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Distribution in Tissue and Seasonal Variation of Norovirus Genogroup I and II Ligands in Oysters

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Abstract:

Bivalve molluscan shellfish, such as oysters, filter large volumes of water as part of their feeding activities and are able to accumulate and concentrate different types of pathogens, particularly noroviruses, from fecal human pollution. Based on our previous observation of a specific binding of the Norwalk strain (prototype norovirus genogroup I) to the oyster digestive tract through an A-like carbohydrate structure indistinguishable from human blood group A antigen and on the large diversity between strains in terms of carbohydrate-binding specificities, we evaluated the different ligands implicated in attachment to oysters tissues of strains representative of two main genogroups of human norovirus. The GI.1 and GII.4 strains differed in that the latter recognized a sialic acid-containing ligand, present in all tissues, in addition to the A-like ligand of the digestive tract shared with the GI.1 strain. Furthermore, bioaccumulation experiments using wild-type or mutant GI.1 Viruslike particles showed accumulation in hemocytes largely, but not exclusively, based on interaction with the A-like ligand. Moreover, a seasonal effect on the expression of these ligands was detected, most visibly for the GI.1 strain, with a peak in late winter and spring, a period when GI strains are regularly involved in oyster-related outbreaks. These observations may explain some of the distinct epidemiological features of strains from different genogroups.

INTRODUCTION

Bivalve molluscan shellfish, such as oysters, can filter large volumes of water as part of their feeding activities and are able to accumulate and concentrate different types of pathogens from fecal human pollution. We have known for 40 years that bacteria and viruses show differences in terms of concentration, accumulation and depuration from contaminated shellfish (34). As a consequence, absence of virus contamination cannot reliably be deduced from failure to detect bacterial contamination. A better understanding of the virus-specific modes of shellfish contamination is needed. A number of factors, including water temperature, mucus production, glycogen content of connective tissue or gonadal development have been identified to influence enterovirus and phage bio-accumulation in oysters (7, 13).

Among human enteric viruses, noroviruses (NoVs) are recognized as being the leading cause of epidemics or sporadic cases of gastroenteritis in all age groups of humans (15). They are discharged in large amounts in sewage and, being very resistant to inactivation, they have been detected in wastewater treatment plant effluents and in surface waters (44). The sanitary consequences are contamination of drinking water and foods such as shellfish, leading to outbreaks among consumers (57). Improved understanding of norovirus behavior in shellfish may lead to increased sanitary quality of shellfish on the market.

Many NoV strains bind to histo-blood group antigens (HBGAs) (50). HBGAs are complex glycans present on many cell types including red blood cells and vascular endothelial cells, as well as on the epithelia of the gastrointestinal, uro-genital and respiratory tracts. They are synthesized from a series of precursor structures by stepwise addition of monosaccharide units via a set of glycosyltransferases (31). Evidence accumulated from volunteers studies and from analysis of outbreaks indicate that binding to these carbohydrates is required for infection (20, 25, 27, 49). Moreover, various human NoV strains that bind to HBGAs present distinct specificities for HBGAs. As a result, most strains infect only a subset of the population based on HBGAs expression (26, 51). It was proposed that NoVs carbohydrate binding properties could be used to improve detection in waters and other complex samples (9).

We previously demonstrated specific binding of the Norwalk virus strain to the oyster digestive tract through an A-like carbohydrate structure indistinguishable from human blood group A antigen (23). Subsequently, this observation was confirmed in different oyster species and for other NoV strains (52, 54). Human A blood group antigen is one of the HBGA ligands of NoVs that are involved in the infection process (26, 48), suggesting that oysters may have the ability to specifically accumulate and concentrate a human pathogen based on the presence of a shared ligand between the two species rather than through non-specific interactions only. Since, different NoV strains show different specificities for HBGAs in humans, all strains may not be captured equally well by oysters.

This new concept where the relationship between the shellfish carbohydrate ligands and the virus strain specificity is taken into account may be used as a tool to discriminate within different

viruses, bivalve molluscan shellfishes and seasons both in terms of risk analysis and for shellfish producers. The objectives of the present study are to develop an ELISA approach for virus binding quantification of two NoV strains representative of the two main genogroups (GI.1 and GII.4) to various oysters tissues, to characterize the ligands involved in binding, to evaluate their relationship with bio-accumulation and finally to assess the influence of seasonal variations on virus binding.

MATERIALS AND METHODS

Reagents and Virus like particles (VLPs). The *Helix pomatia* (HPA) lectin, which is specific for terminal N-acetylgalactosamine residues in alpha linkage, and biotinylated *Maackia amurensis Agglutinin* (MAA), specific for sialic acid residues in α2,3 linkage, were obtained from Biovalley SA, (Marne la Vallée, France) and Oxford Glycosystems (Abingdon, UK), respectively. The CSLEX-1 monoclonal antibody was obtained from BD Pharmingen (San Diego, CA). Peroxidase-labeled avidin was purchased from Vector Labs (Burlingame, CA) and the neuraminidase from *Arthrobacter tumefaciens* was purchased from Roche Diagnostics GmbH (Mannhein, Germany). Synthetic oligosaccharides as polyacrylamide conjugates (PAA) or coupled to human serum albumin (HSA) were obtained from Dr. N. Bovin (Moscow, Russia) and IsoSepAB (Tullingen, Sweden), respectively. The neoglycoconjugates examined in this study were: H type 1-PAA (Fucα2Galβ3GlcNAcβ-R), H type 2-PAA (Fucα2Galβ4GlcNAcβ-R), H type 3-PAA (Fucα2Galβ3GalNAcα-R), Le^y-PAA (Fucα2Galβ4[Fucα3]GlcNAcβ-R), alphaGal-PAA (Galα3Galβ4GlcNAcβ-R), A tri-PAA (GalNAcα3[Fucα2]Galβ-R), Le^x-HSA (Galβ4[Fucα3]GlcNAcβ-R), Sialyl-Le^x-HSA (NeuAcα2,3Galβ4[Fucα3]GlcNAcβ3Galβ4Glcβ-R), and Sialyl-LNnT-HSA (NeuAcα2,3Galβ4GlcNAcβ3Galβ4Glcβ-R).

Constructs containing open reading frames 2 and 3 were used to produce recombinant VLPs for genogroup I.1 (Norwalk virus, strain 8FIIa, Genbank accession number M87661.1) and genogroup II.4 (Houston strain, Genbank accession number EU310927) as previously described (6). Mutants in the P2 subdomain of VP1 were generated by Ala point substitution and used to produce recombinant VLPs His 329 (H329A), Asn 331 (N331A), and Trp 375 (W375A) (11). After purification, the protein concentrations were determined and the number of VLPs was calculated based upon the VP1 molecular weight and 180 copies of VP1 per virus particle (18, 39). Corresponding antibodies were produced by immunizing rabbits with the GI.1 or the GII.4 purified VLPs, respectively.

