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Effect of ploidy level on accumulation and depuration of *Vibrio parahaemolyticus* in Pacific oyster *Crassostrea gigas*

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Abstract :

Triploid cupped oysters represent an advantage over diploid oysters due to their faster growth and their whole year marketable span, especially during the spawning season occurring in the summer. Thus, their commercialization during this warm season could present a risk for human health due to the proliferation of human-pathogenic Vibrio spp. such as Vibrio parahaemolyticus (Vp). The purpose of this study was to evaluate the impact of Crassostrea gigas oyster ploidy on contamination with indigenous Vp in an oyster farm, and on the accumulation and depuration of Vp in laboratory experiments. The study was conducted from May through November 2021 using three batches of diploid oysters and three batches of triploid oysters. We observed that ploidy did not significantly influence contamination by indigenous Vp although the contamination with indigenous Vp trended to be lower in triploids (0.93 log MPN.g−1) in comparison with diploids (3.08 log MPN.g−1) in November. These results could suggest a safer consumption of triploid oysters over diploid oysters in autumn concerning Vp infection risk in the site of this study. Moreover, pathogenic profiles of indigenous Vp varied significantly between July (8% of tdh+ and 100 of trh2+) and November (98% of tdh+ and 0% of trh2+) with no significant difference between ploidies. Ploidy did not significantly influence experimental accumulation or depuration at any time. However, depuration was higher during summer (June to August) than during the other months. In conclusion, our study suggests that Vp levels in C. gigas are not influenced by oyster ploidy. In addition, seasonal variations of indigenous Vp pathogenic profiles and Vp experimental depuration were observed.

Highlights

► Accumulation of indigenous *Vp* are similar between ploidy levels except in November. ► Oyster ploidy did not significantly impact experimental *Vp* accumulation and depuration. ► Pathogenic *Vp* and profiles of experimental depuration varied according to the seasons.

Keywords : Vibrio spp., contamination, diploid, triploid, season

1. INTRODUCTION

Vibrio parahaemolyticus (*Vp*) is a natural inhabitant of coastal and estuarine waters worldwide. This bacterium is the principal pathogenic agent implicated in foodborne infections by the consumption of raw or insufficient cooked seafood causing acute gastroenteritis in humans (Center for Disease Control and Prevention, 2019). These foodborne cases of vibriosis peak during the summer (Baker, 2016), following the seasonal cycle of *Vp* observed in waters and oysters with proliferation occurring when temperatures exceed 15˚C (Parveen et al., 2008). Pathogenicity has first been characterized by the Thermostable-Direct haemolysin (TDH) (Sakurai et al., 1973), the TDH-Related Haemolysin (TRH) (Honda et al., 1988) and more recently by the type III secretion system (Makino et al., 2003). Both pathogenic and non-pathogenic strains can be isolated from seawater and bivalves with seasonal dynamics (Deter et al., 2010; Zimmerman et al., 2007).

The highest prevalence of *Vp* was found in oysters (63 %), followed by clams (53%), fish (51%) and shrimps (48%) (Odeyemi, 2016). Oyster aquaculture is growing worldwide and is widely dominated by China which represented 85% of global production by weight and 79% by value in 2019 (FAO, 2021). Within Europe, 77% of the oyster production occurs in France representing 85,947 tonnes and $445 million in 2019, thus positioning this country as the 5th producing country by weight and the 2nd country by value worldwide (FAO, 2021). The main oyster species cultivated in France is the Pacific oyster *Crassostrea gigas*, present and cultivated worldwide (FAO, 2009). Oysters cultured are either diploid or triploid. Triploid oysters were originally developed in the United States in the 80's (Stanley et al., 1981), and their production increased over the years, especially in France (Boudry et al., 1997; Gérard et al., 1999). The amount of triploid spat produced by French commercial hatcheries has increased regularly each year since 2000, reaching nearly 3 billion units in 2012 (Dégremont

and Benabdelmouna, 2014). Triploid oysters are associated with faster growth than diploids, and are of particular interest during the summer as a result of their partial sterility (Allen and Downing, 1986). Indeed, the partial sterility results in better meat quality compared to diploid oysters, enabling to expand the market for oysters to the warmer months. However, the fact that triploid oysters are marketable during summer raise the public health issues associated with human pathogenic bacteria that proliferate in summer such as *Vibrio* spp. (Di et al., 2017).

Investigations about the effect of ploidy level and disease resistance have been broadly investigated in oyster species, as one indirect mechanism for triploids to avoid disease. Indeed, the faster growth limits the span of time when oysters might be exposed to disease (Dégremont et al., 2015). Previous study showed that triploidy confers neither advantage nor disadvantage over diploidy for OsHV-1 infection in *C. gigas* (Dégremont et al., 2016), while other studies showed higher disease resistance of either triploids, as observed for *Perkinsus marinus* in *C. virginica* (Dégremont et al., 2012) and for *Bonamia roughleyi* in *Saccostrea glomerata* (Hand et al., 1998), or diploids, as found for *Vibrio aestuarianus* in *C. gigas* (Azéma et al., 2016). Moreover, diploid and triploid oysters were shown to be physiologically different throughout the season according to their reproductive patterns (Goulletquer et al., 1996; Jeung et al., 2018; Normand et al., 2009). Previous studies showed that ripe *C. gigas* oysters or at the postspawning stage were more susceptible to the summer mortality phenomenon (Huvet et al., 2010; Samain et al., 2007), to *Vibrio splendidus* and *Vibrio aestuarianus* (De Decker et al., 2011), and OsHV-1 (Dégremont et al., 2013) infections. Altogether, these observations highlighted the importance of the physiological status of oysters on *Vibrio* spp. infections for diploid and triploid oyster comparisons. Concerning human pathogenic *Vibrio*, recent studies did not show any differences in *Vp* contamination

between diploid and triploid *C. virginica* oysters (Grodeska et al., 2019; Jones et al., 2020; Walton et al., 2013). To our knowledge, this has not been studied in *C. gigas*.

The aim of this study was to investigate the impact of ploidy level of *C. gigas* oysters on natural contamination of *Vp* in field condition, and on experimental accumulation and depuration of *Vp* in laboratory condition throughout seasons. For each ploidy, three replicated spawns were used to disentangle the ploidy and batch effects, as significant variation for disease resistance exists among spawns in oysters as demonstrated by Dégremont *et al.* (2012). Oysters were sampled once a month from May to November 2021, and were directly analysed for indigenous *Vp* contamination (May, July, September and November) and exposed to the *Vp* strain IFVp201-*gfp*$^+$ (environmental strain, *tdh*$^+$ *trh*$^+$) to investigate experimental accumulation and depuration (May, June, July, August, September and November).

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

For this study, the *Vp* IFVp201 strain isolated from mussels (*Mytilus edulis*) in December 2009 in Poitou Charentes (France) was used. This environmental strain was characterized as *tdh*$^+$ *trh*$^+$ by qPCR (Lozach et al., in preparation)(Bej et al., 1999), which are virulence genes widely present in clinical isolates but rarely in environmental strains. IFVp201 was previously electroporated with pFD086 plasmid harbouring *gfp* gene and trimethoprim-resistance expression cassettes named thereafter IFVp201-*gfp*$^+$ (Sorée et al., 2022).

