightly linked to the first since diploid and triploid oysters differ in their gonadal development, with triploid oysters generally being characterized by reduced gonadogenesis (Allen & Downing 1986). Because ploidy and oyster lines were correlated to each other, of the two variables, we chose to include oyster line in the models and not ploidy. Oyster lines indeed carried not only information relative to ploidy, but also allowed us to evaluate the potential influence of oyster genetics (i.e., genomic differences between lines) on levels of these pathogens. Our analysis did not reveal an influence of ploidy, however, it unexpectedly did suggest that **an influence of oyster genetics was possible and should be further evaluated**.

Models including oyster line consistently ranked among the two best *oyster* models based on AIC and BIC. Model predictions highlighted a pattern that was consistent among three of our gene targets (*vvha*, *tlh* and *tdh*): a tendency for levels of these bacteria to be more elevated in 2N LOLAs compared to 3N LOLA and 3N XB oyster lines. Higher concentrations of total *V. vulnificus* and *V. parahaemolyticus tdh*+ strains were observed in 2N LOLA oysters compared to oysters from the three other studied oyster lines. Concentrations of total *V. parahaemolyticus* were similar in 2N LOLAs and 2N XBs oysters but these concentrations were more elevated than concentrations observed in 3N LOLAs or 3N XBs. In addition, in our study, 2N LOLAs were associated with higher prevalence and higher infection intensity of both *P. marinus* and *H. nelsoni* compared to the other lines. With this in mind, we hypothesize that 2N LOLAs were under stress and that **their immune system was compromised, favoring an increase in the concentrations of certain *Vibrio* spp.** Although 2N LOLAs are bred specifically for low salinity waters and for *P. marinus* resistance, with at least some resistance inherent as well to *H. nelsoni,* it was clear in this study that they were more heavily impacted by these endemic parasites than the XB line that was more purposefully selected for dual disease resistance to both parasites.

Infections with *P. marinus* or *H. nelsoni* were not identified as significant predictors in our analysis, however, if indeed the overall state of the oyster immune system is important with regard to *Vibrio* spp. concentrations in tissues, we hypothesize that these parasite-related measurements failed to capture the weakened immune system of these oysters. Associations between weakened immune system and increased *Vibrio* spp. load and infections are well-known. A majority of infections by *Vibrio* spp. in humans can remain self-limited, however in immunocompromised patients, the associated disease can become severe and even fatal (CDC 2019). Similarly, oysters weakened by infections by oyster herpesvirus (OsHV-1) have been found to be associated with an increase in tissue colonization by *Vibrio* spp. and other bacteria, which eventually causes the death of the animals (Lorgeril et al. 2018).

Oyster models also pointed to oyster sex as a potential predictor variable of importance for human-pathogenic *Vibrio* spp. concentration in oyster. Mean predictions for *tlh* and *tdh* were more elevated in females compared to indifferent or males, while there was a tendency for males to be associated with more abundant *vvha*, and sex had a minimal impact of predicting *trh* means. However, the range of predicted means for these species or strains across different female, male and *indifferent* oysters was reduced compared to what we observed when the effect of oyster line was assessed. With this in mind, we hypothesize that oyster sex has minimal influence on the concentrations of human-pathogenic *Vibrio* spp. in oysters.

We acknowledge that the increase in predicted mean *Vibrio* spp. concentrations observed in 2N LOLAs compared to other lines remained relatively small (~ 1.5 fold increase in *vvha* concentrations and ~ 4 fold increase in *tdh*). In addition, the confidence intervals associated with the mean predictions suggest that some caution should be used when interpreting our results. However, as discussed above, several arguments suggest that these observations warrant further consideration: 1/ this trend was observed in three of our gene targets; 2/ the oyster line associated with higher predicted means of these *Vibrio* spp. was under higher parasite pressure, and consequently under higher stress, compared to the other lines; and 3/ stress and an associated weakened immune system can favor the proliferation of bacteria including *Vibrio* spp.

In conclusion, our study suggests that oyster gonadal development and oyster ploidy have little to no influence on levels of human-pathogenic *Vibrio* spp. in *C. virginica*. However, we hypothesize that oyster health may influence levels of these bacteria. More specifically we hypothesize that stressful conditions, either due to poor adaptation of the oyster to an environment (as seen in this study), or non-optimal environmental conditions depressing the oyster immune system, may favor increased levels of human-pathogenic *Vibrio* spp. in oyster tissues.

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