Oyster samples. A large batch of oysters (*Crassostrea gigas*) was purchased at the beginning of the study and placed in a clean area in Brittany. These oysters were used for ELISA assay, immuno-histochemistry and bioaccumulation experiments. All samples, each including at least eight oysters were then randomly collected from this batch and shipped within 24 hours to the laboratory at 4°C. Environmental data such as water temperature and salinity were monitored on a daily basis using a Marel smatch TPS (NKE, Hennebont, France), located exactly on the same point as the oysters.

ELISA-based carbohydrate microtiter plate assays. Every month, eight oysters were shucked; digestive tissue including stomach, gills and mantle were dissected separately. One gram of each tissue was homogenized in 2 ml of PBS (pH 7.4), heated for 10 min at 95°C, centrifuged at 13,000 x *g* for 7 min at 4°C and the supernatant recovered. Protein concentration was estimated with BC Assay kit using bovine serum albumin as a standard (Uptima, Montluçon, France). After adjustment at 40 µg/ml, tissues extracts were coated onto NUNC Maxisorp immunoplates (ThermoFischer Scientific, Roskilde, Denmark) in 100 mmol/L carbonate buffer (pH= 9.6) by overnight incubation at 4°C. After blocking with 10% no-fat dried cow's milk in PBS for one hour, VLPs were added at a final concentration of 0.2 µg/ml (10^9 particles per well) and incubated for 1 hour at 37°C. Plates were then incubated with VLP-specific antibodies at 1/1000 dilution in PBS 5% milk for 1 hour at 37°C, and then peroxidase anti-rabbit IgG (Uptima) at 1/2000 in PBS 5% milk were added and incubated for 1 hour at 37°C. Between each step, plates were washed 3 times with PBS containing 5% Tween 20 (Sigma Aldrich, France). The enzyme signals were detected with TMB (3,3′,5,5′-tetramethylbenzidine; BD Bioscience, San Jose, CA) as substrate and read at 450 nm with a spectrophotometer (Safire, Tecan).

Sodium periodate treatment was performed on the coated material using 10 mmol/L sodium periodate in 50 mmol/L sodium acetate buffer, pH 5.0, for 30 min at RT, followed by a 10 min incubation with 1% glycine in water. Control wells were treated similarly without sodium periodate. Inhibition of VLP binding with the HPA lectin was performed by preincubation with the lectin diluted to 50 µg/ml in PBS for 30 min at RT. After washings, VLPs were added, and their binding detected as described above.

For each tissue extract, negative controls without VLPs but with primary or secondary antibodies were included. Human secretor type A, B and O saliva samples coated in individual wells on the ELISA plate served as positive controls in each plate. The GI.1 VLPs are known to bind well to A and O secretor-positive saliva but poorly to B secretor-positive saliva (31), the GII.4 VLPs used here bind well to secretor-positive saliva irrespective of the ABO type (unpublished results). For the HPA inhibition test, positive (without HPA preincubation) and negative controls (without VLPs), as well as positive controls on saliva samples were performed on the same plate. In all cases, after validation of the saliva controls and the negative controls, OD values were measured for all samples. All samples were analyzed in duplicate on the same plate and discordant results between the two replicates (more than 0.1 OD value difference) were not accepted. The test sample ratio was determined by dividing the OD value of the test sample by the OD value of the negative control (same tissue without VLPs). A sample was considered positive if this ratio was ≥ 2.

Binding of VLPs to immobilized neoglycoconjugates. Briefly, oligosaccharides conjugated to either PAA or HSA were coated onto NUNC Maxisorp immunoplates as previously described (31). After blocking with 5% defatted dry cow's milk, VLPs at 4 µg/ml (2×10^{10} particles per well) were added for 2 hours at 37°C. VLP binding was detected by incubation with the respective anti-GI.1 or

anti-GII.4 rabbit antisera diluted at 1/1000 followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (Uptima). The peroxidase substrate TMB (BD Bioscience, San Jose, CA) was used and OD values determined at 450 nm. OD values twice above background were considered positive.

Bioaccumulation of VLPs. GI.1 wild type and H329A, N331A, W375A mutants VLPs or GII.4 VLPs were added at various concentrations to 500 mL of clean seawater and homogenized for 5 min. Four clean live oysters were added to the sea water and incubated for 24 hours at 15°C (water temperature) under oxygenation. All VLPs were bioaccumulated twice (GI.1 mutants), five (GI.1 wild type) and seven (GII.4) times, the different bioaccumulations were conducted from October to April (except December).

Immunohistochemical analysis. Oysters (uncontaminated or after bioaccumulation) were shucked and the body was cut horizontally so as to visualize all organs (from the mouth to the anus) on a single section. Sections were fixed in 10% formaldehyde (Gurr, VWR, France) for 24 hours, paraffin embedded, and sliced into thin sections (5 µm). After preparation of tissue sections as described previously (32), sections from uncontaminated oysters were covered with 2 µg GI.1 or GII.4 VLPs/ml (10^{11} particles/ml) of and left overnight at 4°C before being washed three times for 5 min in PBS at room temperature. The presence of VLPs bound to the oyster tissue was detected using respective antibodies as previously described (32). Negative controls included sections from uncontaminated samples not exposed to VLPs, and exposed sections without primary antibody. Staining with MAA was performed by incubating sections with the biotinylated lectin diluted at 10 µg/ml overnight at 4°C, followed by a second incubation with peroxidase-labeled avidin at 1/1000 for 45 min at room tmperature. Immunoreactivity, detected under microscopic analysis, was considered as strong (intense red coloration), weak (pale red coloration) or negative (no coloration).
For bioaccumulation experiments, brightfield images captured with an Olympus color CCD camera were analysed using a deconvolution algorithm to separate the dye contribution at each pixel (41). Regions of interest (covering hemocytes surfaces) were selected for each image through a markup algorithm in Image-J.

Neuraminidase treatment was performed on oyster sections by incubation at 37°C with 15 mU neuraminidase in 50 mmol/L sodium acetate buffer pH 5.3 for a total of 18 hours with replacement of the neuraminidase solution every 6 hours. Serial control sections were incubated in parallel in the same buffer without the enzyme. Blocking of VLP binding by the MAA lectin was performed by preincubating tissue sections with the lectin diluted at 50 µg/mL in 1% PBS/ BSA at 4°C overnight. Treatment with sodium periodate at 10 mmol/L and 1 mmol/L in 50 mmol/L sodium acetate buffer, pH 5.0, was performed on tissue sections for 30 min at room temperature. Serial control sections were preincubated in the same conditions in absence of the lectin or sodium periodate prior to addition of the VLPs. Then sections were rinsed 3 times with PBS, incubated with GI.1 or GII.4 VLPs for 1 hour at room temperature, and the detection of binding was performed as above.

Statistical analysis.

Means were compared using the Student's t test, and a P value of less than 0.05 was considered to be significant (Origin software, Paris, France).