IFVp201-*gfp*$^+$ was grown overnight at 37°C on agar plates of Luria Bertani containing 3% NaCl supplemented with 50 µg.mL^{-1} of trimethoprim (LBS50). A single colony formed on LBS50 agar plate was transferred into LBS50 liquid medium and incubated at 37°C overnight at 50-70 rpm. Overnight cultures were washed twice with buffered-physiological water (BPW, 0.4 g.L^{-1} of $K_2H_3PO_4$; 4.5 g.L^{-1} of Na_2HPO_4, $12H_2O$; 7.2 g.L^{-1} of NaCl) before

inoculation in filtered and sterile seawater (FSSW, natural seawater filtered with 1 µm polypropylene mesh and treated with UV). This washed-culture of IFVp201-*gfp*$^{+}$ was analysed using Cyflow Space flow cytometer (Sysmex-Partec, Munster, Germany) to determine the bacterial concentration (log bacteria.mL^{-1}).

2.2. Oyster batches and sampling site

Three batches of diploid *C. gigas* oysters (called thereafter D1, D2 and D3) and three batches of triploids (called thereafter T1, T2 and T3), were bought at a commercial hatchery in November 2020. Each batch was produced using common practices developed by commercial hatcheries, by mating several dozens of females with a few males from broodstocks developed by the commercial hatchery. For diploid batches, all parents were diploids, while triploids batches were produced by mating diploid females with tetraploid males, as usually done by French commercial hatcheries (Dégremont et al., 2019). Batches used in this study are representative of hatchery-produced oysters used by oyster farmers in France. For each batch, oysters weighted around 30 g in November 2020, which is the beginning of the market-size in France. For each batch, oysters were deployed in six bags fixed on racks in the experimental farm at Agnas on November 16th 2020 (1°10′35″ W, 45°52′14″ N), only accessible by boat. Due to COVID issues, the boat operation was restricted, and so, oysters were transferred to La Floride (1°09′15″ W, 45°48′12″ N) on April 12th 2021, accessible from the laboratory by walking. Both sites are located in the Marennes-Oléron Bay which is the most important area of shellfish culture in Europe, mainly based on cupped oysters and mussels, and both sites are in the intertidal area as most of the oyster farms (Goulletquer and Le Moine, 2002). For each batch and sampling date, 75 to 85 oysters were collected and then transferred to Ifremer mollusc experimental platform at Bouin (France) on May 17th, June 21st, July 26th, August 23rd, September 20th and November 22nd 2021. Experimentations were performed less than 24 h after oyster collection except in June when a delivery issue led to a

24 h delay. Indigenous *Vp* presence in oysters was investigated every two months, in May, July, September and November 2021. Experimental accumulation and depuration were performed in May, June, July, August, September and November 2021. For each sampling date, shell length and wet tissue weight were recorded from 30 oysters for each diploid and triploid batch to estimate their growth kinetics. One of the triploid batches (T2) underwent severe mortalities in the environment to the point that this batch was missing in September and November 2021 experimentations.

Environmental parameters were measured regularly by IFREMER LER/PC for the "Reseau d'Observatoires de Microbiologie Environnementale intégrée" at the sampling point (1° 09' 17" O, 45° 47' 53" N). Seawater temperature (in °C) and salinity (ppt) were measured every 15 min by STPS sensors (NKE, France) placed in proximity of the sampling site during the entire period of experimentation. In addition, a Thermobutton sensor measured the temperature (°C) every 30 min inside the oyster bags between 22nd July and 30th November 2021.

2.3. Ploidy verification

Due to a risk of contamination among ploidy before purchasing the oysters, the ploidy of each batch was checked. For each ploidy, six pools of five individuals were analysed, using small pieces of gills (1 mm²) transferred into 2 mL of Cystain UV Ploidy solution (Sysmex, Japan). Gills were disrupted by pipetting and the mix was filtered on 30 µm Celltricks (Sysmex, Japan) into a new tube and 1 mL of Cystain UV Ploidy solution was added before analyses using Cyflow Space flow cytometer (Sysmex-Partec, Munster, Germany) with UV LED (450/50 nm). A diploid oyster was used as standard before analyses.

2.4. Quantification and characterization of indigenous *Vp* contamination

2.4.1. Preparation of samples, culture enrichment and DNA extraction

For each batch and each sampling date (in May, July, September and November 2021), one pool of 10 oysters was analysed for indigenous *Vp*. The Most Probable Number (MPN)-qPCR protocol of (Luan et al., 2008), used to estimate the viable number of bacteria in a sample, was adapted to 96-deep well plates. Briefly, the pool of 10 oysters was blended three times for 30 sec with Laboratory Waring Blender. 25 g of oyster homogenate were transferred into 225 mL of alkaline peptone water 2% NaCl (APWS: 20 $g.L^{-1}$ of bacteriological peptone, 20 $g.L^{-1}$ of sodium chloride). This suspension, corresponding to the $1/10^{e}$ dilution of the homogenates, was serially-diluted until the $1/10,000^{e}$ dilution and 1.5 mL of each dilution was transferred into a 96-deep well plate (six replicates per dilution) and incubated at 36 ± 2°C for 20 h. Three wells of APWS (1.5 mL) were used as negative controls. After incubation, 1 mL of the bacterial suspension from each well was transferred into a new 96-deep well plate. These suspensions were washed twice with BPW followed by centrifugation. Pellets were re-suspended in 150 µL of DNAse free water (Merck Millipore™, Germany) and transferred into 96-well microplate. Nucleic acids were extracted by thermal lysis (15 min at 95°C) using a TC5000 96X 0.2ML thermocycler (Techne, UK). Plates were centrifuged and supernatants were transferred into new 96-well microplate and maintained at -20°C. DNA concentrations in each well were determined using Epoch Microplate Spectrophotometer (BioTek Instrumentals, Inc., Winooski, VT, USA) and concentrations were adjusted if necessary at approximatively 50 $ng.\mu L^{-1}$ with DNAse free-water (Merck Millipore™, Germany).

2.4.2. Quantification and characterization of indigenous *Vp*

Firstly, affiliation to the species level (*Vp*) was determined by qPCR targeting *toxR* gene using the Platinum™ Quantitative PCR SuperMix-UDG Kit (Invitrogen™, CA, USA) and the TaqMan™ Exogenous Internal Positive Control (IPC) Reagents (Applied Biosystems ™, CA,

USA) to detect qPCR inhibition (Lozach *et al.*, in preparation). The PCR reaction mixture (25 µL) consisted of 2 µL of DNA, 12.5 µL of the reaction buffer (2X SuperMix-UDG PCR quantitative Platinum ™), 0.5 µL of IPC DNA (50X Exo IPC DNA), 1 µL of $MgCl_2$ (50 mM), 2.5 µL of IPC mix (10X Exo IPC mix), 5.625 µL of DNAse water, 0.375 µL of probe (20mM), 0.125 and 0.375 µL of reverse and forward primers (20mM), respectively. DNA was replaced by 2 µL of block IPC (10X block IPC) for non-amplification control wells and by DNAse free-water for non-template control. A standard was performed by dilution of a reference plasmid in DNAse free-water from 10^5 to 10^0 copies per well and was used to validate positivity of wells when the concentration was superior to 10 copies. The amplification was carried out with a Mx3000P QPCR System (Agilent™ Technologies, CA, USA). Most probable number (MPN) enumeration (in log $MPN.g^{-1}$ of oyster tissues) was performed for *toxR* detection following https://standards.iso.org/iso/7218/. *toxR*$^+$ wells were further characterized for the genetic markers *tdh, trh1* and *trh2* genes. Previous studies performed on French coasts (Cantet et al., 2013; Deter et al., 2010; Esteves et al., 2015) showed a high prevalence of *trh*$^+$ strains that were further characterized according to the presence of *trh1* and *trh2* genes. The qPCR were performed using the Platinum™ Quantitative PCR SuperMix-UDG Kit (Invitrogen™, CA, USA) (Lozach *et al.*, in preparation) and with a PCR reaction mixture (25 µL) consisted of 2 µL of DNA, 12.5 µL of the reaction buffer (2X SuperMix-UDG PCR quantitative Platinum ™), 0.5 to 1 µL of $MgCl_2$ (50 mM), 8 to 8.375 µL of ultrapure water, 0.375 to 0.625 µL of each primer (20 mM). Results were expressed for each batch as a ratio of the number of positive wells for the gene (*tdh, trh1* and *trh2*) over the total number of *toxR*$^+$ wells.