RESULTS

Comparison of the genogroups binding capacity to oysters tissue extracts. By using representative VLPs GI.1 and GII.4, the two main human NoV genogroups were compared in their capacity to bind to different oyster tissues, namely the digestive tissue, the gills and the mantle (Table 1). GI.1 VLPs bound readily to digestive tissues but not to the gills or the mantle. With GI.1 VLP, the mutants H329A and W375A lost their capacity to bind to digestive tissues whereas the N331A mutant bound to digestive tissues. The ratio observed for this mutant was slightly higher than for the GI.1 prototype for all tissues tested, but the differences were not significant (Table 1). GII.4 VLPs bound strongly to digestive tissues but also to the gills and the mantle. Although the mean binding to the gills was somewhat lower than to the digestive tissues or the mantle, the difference did not reach statistical significance ($p>0.3$).

In a previous study we demonstrated on tissues sections that GI.1 VLPs bind specifically to carbohydrate ligands of oysters digestive tissues (23). Since periodate oxidation cleaves C-C bonds with vicinal hydroxyl groups of carbohydrates, we treated the coated tissue extracts in the ELISA plate with 10 mmol/L sodium periodate and observed a total loss of binding capacity for GI.1 VLPs (Fig. 1A). Similarly, periodate treatment completely abolished binding of GII.4 VLPs to all oysters tissue extracts, indicating that GII.4 binding was carbohydrate-dependent, similar to that of GI.1 (Fig. 1B).

Since the HPA lectin, that recognizes α-linked N-acetylgalactosamine terminal residues prevented binding of GI.1 to tissues sections (23), the ELISA plate was incubated with this lectin prior incubation with GI.1 or GII.4 VLPs. As expected, the GI.1 binding to digestive tissues was completely inhibited (Fig. 1C) ($p= 0.0124$). In contrast, the binding of GII.4 VLPs to oyster tissues (Fig. 1D) was only inhibited by approximately 50% for digestive tissues ($p= 0.0182$), and no significant inhibition was observed on mantle and gills ($p= 0.453$ and $p= 0.315$, respectively). This suggested that GII.4 VLPs bind to two distinct ligands, one primarily or exclusively present in digestive tissues and shared with both those of HPA and GI.1 VLPs and the other present in all three tissues, but not shared with either HPA or GI.1 VLPs.

Identification of the VLP ligands.

In order to get insights into the structure of the second carbohydrate ligand of GII.4 VLPs, we first tested the binding of the two strains to a selected set of neoglycoconjugates (Fig. 2). The GII.4 VLPs attached to H type 1, H type 3 and Le^y as well as to the A epitope, consistent with the recognition of a ligand shared with the HPA lectin on oyster digestive tissues. In addition and similar to what was previously observed with other GII strains (42), the GII.4 VLPs used in this study attached to sialylated structures, Sialyl-Le^x and to a lesser extend its non fucosyl counterpart Sialyl-

LNnT (Fig. 2). These results suggested that the GII.4 VLPs may recognize a sialylated carbohydrate motif present in various oyster tissues.

To test this hypothesis, we used an immunohistochemistry approach. Oriented tissue sections with all organs present on the same slide were incubated with the different VLPs. The GI.1 VLPs bound principally to oesophagus, midgut, primary and secondary ducts of the digestive diverticula and tubules, as previously described (23, 54) (Fig. 3A). No binding was observed to gills, mantle or labial palps (Fig. 3A and C). The GII.4 VLPs also bound to the different digestive organs, but strong binding was additionally observed to labial palps, gills and mantle (Fig. 3B, D and E). Within contrast to what was observed with GI.1 VLPs, those from the GII.4 strain bound to non-epithelial histological structures from all organs in addition to the digestive epithelial cells. This set of observation is in accordance with the results obtained by ELISA and shown in Table 1.

After treatment of tissue sections with 1 mmol/L sodium periodate, GI.1 binding was not clearly decreased (not shown) and some binding was still observed on the epithelial part of the digestive tissues for GII.4, although it was no longer detectable on gills or other tissues (Fig. 3F and G). However, when tissues sections were treated with a higher periodate concentration (10 mmol/L), both GI.1 (data not shown) and GII.4 binding were completely lost (Fig. 3H). This result is consistent with the possible presence of two GII.4 ligands on oysters tissues, one localized in all tissues and sensitive to 1 mmol/L periodate, as expected for a sialic acid-containing epitope and the second, restricted to the digestive tissues and only sensitive to a higher periodate concentration, as expected for a neutral carbohydrate epitope. To determine whether sialic acid residues in α2,3 linkage were indeed present in *Crassostrea gigas* oysters, tissue sections were first incubated with the MAA lectin and an anti-sialyl-Le^x mAb. Although no reactivity was detected using the anti-sialyl-Le^x, a strong staining, largely overlapping with that of the GII.4 VLPs, was observed in most tissues with MAA (Fig. 3I). In addition, following treatment of the tissue sections with neuraminidase, MAA binding was completely lost, except on the mantle single epithelial cell layer, indicating that on most histological structures, the lectin recognized neuraminidase-sensitive sialylated structures, as expected (Fig. 3J). When tissue sections were incubated with the MAA lectin prior to GII.4 VLPs incubation, the VLP binding was drastically reduced, showing a competition between the lectin and the VLPs for their binding sites (Fig. 3K and L). To confirm the involvement of sialic acid residues in GII.4 VLPs binding, tissues sections were then treated with neuraminidase before incubation with VLPs. The attachment of GII.4 VLPs was largely decreased (Fig. 3M and N). In contrast, no effect was observed on GI.1 VLPs binding (Fig. 3O).

Bioaccumulation. In order to evaluate biological impact of live oysters, bioaccumulations were performed using the different GI.1 VLPs, using final concentrations ranging from $5x10^5$ to $5x10^8$ VLPs/ml of seawater. GI.1 VLPs were bio-accumulated very efficiently and were detected only in digestive and connective tissues. An intense and clear red staining of digestive tubules (Fig. 4F) or some cells within connectives tissues (Fig. 4D) was observed. Even at 10^5 VLPs/ml of seawater, VLPs

could be detected after thin layer sectioning. Surprisingly, GII.4 VLPs were not found in oyster tissues following bioaccumulation, despite several assays and high concentrations (10^9 VLPs/ml of seawater) used. Subsequent studies demonstrated that the GII.4 VLPs lost their structural integrity when suspended in sea water (electron microscopy observation, data not shown). As differences were observed between GI.1 and mutants by ELISA, bioaccumulations were conducted using the three GI.1 mutant VLPs. All three mutants could be detected within shellfish tissues, but only when higher concentrations were used (at least 10^9 VLPs/ml of seawater). Specific staining was seen mainly on hemocytes localized in the connective tissue of all organs. No red or brown color was seen in the negative controls (Fig. 4A and B). Nevertheless, different color intensities were clear under microscopic examination following bioaccumulation of either the wild type and N331A mutant or the H329A and W375A mutants: the H329A and W375A mutants showed pale brown coloration (Fig. 4C and E), whereas the wild type and N331A mutant showed a clear red coloration (Fig. 4D and F), suggesting a higher bioaccumulation efficiency of the latter. Dye color intensity was computed for 307 hemocytes from 9 individual fields from tissue sections of oysters bioaccumulated with GI.1 wild type VLPs at 10^7 VLPs/mL, 826 hemocytes from 13 fields from tissue sections of oysters bioaccumulated with the GI.1 H329A mutant VLPs at 10^9 VLPs/mL, and for 148 hemocytes from 6 fields of control oysters. Mean intensities showed a statistically significant difference ($p< 0.05$), between the GI.1 wild type and H329 VLPs (Fig. 5). Although dye intensity cannot be directly correlated with the number of bioaccumulated VLPs, this result clearly shows that the lack of ligand recognition drastically reduces bioaccumulation. This is particularly obvious when considering that mutant VLPs were detected only when seeded at a concentration a hundred times higher than that of the GI.1 prototype VLPs.

Seasonal variations. Oysters collected every month were assayed in the ELISA test at least three times each and in separate experiments. A clear seasonal effect was observed for GI.1 VLPs, with an increased binding capacity during the months January to May that corresponds to the end of winter and most of spring (Fig. 6A). A comparison of the two defined periods, period A (January-May) (mean signal to noise ratio values 10.9 ±3.6) vs period B (June-December) (4.4 ±1.1), revealed a highly significant different level of GI.1 binding to oysters digestive tissues ($p=4.8x10^{-12}$). In contrast, for GII.4 VLPs, seasonal variation of binding to tissue extracts was far less apparent (Fig. 6B), although the difference between these two periods (12.3 ±3.7 compared to 9.1±2.8), were statistically significant in the digestive tissues ($p= 0.001$), mantle ($p=0.004$) and gills ($p=0.01$). No significant differences were observed between the binding of GI.1 and GII.4 VLPs to the digestive tissues during period A ($p= 0.167$), but significant difference was observed for binding to digestive tissues during period B ($p=1.28x10^{-10}$). Seawater temperature varied from 6°C to 20°C, and showed statistically significant different values between the two seasons considered (11.56±2.8 compared to 16.8±2.9, $p<0.001$), suggesting a potential inverse association with high expression of GI.1 binding sites on

oysters gut. However, water temperature began to decrease about two months prior appearance of strong GI.1 binding, showing a loose association (Fig.7). Salinity presented a more complex pattern of variation with its highest level being in summer. Although it was also lowest during cold months (December, January), presumably linked to other environmental parameters such as rain, this was not clearly associated with virus binding.

DISCUSSION

Noroviruses are genetically and antigenically diverse (3, 15). Since the first characterization of the Norwalk virus in 1990 (17), a large number of strains have been described worldwide. The genetic classification system is based on relatedness of the complete VP1 capsid protein, and currently there are five recognized genogroups (58). Among these five genogroups, humans may be infected by GI, GII and GIV strains, while GIII and GV strains infect cows and mice, respectively. For some years the GII strains, particularly those of the GII.4 cluster, have been the predominant viruses detected in different parts of the world (29, 46). Beside this high prevalence of GII.4 NoV in the human population, another important distinctive feature of GII.4 is their manner of transmission since they appear to be mainly transmitted via person-to-person contact in community outbreaks (47). Other strains, and importantly GI strains, are more often transmitted via food or environmental contamination (30, 36). Fecal viral load, which is higher for GII compared to GI strains (10), shedding by asymptomatic subjects (2), and distinct behaviors during waste water treatments (12) or food processing (8) may partially explain some of the observed differences between GI and GII epidemiology. More general parameters such as global climate change may also have an impact on outbreak seasonality and strain transmission (40) and the overall impact of environment on infectious diseases needs to be considered (43). However, the epidemiological difference between genogroups implicated in outbreaks is even clearer when considering oyster-related outbreaks where NoV GI may constitute up to 30% of strains detected in patients stools or shellfish samples (14, 19, 21,24, 35). For a long time, oysters were believed to act as filters or ionic traps, passively concentrating particles. However depuration failure, long-term persistence in shellfish and the above mentioned difference in strain transmission argue in favor of more specific mechanisms for NoV bioaccumulation in oysters. Specific binding of Norwalk virus via a carbohydrate structure very similar to human histo-blood group A antigen in *Crassostrea gigas* oysters (23), subsequently confirmed to occur in another oyster species (*Crassostrea virginica*) (52), may explain these differences. Interestingly, characterization of a blood group A activity in the acidic polysaccharide fraction from *Craossostera gigas* viscera was reported quite a long time ago (38). Data presented here confirmed that GI.1 VLPs bind mainly to digestive tissues but not to other organs, and were consistent with the results of bioaccumulation studies, performed with Norwalk virus (GI.1) and RT-PCR detection (4). However, genetic diversity of NoVs is also reflected in their binding capacity to various HBGAs structures (26, 54). Differences observed between GI.1 and GII.4 binding to human HBGAs are also present in oyster tissues. Our data

demonstrate that the distribution of GII.4 is not restricted to digestive tissues as for GI.1, in accordance with reports demonstrating the presence of GII.4 in gills, albeit to a lower extent than in digestive tissues (33, 56). Our quantitative analysis is consistent with a lower expression of GII.4 binding sites in gills as compared to the digestive tissues, although the difference was not statistically significant. In addition, we demonstrate here that the binding to gills and mantle tissue sections involves a sialic acid in α2,3 linkage, whereas in digestive tissues the interaction involves both the sialic acid and an A-like carbohydrate ligand. Very little is known on sialic acid and their distribution in oyster tissues (55). Some differential recognition of these ligands by GI and GII strains may lead to distinct outcomes in terms of the persistence of viral particles within the different organs. In other words, recognition of the sialylated ligand by GII strains may lead to a quicker degradation or release, whereas recognition of the A-like ligand results in virus persistence. Following accidental contamination by sewage of a producing area few years ago, we found that after one week the number of shellfish containing GII NoVs was higher than for GI strains, whereas after three weeks the converse was true (24). Although this hypothesis remains somewhat speculative and requires further evaluation, it may partly explain why GII strains that are shed in the environment in far larger amounts than GI strains are relatively less frequent causes of oyster-related outbreaks (14, 19, 21, 24, 35).