2.5. Oyster bacterial challenge with IFVp201-*gfp*$^+$

2.5.1. Accumulation of IFVp201-*gfp*$^+$

Oyster bacterial challenge was performed as previously described in (Sorée et al., 2022) with slight modifications. Briefly, IFVp201-*gfp*$^+$ was inoculated in six 20 L tanks of FSSW (33.5 ± 0.8 ppt) with initial concentration of 10^6 CFU.mL^{-1}. For each diploid and triploid batch, 50 oysters were placed into one contaminated tank and 25 oysters were held as controls in a 6 L tank of FSSW without IFVp201-*gfp*$^+$. The 12 tanks were maintained at 19°C for 24 h and aeration was provided to all tanks. After 24 h, IFVp201-*gfp*$^+$ concentration was assessed by flow cytometry in triplicate samples of contaminated and control seawater. Oysters were then transferred one hour into "fresh" FSSW. Haemolymph was collected as described in 2.5.3 from two pools and one pool of five oysters from each contaminated and control tank, respectively. IFVp201-*gfp*$^+$ was quantified by flow cytometry from two analytical replicates per haemolymph pool. These data represented the 24 h-accumulated concentration of IFVp201-*gfp*$^+$ in oysters.

2.5.2. Depuration of Vp201-*gfp*$^+$

Remaining oysters exposed to IFVp201-*gfp*$^+$ (n = 40) and control oysters (n = 20) were transferred into new separate tanks with 20 L and 6 L of FSSW, respectively, and maintained in the same conditions as for accumulation. After 24 h and 48 h, haemolymphs were collected from three haemolymph pools of five oysters previously exposed to IFVp201-*gfp*$^+$, and from one haemolymph pool of five oysters for the control. IFVp201-*gfp*$^+$ was quantified by flow cytometry in two analytical replicates per pool. In June 2021, data could not be collected at the 48 h of time point due to a delay in the oyster delivery to the laboratory processing the samples. Log reduction was calculated as the difference between the accumulated concentration and concentrations after 24 h (24 h log reduction) and 48 h of depuration (48 h log reduction).

2.5.3. Sample preparation and analysis

At each step (accumulation and depuration), oysters were shucked using a sterile shucking knife on a sterile surface. Abductor muscles were cautiously cut with sterile scalpel blades to enable shell opening. Haemolymphs were collected using a sterile 25G needle and sterile 1 mL insulin syringe from the pericardial cavity. Collected haemolymphs were filtered on a 30 µm-sterile nylon mesh to remove aggregates. Samples were then analysed by flow cytometry using a Cyflow Space (Sysmex-Partec, Munster, Germany) by detection of their green fluorescence (FL1 detector of the flow cytometer) and their relative size and granularity (Forward SCatter [FSC]; Side SCatter [SSC]). The bacterial counting was performed according to the number of cells detected by the flow cytometer in a volume of 200 µL of sample and was expressed in log bacteria.mL^{-1} (Soré et al., 2022).

2.6. Statistical analyses

All statistical analyses were performed using RStudio 2021.09.0+351 "Ghost Orchid" Release (2021-09-20) for Windows. Data are presented as means ± standard error and the significance level was set to $p < 0.05$.

2.6.1. Growth of oysters

For each sampling date, wet tissue weight and shell length were analysed using non-parametrical Kruskal-Wallis test and post hoc Dunn test between ploidies and batches nested within ploidy.

2.6.2. Quantification and characterization of indigenous *Vp* in oysters

Contamination with indigenous *Vp* and pathogenicity characterization of indigenous *Vp* in oysters were analysed using an ANCOVA with wet tissue weight as covariables, and with ploidy (diploid *vs* triploid), sampling date (July and November 2021, when *Vp* was detected) and their interaction as factors. Absence of replicates for each batch nested within ploidy did

not allow to include this factor in the analysis. Differences among factors were estimated using a post hoc Emmeans test.

2.6.3. Experimental IFVp201-*gfp*$^{+}$ concentrations in seawater

For each sampling date, concentrations of IFVp201-*gfp*$^{+}$ (in log bacteria.mL^{-1}) in seawater before accumulation by oysters were analysed using an ANOVA with tank as factor. For each sampling date, log reductions of IFVp201-*gfp*$^{+}$ concentrations in seawater before and after accumulation by oysters were analysed using an ANCOVA with wet tissue weight as covariables and ploidy as factor. Absence of replicates for each batch nested within ploidy did not allow to include this factor in the analysis. To evaluate differences of log reduction in seawater among months of experimentation, an ANCOVA and a post hoc Emmeans tests were performed with wet tissue weight as covariable and sampling date as factor.

2.6.4. IFVp201-*gfp*$^{+}$ accumulation and depuration in haemolymph

For each sampling date, IFVp201-*gfp*$^{+}$ accumulation in haemolymph (in log bacteria.mL^{-1}) was analysed using an ANCOVA with wet tissue weight as covariable, and ploidy and batch nested within ploidy as factors. For each sampling date, log reductions of IFVp201-*gfp*$^{+}$ concentrations after 24 h and 48 h of depuration were analysed using an ANCOVA with wet tissue weight as covariable and ploidy as factor. Absence of replicates of log reduction for each batch did not allow analysis with batches nested within ploidy as factor.

To evaluate differences in accumulation and log reduction in haemolymph among the months of experimentation, an ANCOVA and post hoc Emmeans test were performed with wet tissue weight as covariable and sampling date as factor.

3. RESULTS

3.1. Ploidy validation and environmental condition

Analyses of oyster ploidy level by flow cytometry confirmed the ploidy of each batch of oysters used for this study.

Seawater temperature measured at the sampling site ranged from 11°C in November 2021 to 23°C in July 2021 (**Fig. 1A**). Temperatures were higher than 15°C between May 17th and October 30th, and a mean of 19°C was observed during the sampling period. Salinity ranged from 30 ppt in July 2021 to 34 ppt in September 2021 with a mean of 33 ppt during the sampling period (**Fig. 1B**).

3.2. Growth of diploid and triploid oysters

Wet tissue weights and shell lengths for each ploidy from May to November 2021 are shown in **Fig. 2A** and **2B**, respectively. Details of each growth trait for each batch are given in **Table S1**.

In May 2021, similar wet tissue weights were observed for diploid (5.33 ± 0.21 g) and triploid (5.32 ± 0.15 g) oysters (**Fig. 2A**). The wet tissue weight increased until July for both diploid (7.70 ± 0.22 g) and triploid (7.84 ± 0.26 g) oysters with no significant differences from May to July but with significant difference among batches in May ($p = 0.013$), June ($p = 0.003$) and July ($p < 0.001$). For these three months, one diploid batch was lighter than the two others batches. Moreover, in July, one triploid batch was lighter than the two other batches (**Table S1**). Triploid wet tissue weight continued to increase until November (10.04 ± 0.29 g) while diploid wet tissue weights decreased until September (6.44 ± 0.24 g) followed by a new increase until November (7.24 ± 0.26) (**Fig. 2A**). The difference of wet tissue weights between diploid and triploid oysters was significant in August ($p = 0.015$), September ($p < 0.001$) and November ($p < 0.001$) (**Fig. 2A**) and among batches in August ($p = 0.002$) with one diploid batch heavier than the two others (**Table S1**). The shell lengths were not significantly different between diploid and triploid oysters in May (6.71 and 6.67, respectively) and in July (7.6 and 7.9 cm, respectively) while triploids exhibited a significantly longer shell length than diploids in June (7.4 cm vs 7.1 cm, $p < 0.01$), August (8.3 cm vs 7.6 cm, $p < 0.001$), September (8.4 cm vs 7.5 cm, $p < 0.001$) and November (8.1

cm vs 7.3 cm, $p < 0.001$) (**Fig. 2B**). Differences among batches were significant in June ($p = 0.027$) and in July ($p = 0.049$). Thus, one batch of diploid oysters was shorter than the two other batches in June, and one batch of diploids and one batch of triploids were shorter than the two others in July (**Table S1**).

3.3. Indigenous *Vp* contamination

Vp natural concentration (*tox*R$^+$, in log MPN.g^{-1}) in tissues of diploid and triploid oysters deployed at this study site are reported in **Fig. 3A**, whereas **Fig. 3B** shows the prevalence (in %) of *tdh*, *trh1* and *trh2* genes among the *toxR*$^+$ samples collected in July and November 2021.

Indigenous *Vp* were detected in oysters in July and November but not in May and September 2021 (**Fig. 3A**). The interaction between ploidy and dates was not significant ($p = 0.135$). Concentrations of indigenous *Vp* tended to be higher in diploid than in triploid oysters ($p = 0.0531$) especially in November with 0.93 ± 0.93 log MPN.g^{-1} in triploid and 3.08 ± 0.70 log MPN.g^{-1} in diploid oysters (**Fig. 3A**). No significant difference in the indigenous *Vp* contamination was observed between sampling dates ($p = 0.225$).

Concerning the prevalence of virulence genes, the interaction between ploidy and dates was not significant ($p > 0.05$). No significant difference was observed between diploid and triploid oysters for the prevalence of *tdh* ($p = 0.37$), *trh1* ($p = 0.82$) and *trh2* ($p = 0.79$) genes (**Fig. 3B**) while it was significant between sampling dates for *tdh* ($p < 0.001$) and *trh2* ($p < 0.001$) genes but not for *trh1* ($p = 0.416$). Thus, prevalence of pathogenic genes from July to November decreased for *trh2* (100% to 0%, respectively, $p < 0.001$) and increased for *tdh* (9% to 98%, respectively, $p < 0.001$) genes (**Fig. 3B**).

3.4. Experimental IFVp201-*gfp*$^+$ accumulation and depuration in diploid and triploid oysters

3.4.1. Experimental IFVp201-*gfp*$^+$ concentrations in seawater

Details of concentrations of IFVp201-*gfp*$^+$ in seawater before and after accumulation with oysters. and log reductions for each batch are given in **Table S2.**

For each sampling date, IFVp201-*gfp*$^+$ concentrations in seawater before accumulation ranged from 4.89 to 6.10 log bacteria.mL^{-1} (mean: 5.80 ± 0.44 log bacteria.mL^{-1}) with no significant difference among tanks ($p > 0.05$) (**Table S2**). For each sampling date, the log reduction of IFVp201-*gfp*$^+$ concentrations in seawater after accumulation by oysters ranged from 0.34 to 1.71 log with no significant difference between diploid and triploid oysters ($p > 0.05$) (**Table S2**). The log reduction of IFVp201-*gfp*$^+$ concentrations in seawater was significantly different among sampling dates ($p < 0.001$) with the highest value in July (1.19 log), intermediate in May (0.71 log), August (0.85 log) and September (0.71 log), and the lowest in June (0.58 log) and November (0.46 log) (**Fig. 4**).

3.4.2. Experimental IFVp201-*gfp*$^+$ accumulation and depuration in haemolymph

Fig. 5 represents the quantification of IFVp201-*gfp*$^+$ in haemolymph by flow cytometry after 24 h of accumulation (0 h), and after 24 h and 48 h of depuration for diploid and triploid oysters for each month. Details of accumulation and depuration at 24h and 48h for each batch are given in **Table S3**.

Accumulation of IFVp201-*gfp*$^+$ in haemolymph varied between 2.10 and 3.70 log bacteria.mL^{-1} for diploid oysters, and between 2.17 and 3.97 log bacteria.mL^{-1} for triploids for all the sampling dates (**Fig. 5**). For each sampling date, no significant difference in accumulation of IFVp201-*gfp*$^+$ was observed between diploid and triploid oysters ($p > 0.05$), whereas significant differences were observed among batches only in July ($p = 0.021$) and August ($p < 0.01$). Thus, accumulation for one batch of diploids and one batch of triploids was lower than in the two other batches in July, while it only concerned one batch of triploids

in August (**Table S3**). Moreover, concentration of IFVp201-*gfp*$^+$ in haemolymph decreased for both ploidies at 24h of depuration for each sampling date, and in a lesser extent at 48h of depuration except in September, where it remains stable, and in November, where it increased from 1.44 log bacteria.mL^{-1} at 24h to 2.41 log bacteria.mL^{-1} at 48h (**Fig. 5**). Reduction of IFVp201-*gfp*$^+$ concentrations in haemolymph after 24 h of depuration (24 h log reduction) varied between 0.12 and 2.34 log for diploid oysters, and between -0.05 and 2.48 log for triploid oysters for all the sampling dates (**Fig. 5**). For each sampling date, no significant difference in 24 h log reduction was observed between diploid and triploid oysters ($p > 0.05$). Similar finding was observed for the reduction of IFVp201-*gfp*$^+$ concentrations in haemolymph after 48 h of depuration ranging from -0.13 to 2.62 log for diploid oysters, and 0 to 3.07 log for triploids ($p > 0.05$) (**Fig. 5**).

The date of sampling had a significant impact on accumulation ($p < 0.01$), and on 24 h ($p < 0.001$) and 48 h log reduction ($p < 0.001$) of *Vp* in oysters. Thus, accumulation of IFVp201-*gfp*$^+$ was the highest in June (3.20 log bacteria.mL^{-1}), intermediate in May (2.98 log) and July (3.02 log), and the lowest in August (2.76 log), September (2.54 log) and November (2.56 log) (**Fig. 5** and **Table S4**). The 24 h log reduction was the higher in June (1.72 log) and July (1.64 log), intermediate in August (1.31 log), and the lower in May (0.91 log), September (0.27 log) and November (1.13 log) (**Fig. 5** and **Table S4**). The 48 h log reduction was the higher in July (2.42 log), intermediate in August (1.57 log) and May (1.07 log), and the lower in September (0.46 log) and November (0.12 log) (**Fig. 5** and **Table S4**).

4. DISCUSSION

Vibrio parahaemolyticus is the primary bacterial cause of gastroenteritis associated with seafood consumption, and is naturally present in coastal and estuarine marine environments worldwide. *Vp* is isolated more frequently in oysters (63%) than in other marine organisms (Odeyemi, 2016). In France, consumers prefer triploid *C. gigas* during the summer due to

their higher meat quality compared to diploids which are gravid. Since the summer is also the season during which *Vp* are more likely to proliferate (Baker, 2016), defining the potential human health risks associated with consumption of triploid oysters during this season is critical. In our study, we assessed contamination by indigenous *Vp* naturally present in the environment in diploid and triploid oysters. Additionally, oysters were experimentally exposed to a GFP-tagged *Vp* strain to investigate the impact of oyster ploidy level on accumulation and depuration of the bacteria. Finally, *Vp* accumulation, both natural and experimental, and depuration were investigated temporally throughout the study to determine if a higher risk for human consumption would be observed.