In addition to ligand-specific recognition, binding of NoVs to oyster tissues may involve non-specific interactions, as suggested in the past for enterovirus or reovirus (5, 7, 13). However, it is likely that these non-specific mechanisms of attachment are less efficient than specific ligand-mediated binding and we may predict that viruses captured through non-specific interactions only will be less efficiently concentrated and/or that their persistence within oyster bodies will be shorter. The demonstration that GI.1 mutant VLPs were accumulated confirmed this hypothesis as the two mutants that did not recognize the type A antigen were detected in tissues sections albeit to a far less extent than the GI.1 controls VLPs. These mutants were made because the individual amino acids make up (H329, W375) or are in proximity (N331) to the site of the VLP responsible for binding to HBGAs for Norwalk virus, based upon studies performed at an atomic resolution scale. Mutation of His-329 or Trp-375 chains completely abrogates carbohydrate binding (11). Similarly, these mutations induced a total lack of recognition of oyster DT, and a less efficient bioaccumulation. Another consequence of specific ligand-mediated bioaccumulation may be its effect on viral persistence within the shellfish body. This may be the objective of future studies, as VLPs being non infectious, can be used to bioaccumulate oysters then re-located in the environment for a follow-up analysis (28). Unfortunately, the GII.4 VLPs used in this study were not stable after dilution into seawater (data not shown), so bioaccumulation could not be performed. Although VLPs are useful surrogates of the noncultivatable NoVs, control experiments must be performed before use of the VLPs in environmental studies to avoid misinterpretation of negative results. The bioaccumulation experiments showed that VLPs are localized in hemocytes within connective tissues. Two subtypes of these cells have been defined, the granulocytes and the hemoblast-like cells. Granulocytes have important physiological functions

including nutrient transport, digestion, wound healing, shell mineralization and excretion, suggesting that viral particles could be destroyed rapidly if ingested by this cell type (1). However, hemoblast-like cells do not contribute to defensive responses like phagocytosis or encapsulation and lack the common intracellular enzyme systems associated with host defense (1). Further characterization of these cells will be important to evaluate viral particle behavior.

Another major interest of the data presented here concerns the observed seasonal variation. Oysters are able to bind much more efficiently to GI.1 VLPs during the first five months of the year (January to May) as compared to the rest of the year. At variance with our results, a lack of seasonal variation was reported for three oyster species using GI.1 VLPs (53). Different parameters such as the number of replicates, sensitivity of the test, or environmental conditions may explain the different results. The differences observed here may be linked to the water temperature, lower during the end of winter and beginning of spring. Influence of water temperature is difficult to analyze as many other environmental parameters, such as oyster physiology, chlorophyll a concentration, phytoplankton, may have an impact (16). Interestingly, the end of winter/beginning of spring period corresponds to the highest concentration of NoVs in sewage and to a time when heavy rainfall is common, both of which may increase the risk of oyster contamination following failure of sewage treatment plant or during flooding (12, 37). Although present, the seasonal effect was markedly less apparent for GII.4 VLPs, suggesting that the corresponding strains may be accumulated with more or less similar efficiency all year round. In France, the peak of oyster-related outbreaks occurs at the beginning of the year (21, 22, 24, 25), whereas the peak of consumption lies between December 24-31 (45).

In the present study, we demonstrated that NoVs of distinct genogroups present a different behavior with regard to oyster tissue recognition, similar to findings in human tissues, and that seasonal variation of NoV VLP binding to oyster tissues exists. As fundamental studies progress to understand the behavior of NoVs in humans, basic research on oyster contamination mechanisms will progress, too. Understanding processes and mechanisms of virus uptake by molluscan shellfish, in conjunction with environmental studies, will lead to the development of strategies to prevent oyster contamination in the future. These studies, initialized more than 40 years ago for other enteric viruses, may be more successful now as new tools are developed.

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TABLES

Table 1: GI.1, GII.4 and mutant VLPs binding capacity to oyster tissue extracts evaluated by ELISA assay.

VLPs	Shellfish tissues analyzed[a]			
	# of samples analyzed	Digestive tissues	Gills	Mantle
GI.1	50	7.4 (±4.2)	1.7 (±0.5)	1.8 (±0.9)
H329A	6[b]	0.8 (±0.1)[d]	1.0 (± 0.1)	1.0 (±0.1)
N331A	6[b]	10.1 (±3.4)[c]	2.4 (±1.3)	3.1 (±1.8)
W375A	6[b]	0.8 (±0.1) [d]	0.9 (±0.2)	1.0 (0.2)
GII.4	50	10.8 (±3,7)	8.9 (±4.3)	9.9 (±3.4)

a: Numbers represent the mean values of signal to noise ratio ± SD calculated as described in the Materials and Methods section, obtained for all shellfish samples analyzed.

b: samples were selected from different periods of the year, mean OD values of saliva used as positive controls was 1.3 ±0.3 for GI.1 and mutants and 1.0±0.2 for GII.4

c: comparison with GI.1 $p= 0.31$

d: comparison with GI.1 $p<0.05$

FIGURE LEGENDS

Figure 1: Inhibition of binding of VLPs to oysters tissue extracts.
Binding of GI.1 (A and C) and GII.4 (B and D) VLPs to digestive tissues, gills and mantle (x axis) tissue extracts pretreated with sodium periodate (A and B) or preincubated with the HPA lectin (C and D) expressed as signal to noise ratio +/- SD (y axis). Filled bars are homogenates in the absence of the inhibitory agent and empty bars represent the presence of the inhibitory agents. The positive threshold is indicated by a dashed line.

Figure 2: Binding of recombinant VLPs to neoglycoconjugates.
Binding of GI.1 (black bars) and GII.4 (grey bars) VLPs to either PAA or HSA-conjugated synthetic oligosaccharides (x axis) expressed as OD mean values of duplicates for each neoglycoconjugate (one representative experiment out of three with similar results).

Figure 3: Immunohistochemical analysis of GI.1 and GII.4 recombinant VLPs binding to oyster's tissues. VLPs and the MAA lectin were incubated on tissue sections as described in the Materials and Methods section. Binding of GI.1 VLPs to the esophagus (arrow) and lack of binding to the labial palps (star) (**A**). Binding of GII.4 VLPs to the esophagus (arrow), the labial palps (star) and connective tissue structures (arrow head) (**B**). Absence of binding of GI.1 VLPs to the gills (**C**). Binding of GII.4 VLPs to connective tissue structures of the gills (arrow head) and weak staining of epithelial cells (arrow) (**D**). Binding of GII.4 VLPs to mantle (**E**). Staining of the digestive tissue by GII.4 VLPs on epithelial cells (arrow) and connective tissue (arrow head) in absence of periodate treatment (**F**), after 1 mM periodate treatment (**G**) and after 10 mM periodate treatment (**H**). Staining of the digestive tissue by MAA without (**I**) and with (**J**) neuraminidase treatment. GII.4 VLPs binding to the gills without (**K**) or with MAA preincubation (**L**). GII.4 VLPs binding to the digestive tube without (**M**) and with (**N**) neuraminidase treatment. GI.1 binding to the esophagus after neuraminidase treatment (**O**).

Figure 4: Immunohistochemical analysis of GI.1 and GI mutants recombinant VLPs after bioaccumulation.

Oysters were incubated in seawater seeded with GI.1, or H329A, for 24 hours. Tissues were then fixed and VLPs were detected by immunohistochemistry as described in the Materials and Method section. Negative controls were performed using tissue sections from oysters bioaccumulated with GI.1 VLPs but without primary antibody (A) (arrow shows hemocytes that appear after counterstaining with hemalun), or using tissues sections from non bioaccumulated oysters incubated with both primary and secondary antibodies (B). Wild type GI.1 VLPs after bioaccumulation were detected in connective tissues (D) and around digestive diverticula (F). H329 VLPs were detected in

connective tissues (C) and around digestive divericula (E). Note the difference in red color intensity between D, F and C, E.

Figure 5: Quantitative analysis of immunohistochemical detection of bioaccumulated VLPs. Dye color intensities of hemocytes were computed as described in the Materials and Method section. Mean values +/- SD are shown for hemocytes of oysters bioaccumulated with wild type Norwalk virus VLPs (NVwt) at 10^7VLPs/mL, with mutant H329A Norwalk virus VLPs (H329) at 10^9VLPs/mL, or of a control oyster (control). *p<0.01, **p<0.001.

Figure 6: Seasonal variations of VLPs binding to oysters tissue extracts.
Binding of VLPs from the GI.1 strain (A) and the GII.4 strain (B) to the digestive tract (black bars), the gills (grey bars) and the mantle (white bars) extracts from collected oysters over one year (x axis), expressed as signal to noise ratio +/- SD (y axis). The positive threshold is indicated by a dashed line.

Figure 7: **Salinity and temperature variations.**
Salinity (open square) and temperature (black triangle) were expressed as mean values of daily recorded data +/- SD per month.

FIG. 1

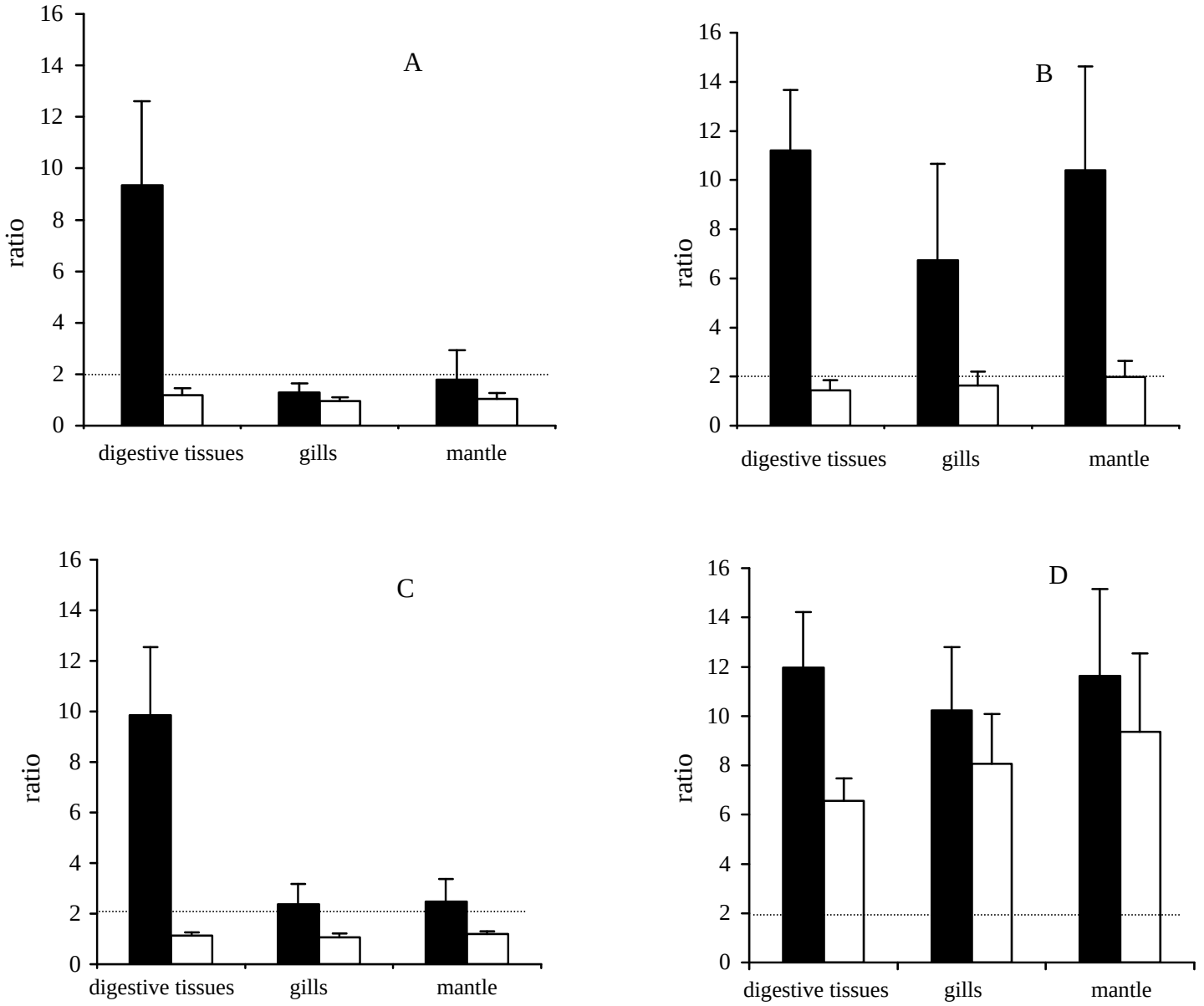
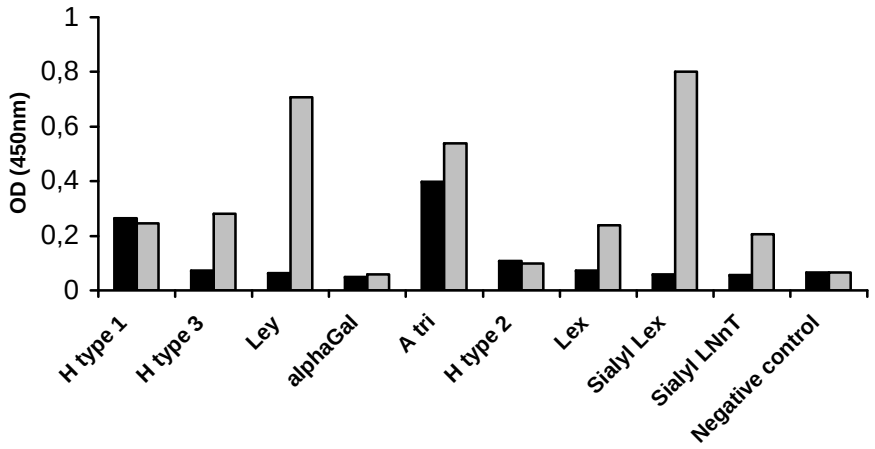


FIG.2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Sialyl LNnT
Negative control