Occurrence and concentrations of indigenous *Vp* in *C. gigas* oysters varied according to the seasons with the exception of the September time point. In our experimental farm, there was no detection of *Vp* in May or September 2021, while it was detected in July and November 2021 for both diploid and triploid oysters. Absence of indigenous *Vp* in May can be explained by the seawater temperature just reaching 15°C, which is the threshold temperature for *Vp* proliferation as shown in previous studies (Deter et al., 2010; Kaneko and Colwell, 1973). In September, however, the seawater temperature was 20 ± 1°C (Fig. 1), thus it did not explain the absence of indigenous *Vp* in oysters. This was particularly surprising since *Vp* had been isolated two months earlier (Fig. 2A), and also from the same geographical area in September in a previous study (Deter et al., 2010). Although human error cannot be ruled out, weather conditions might have played a role in our study. Indeed, extremely dry conditions in August were recorded, together with unusually high air temperatures (air temperature > 35°C), high temperatures inside oyster bags (seawater temperature > 30°C, Fig. S1) and high salinity levels (> 34 ppt, Fig. 1B) in September. High salinity might have affected oyster physiology resulting for example in a decrease of the oxygen consumption rate and enzyme activities, and an increase in glycogen decomposition and lactic acid concentrations (Chen et al., 2022), or in

decrease in haemocyte locomotion (Fisher and Nowell, 1986) as it was shown in *C. gigas* and *C. virginica*, respectively. Moreover, it was shown that postspawning oysters were at higher risk during heat shock compared to prespawning oysters resulting in higher mortality, lower energy for metabolic activities, and reduced haemocyte phagocytosis and haemolymph antimicrobial activity (Li et al., 2007). Furthermore, it was shown that *Vp* proliferation was reduced in high salinity conditions (Parveen et al., 2017). During a drought with particularly high salinity levels in North Carolina from 2007 to 2009, *V. vulnificus* was shown to be undetectable in oysters (Froelich et al., 2012). Taken all together, those results could explain the absence of *Vp* in oysters in September in our study. Our study showed also that ploidy did not influence indigenous *Vp* contamination, suggesting a similar *Vp* infection risk for human consumption, although in November 2021 we observed a tendency for lower indigenous *Vp* contamination to occur in triploid compared to diploid oysters. These results confirmed previous studies showing no significant difference in *Vp* contamination between diploid and triploid *C. virginica* oysters (Grodeska et al., 2019; Jones et al., 2020; Walton et al., 2013). It is interesting to note that profiles of pathogenicity of indigenous *Vp* in oysters did not differed between diploid and triploid oysters even if they differ between months. Thus, we observed less *trh2*$^{+}$ profile strains during autumn in comparison to summer which is in agreement with previous studies (Canteet al., 2013; Esteves et al., 2015).

Experimental accumulation and depuration of *Vp* by oysters did not differ between diploid and triploid oysters, which is consistent with the log reductions of IFVp201-*gfp*$^{+}$ concentration in seawater. Similar results were observed previously (Phuvasate and Su, 2013). This could be explained by similar clearance rate and oxygen consumption for both ploidies (Haure et al., 2021). In addition, we showed that, for both diploid and triploid oysters, *Vp* depuration was higher during summer (June to August) than during the other months. This can be explained by higher filtration rates of oysters during the summer months (Ehrich and

Harris, 2015). Higher depuration in our study was consistent with the reductions of IFVp201-*gfp*$^{+}$ concentration in seawater for both diploid and triploid oysters significantly higher in July than in other months. Another hypothesis explaining our results relies on the reproductive cycle of oysters. A previous study showed that oysters in gametogenesis (May-June) displayed lower haemocyte activities than oysters in ripe gametes and post-spawning periods (Gagnaire et al., 2006). In our study, both diploid and triploid oysters could exhibit low haemocyte activities during gametogenesis (May-June), resulting in less depuration of oysters than during the following months. These differences of depuration could also be explained by niche competition between indigenous *Vp* and the Vp201-*gfp*$^{+}$ strain. Indeed, higher concentrations of indigenous *Vp* in July was correlated with higher experimental depuration in comparison to May and September when the low experimental depuration was associated with the absence of indigenous *Vp* in oyster tissues. Similar results were observed by Froelich *et al.* (2012) after experimental contamination of *V. vulnificus* with oysters free of indigenous *V. vulnificus*. Furthermore, the natural exposition of oysters to *Vp* in July could result in an innate immune priming. Indeed, Zhang *et al.* (2014) showed that a first infection with *V. splendidus* induced a significant increase of the total count of haemocytes and of the immune processes after a second infection with *V. splendidus* (Zhang et al., 2014). Based on these studies, the influence of season and physiological status of oysters on *Vp* accumulation and depuration warrants further investigations.

In our study, triploid oysters displayed faster wet tissue weight gain and shell length increase than diploid oysters which is consistent with previous studies (Dégremont et al., 2012; Wadsworth et al., 2019). The slight decrease in wet tissue weight for diploid oysters (-1 g) between July and September can be explained by the release of gametes at this period. Moreover, the decrease observed in shell lengths for both diploid (0.36 cm) and triploid (0.19 cm) oysters between July and September can be explained by the vulnerability of peripheral

part of shells during growth which could have been damaged during collection or transportation. Our results suggested that the growth advantage of triploid oysters over diploid oysters would not have an effect on human health risks associated with *Vp*.

5. CONCLUSIONS

In conclusion, our study suggests that *Vp* levels in *C. gigas* are not influenced by oyster ploidy similarly to the results obtained on *C. virginica* (Grodeska et al., 2019; Walton et al., 2013). Together, our study and these previous studies suggest that *Vp* infection risk for consumers is not affected by oyster ploidy. However, in November, the lower contamination by indigenous *Vp* of triploid oysters than diploid oysters would suggests a reduced risk of *Vp* infection for consumers, although complementary studies are needed to confirm these observations. In addition, seasonal variations of indigenous pathogenic profiles and *Vp* experimental depuration were observed. This study provides meaningful comparison of diploid and triploid *C. gigas* regarding *Vp* contamination. accumulation and depuration.

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AUTHORSHIP CONTRIBUTION

Marion Sorée: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – Original Draft, Writing – Review & Editing, Supervision. **Anna Le Meleder:** Methodology, Investigation**,** Formal Analysis, Writing – Review & Editing. **Elise Maurouard**: Resources, Writing – Review & Editing. **Mathias Papin**: Methodology, Writing – Review & Editing.

Christophe Stavrakakis: Resources, Funding acquisition, Writing – Review & Editing. **Corinne Audemard**: Writing – Review & Editing. **Dominique Hervio Heath**: Conceptualization, Methodology, Formal Analysis, Writing – Review & Editing. **Lionel Dégremont**: Conceptualization, Resources, Formal Analysis, Writing – Review & Editing.

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TABLE AND FIGURES LEGENDS

Fig. 1. Kinetic of seawater temperature (°C, **A**) and salinity (ppt, **B**) from 17th May through 22nd November 2021. Regressions line were represented in red.

Fig. 2. Weights (g) of wet tissues (**A**) and lengths (cm) of shells (**B**) from May to November 2021 of diploid (grey) and triploid (black) *C. gigas* oysters. For each batch at each sampling date n = 30. Data are represented as mean of the three batches per ploidy ± standard error.

Fig. 3. Mean *Vp* levels (log MPN.g^{-1}, n = 3) (**A**) and prevalence (%) of virulence markers (*tdh. trh1* and *trh2*) among *toxR*$^{+}$ samples (n = 3) (**B**) in diploid (grey) and triploid (black) oysters deployed at La Floride. Prevalence data represent the ratio of number of positive

samples for each of the genes (*tdh*. *trh1* and *trh2*) among the samples in which *toxR* was detected (*toxR*$^+$ samples). Data are represented as means of the three batches per ploidy ± standard error.