Fig. 3

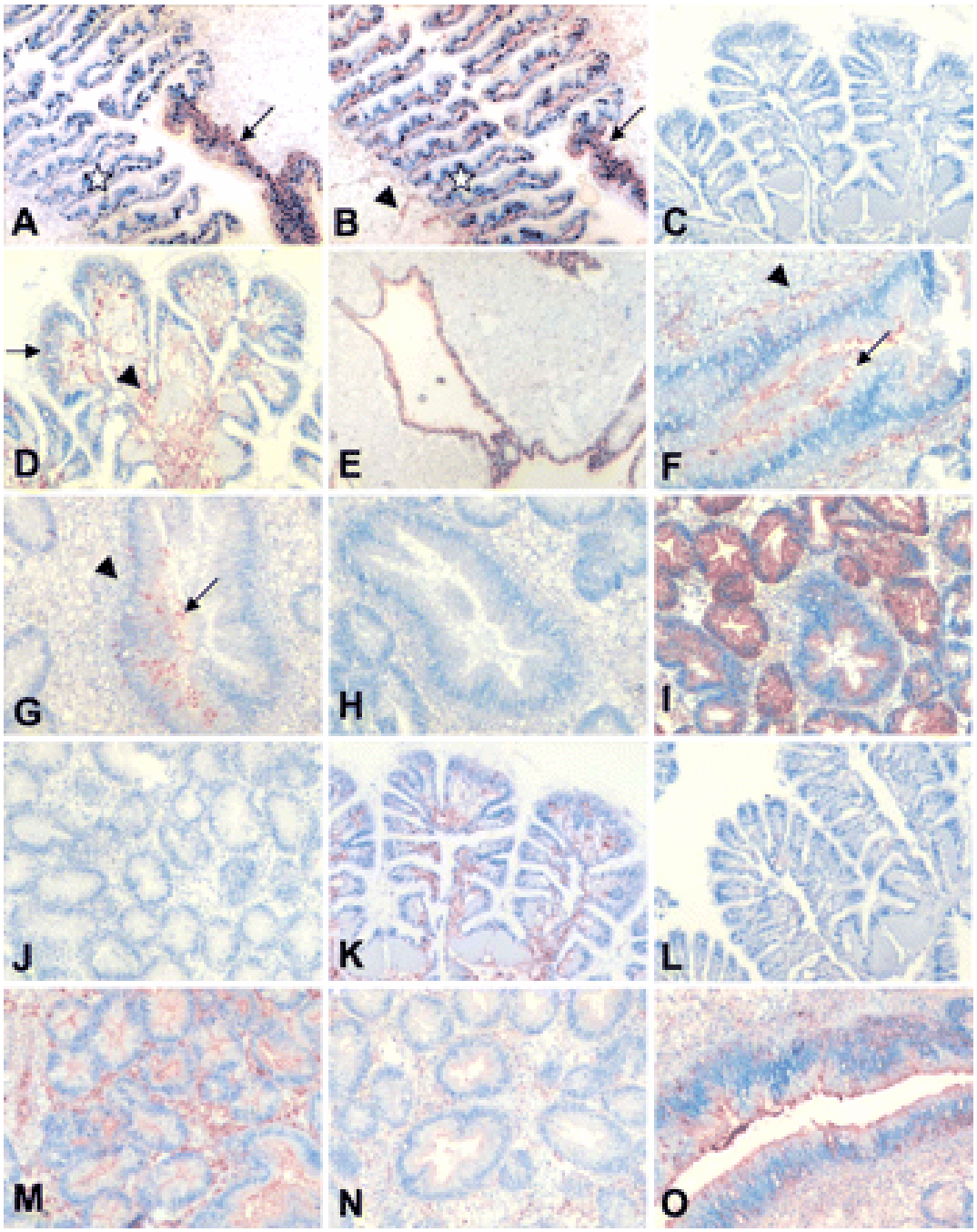

Fig. 4

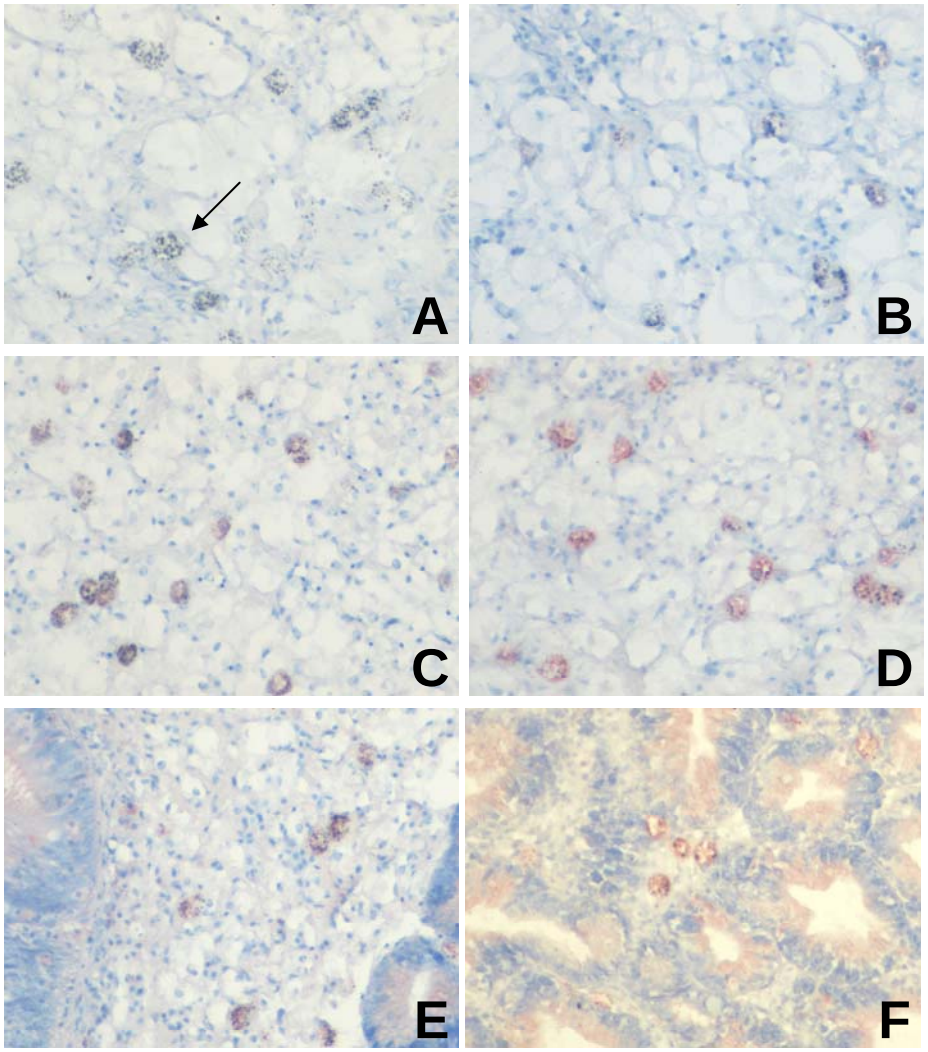

FIG.5

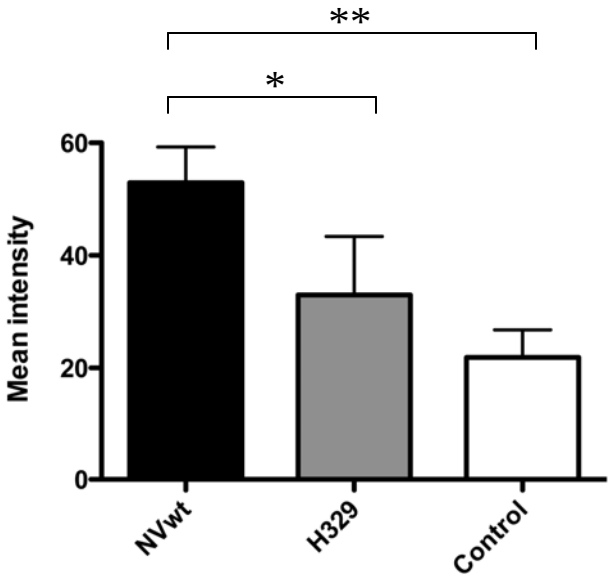

FIG. 6.

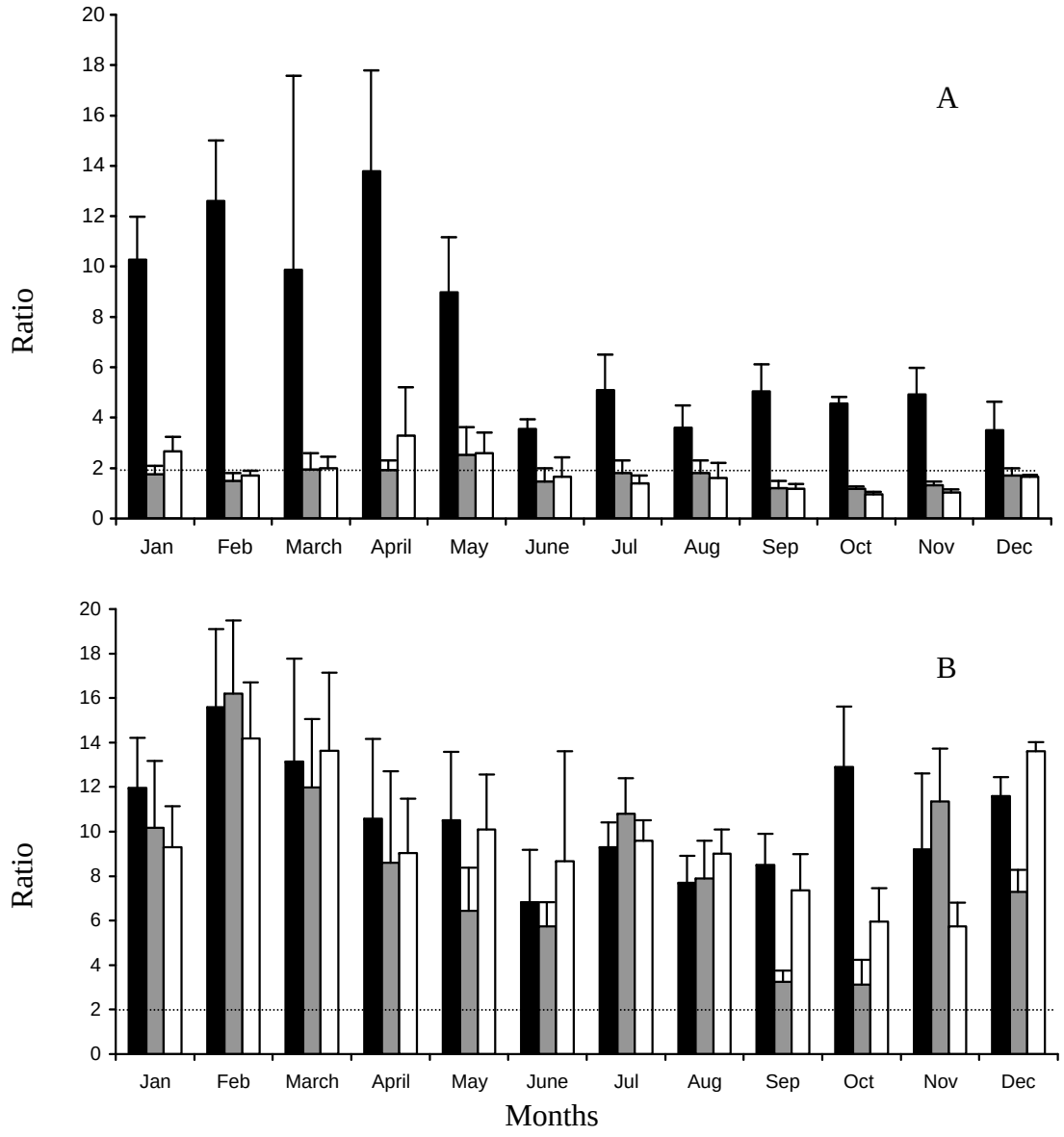
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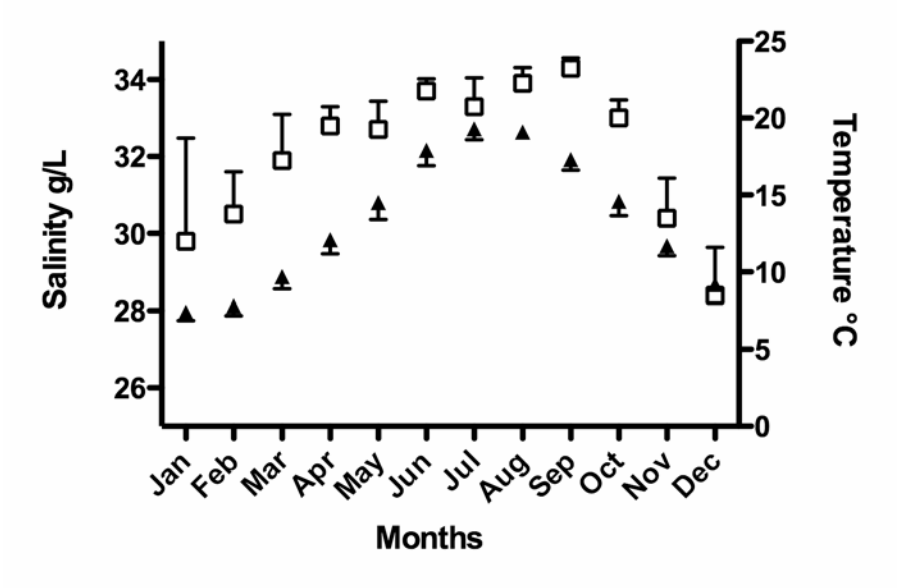
FIG. 7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