Fig. 4. Log reduction of IFVp201-*gfp*$^+$ concentrations in seawater after experimental accumulation by the diploid (grey) and triploid (black) oysters (n = 3). Data are represented as the mean between the three batches for each ploidy ± standard error.

Fig. 5. IFVp201-*gfp*$^+$ quantification (log bacteria.mL^{-1}) in haemolymph of diploid (grey) and triploid (black) oysters by flow cytometry after 24h of accumulation (0 h), and at 24 h and 48 h of depuration. Oysters were exposed to 10^6 CFU.mL^{-1} of IFVp201-*gfp*$^+$. 0 h: mean of two pools of five oysters per batch ± standard error (n = 3); 24 h and 48 h data: mean of three pools of five oysters per batch ± standard error (n = 3).

Table S1. Wet tissue weights (A) and shell lengths (B) from May to November 2021 of diploid and triploid oyster batches (n = 30). D1, D2, D3: batches of diploid oysters. T1, T2, T3: batches of triploid oysters. Data are represented as the mean of 30 oysters per batch ± standard error.

Table S2. Concentrations of IFVp201-*gfp*$^+$ in seawater before (n = 3) and after accumulation (n = 3), and the log reduction (n = 1). D1, D2, D3: batches of diploid oysters. T1, T2, T3: batches of triploid oysters. Data are represented as the mean of three replicates per batch ± standard error.

Table S3. Concentration of IFVp201- *gfp*$^+$ in haemolymph of oysters after 24 h of accumulation (n = 2), after 24 h and 48 h of depuration (n = 3), and log reductions after 24 h and 48 h of depuration for each batch of oysters. D1, D2, D3: batches of diploid oysters. T1, T2, T3: batches of triploid oysters. 24 h of accumulation: data are represented as mean of two pools of five oysters ± standard error. 24 h and 48 h of depuration: data are represented as mean of three pools of five oysters ± standard error.

Table S4. *p*-values of ANCOVA with wet tissue weight as covariable and sampling date as factor for accumulation and log reduction in haemolymph of oysters. Diploid and triploid oysters were analysed together due to absence of significant difference between the ploidies. In red, significant *p*-value.

Figure S1. Kinetic of seawater temperature (°C) from 1st through 30th September 2021 within oyster bags.

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DECLARATION OF CONFLICT OF INTEREST

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

HIGHLIGHT:

- Accumulation of indigenous *Vp* are similar between ploidy levels except in November

- Oyster ploidy did not significantly impact experimental *Vp* accumulation and depuration

- Pathogenic *Vp* and profiles of experimental depuration varied according to the seasons

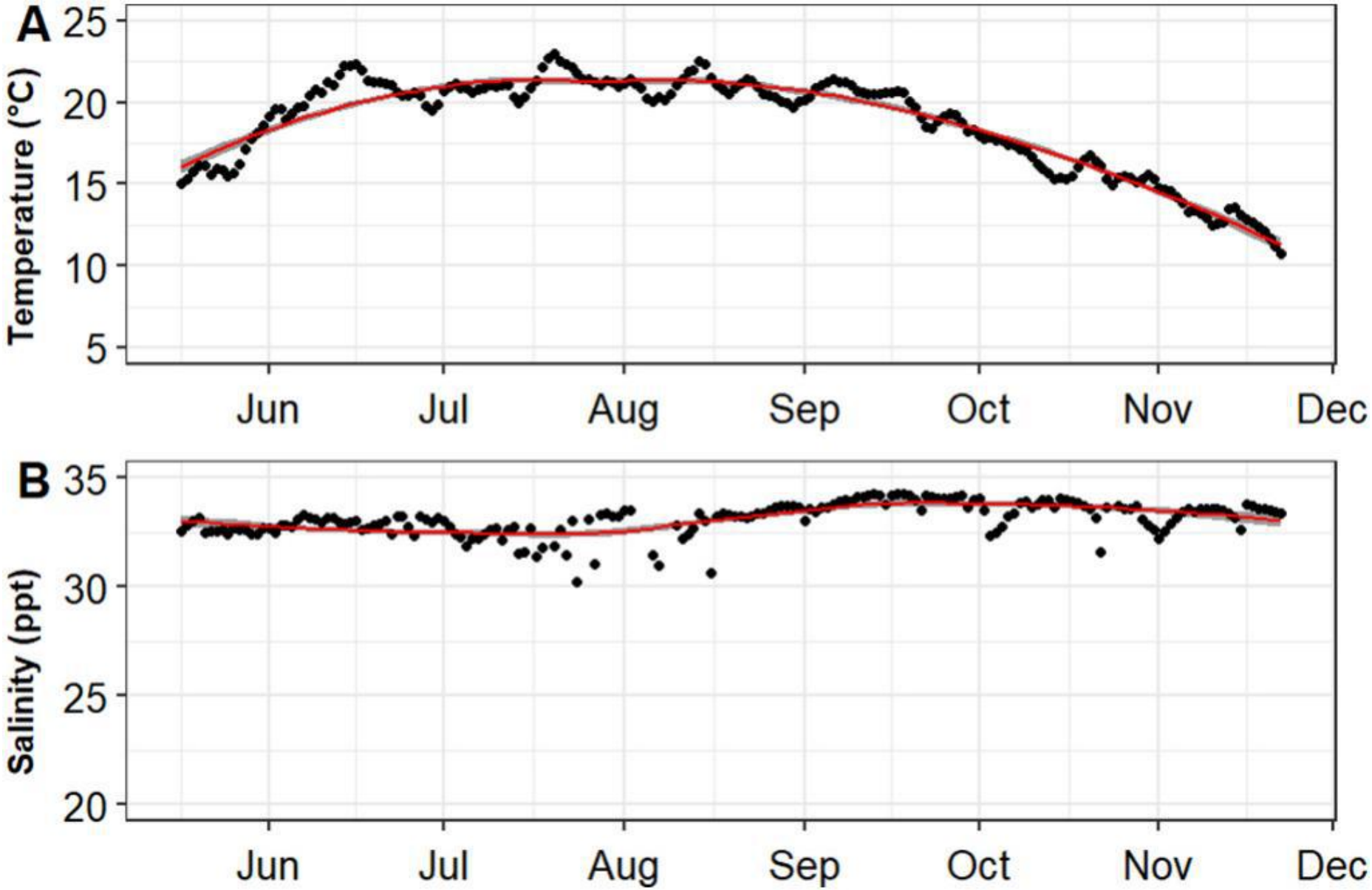
A
Temperature (°C)
25
20
15
10
5
Jun
Jul
Aug
Sep
Oct
Nov
Dec
B
Salinity (ppt)
35
30
25
20
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Figure 1

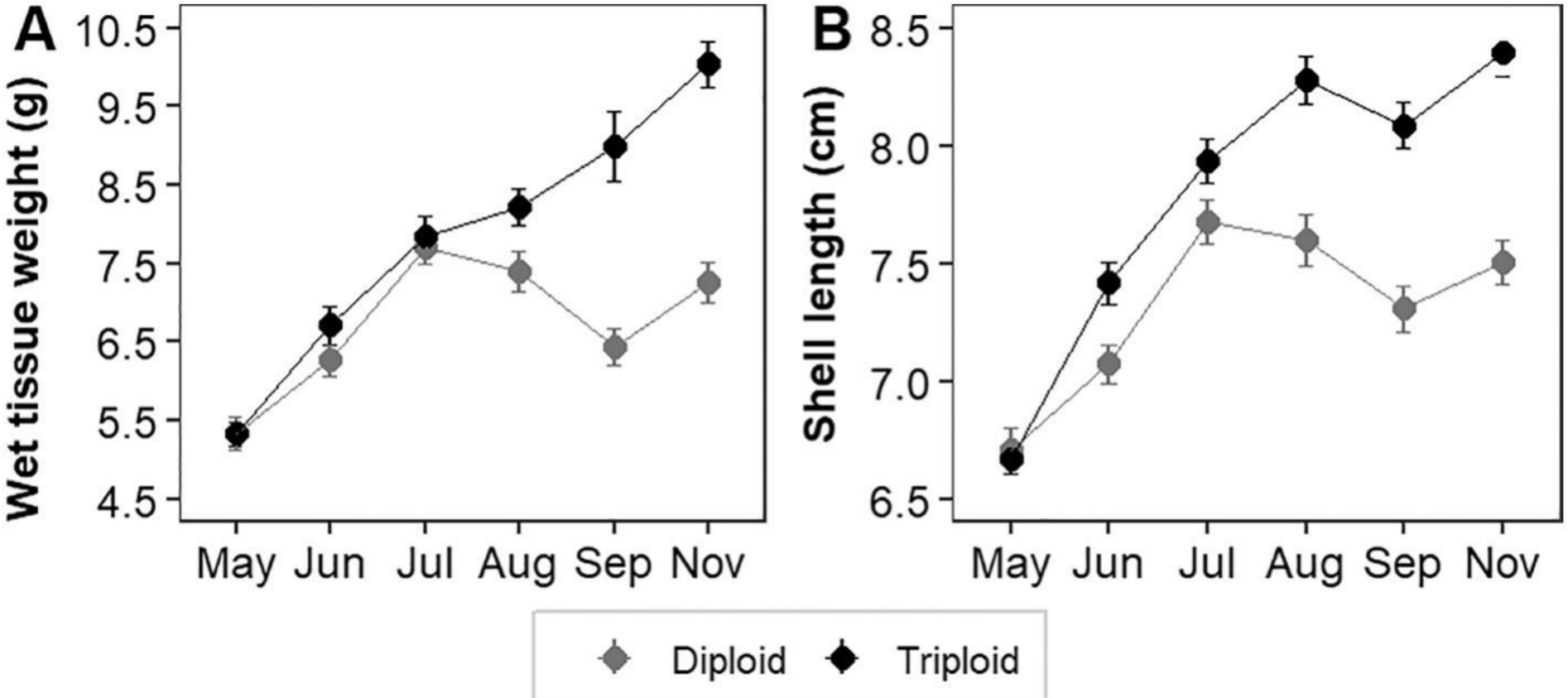
A
Wet tissue weight (g)
10.5
9.5
8.5
7.5
6.5
5.5
4.5
May Jun Jul Aug Sep Nov
B
Shell length (cm)
8.5
8.0
7.5
7.0
6.5
May Jun Jul Aug Sep Nov
Diploid
Triploid

Figure 2

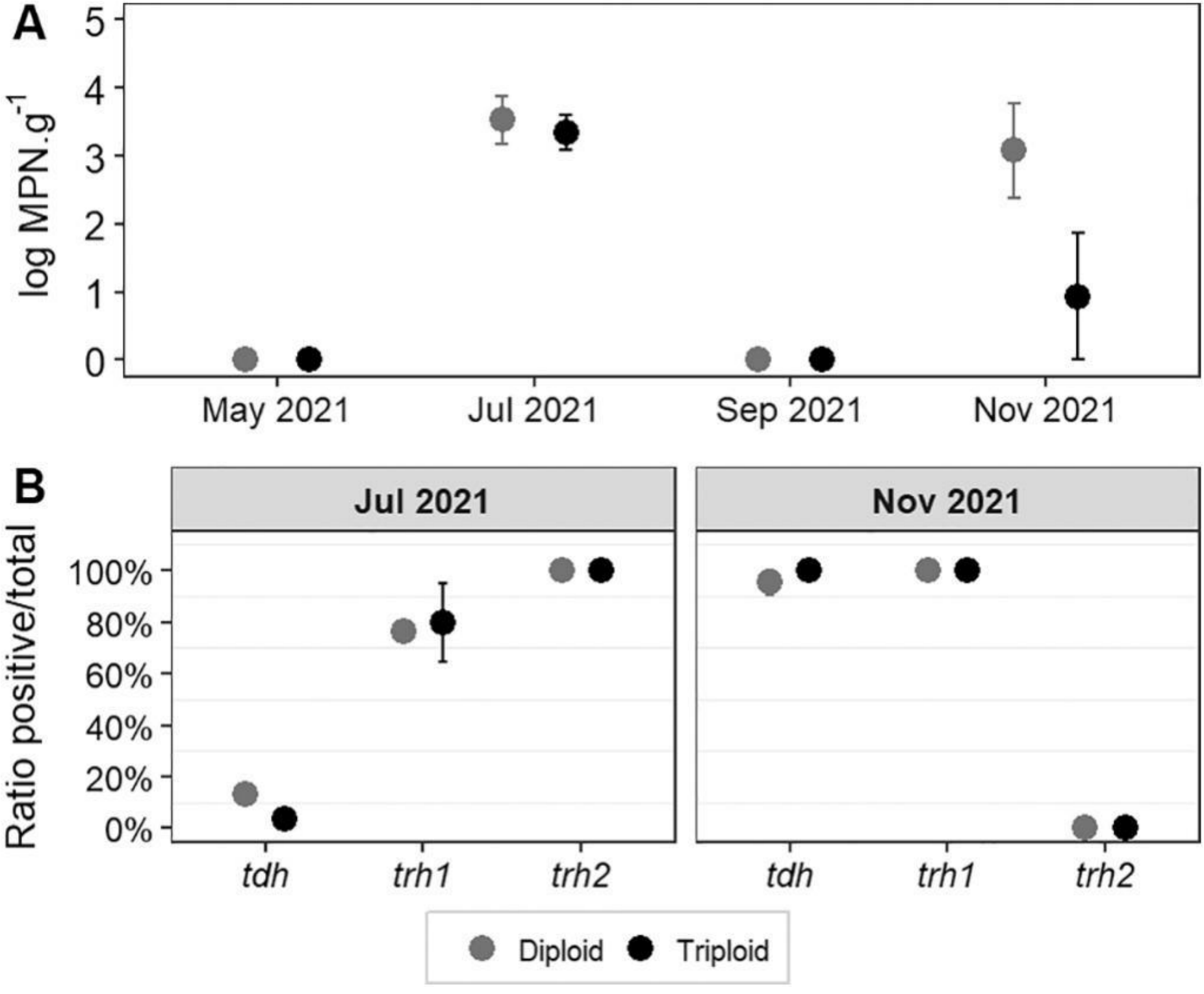
A
log MPN.g-1
5
4
3
2
1
0
May 2021
Jul 2021
Sep 2021
Nov 2021
B
Ratio positive/total
Jul 2021
Nov 2021
100%
80%
60%
40%
20%
0%
tdh
trh1
trh2
Diploid
Triploid

Figure 3

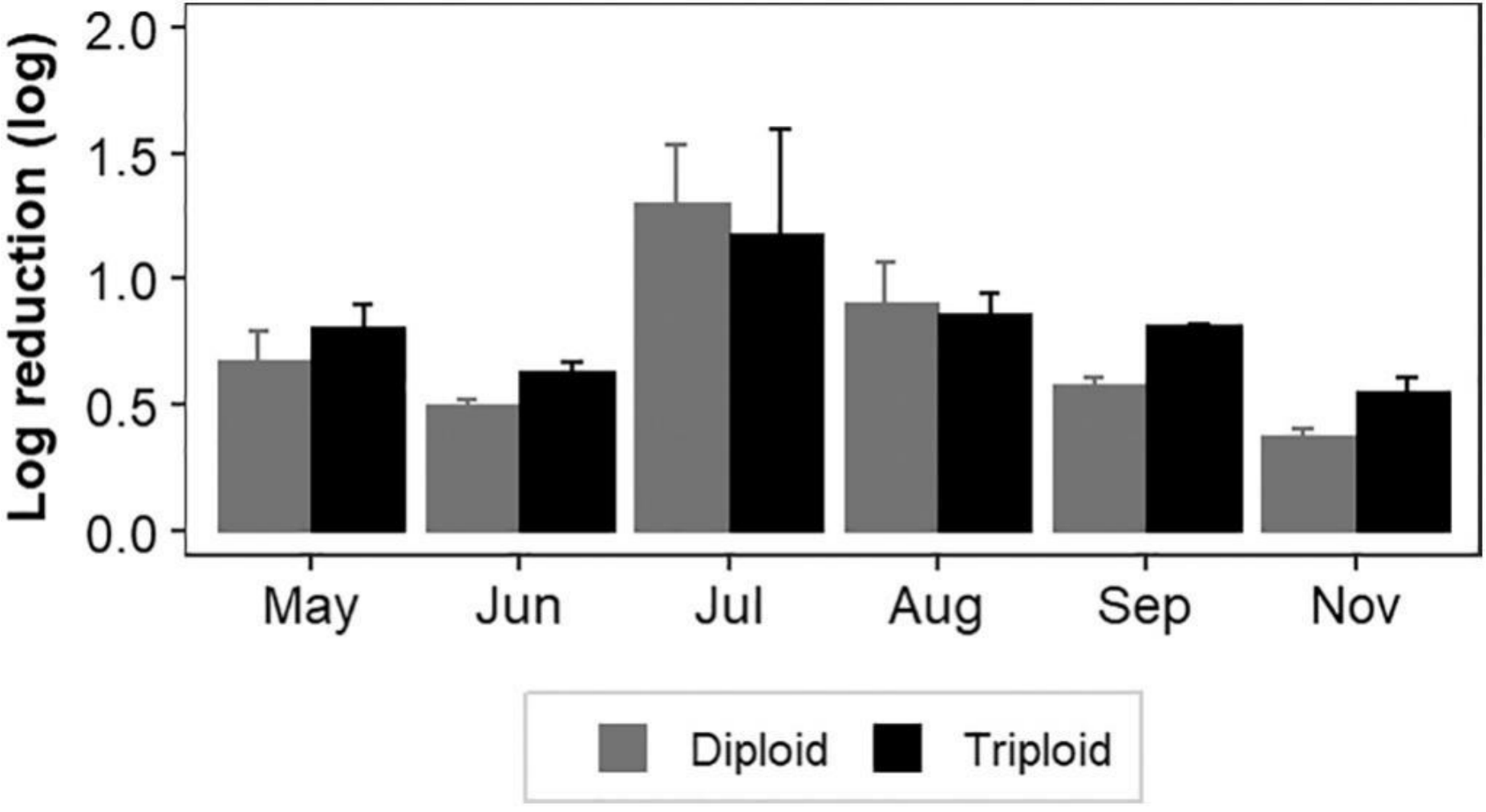
Log reduction (log)
2.0
1.5
1.0
0.5
0.0
May
Jun
Jul
Aug
Sep
Nov
Diploid
Triploid

Figure 4

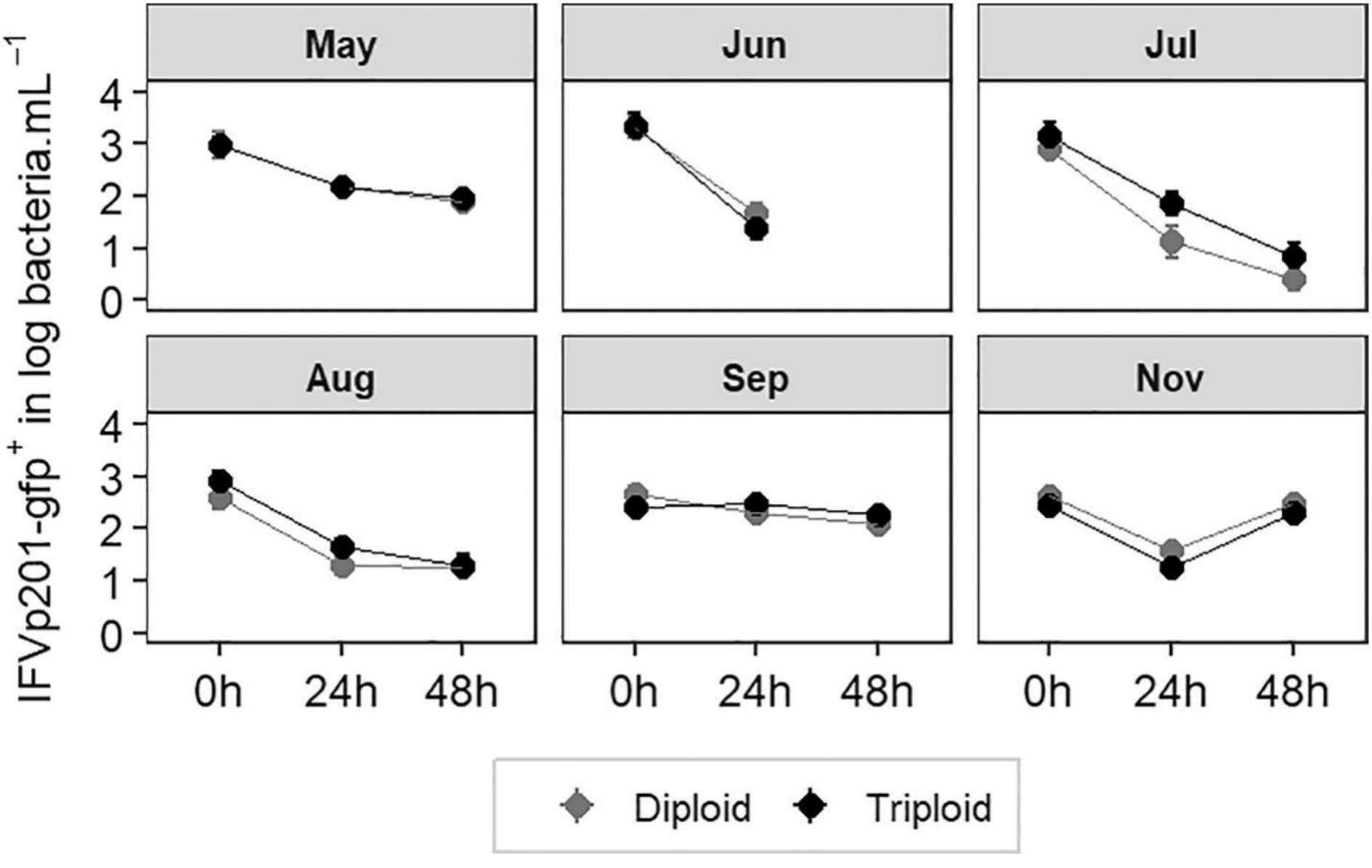
May
Jun
Jul
Aug
Sep
Nov
IFVp201-gfp+ in log bacteria.mL−1
0
1
2
3
4
0h
24h
48h
Diploid
Triploid

Figure 5