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RESEARCH REPORT

Deregulation of the humoral immune response of the oyster (*Crassostrea corteziensis*) exposed to naphthalene

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Abstract

Naphthalene is one of the most abundant polycyclic aromatic hydrocarbons (PAH) in aquatic ecosystems, and it can cause alterations in the immune system of organisms that live there. The oyster *Crassostrea corteziensis* is a species native to the Eastern Tropical Pacific, with economic and ecological importance. In this study, we evaluated the effect of subacute exposure to sublethal concentrations of naphthalene on the parameters of the humoral immune response (lysozyme and phenoloxidase activity, and nitric oxide production) on the oyster *C. corteziensis*. The results indicated that naphthalene, under the conditions tested, significantly deregulated the parameters evaluated. This could increase susceptibility to infections and therefore affect oyster production.

Key Words: naphthalene; oyster; *Crassostrea corteziensis*; immune response

Introduction

The oyster *Crassostrea corteziensis* (Hertlein, 1951), also known as the pleasure oyster or the Cortez oyster, is a species distributed from the Gulf of California (also known as the Sea of Cortez) to Panama (Cáceres-Martínez *et al.*, 2008). Production of the oyster worldwide is ~4.5 million tons/year (FAO, 2010), and specifically in Mexico, around 50,000 tons/year are produced, of which 1,500 tons correspond to the species *Crassostrea corteziensis* (CONAPESCA, 2013). This species possesses great economic and ecological importance; however, due to its sessile nature and feeding through filtration (150 l/24 h), it can accumulate biological and chemical contaminants, substances that can alter the physiology of these molluscs (Zuykov *et al.*, 2013).

Polycyclic aromatic hydrocarbons (PAH) are chemical contaminants that are frequently found in aquatic ecosystems; they are characterized by having high persistence in the environment, high toxicity, and the capacity of bioaccumulation (Ramdine *et al.*, 2012). Naphthalene is one of the most important PAH in this type of ecosystem due to its presence in the soluble fractions of petrogenic oils (Hansen *et al.*, 2007), which constitutes one of the most toxic fractions of petroleum for marine life (Vijayavel *et al.*, 2004; Vijayavel and Balasubramanian, 2006; Zambrano *et al.*, 2012). The mechanisms of toxicity of the PAH are through the bonding of these compounds with the hydrophobic sites of the cell's macromolecules, which causes alterations in the physiology of these, especially in the immune system (Vijayavel and Balasubramanian, 2006; Zambrano *et al.*, 2012).

The immune system of the oysters is composed of cellular and humoral mechanisms such as phagocytosis, a process that involves hemocyte ingestion and destruction, while humoral mechanisms include the following: 1) Phenoloxidase (PO), an enzymatic pathway activated by components of the cell wall of microorganisms, resulting in melanin, a molecule with fungistatic properties (Nappi and Vass, 1993; Vargas-Albores and Barracco, 2001; Luna-Acosta *et al.*, 2011); 2) lysozyme, an enzyme that hydrolyzes the bond between the β-1,4, N-acetylmuramic acid and the N-acetyl glucosamine localized in the peptidoglycan layer of the bacterial cell wall (McDade and Tripp, 1967; Itoh *et al.*, 2010), and 3) Nitric oxide (NO), a molecule produced by hemocytes in the presence of antigens, with high cytotoxic and microbicidal capacity (Rivero, 2006); in addition, NO reacts with the superoxide anion (O_2^-) during the respiratory burst and generates peroxynitrite ($ONOO^-$),

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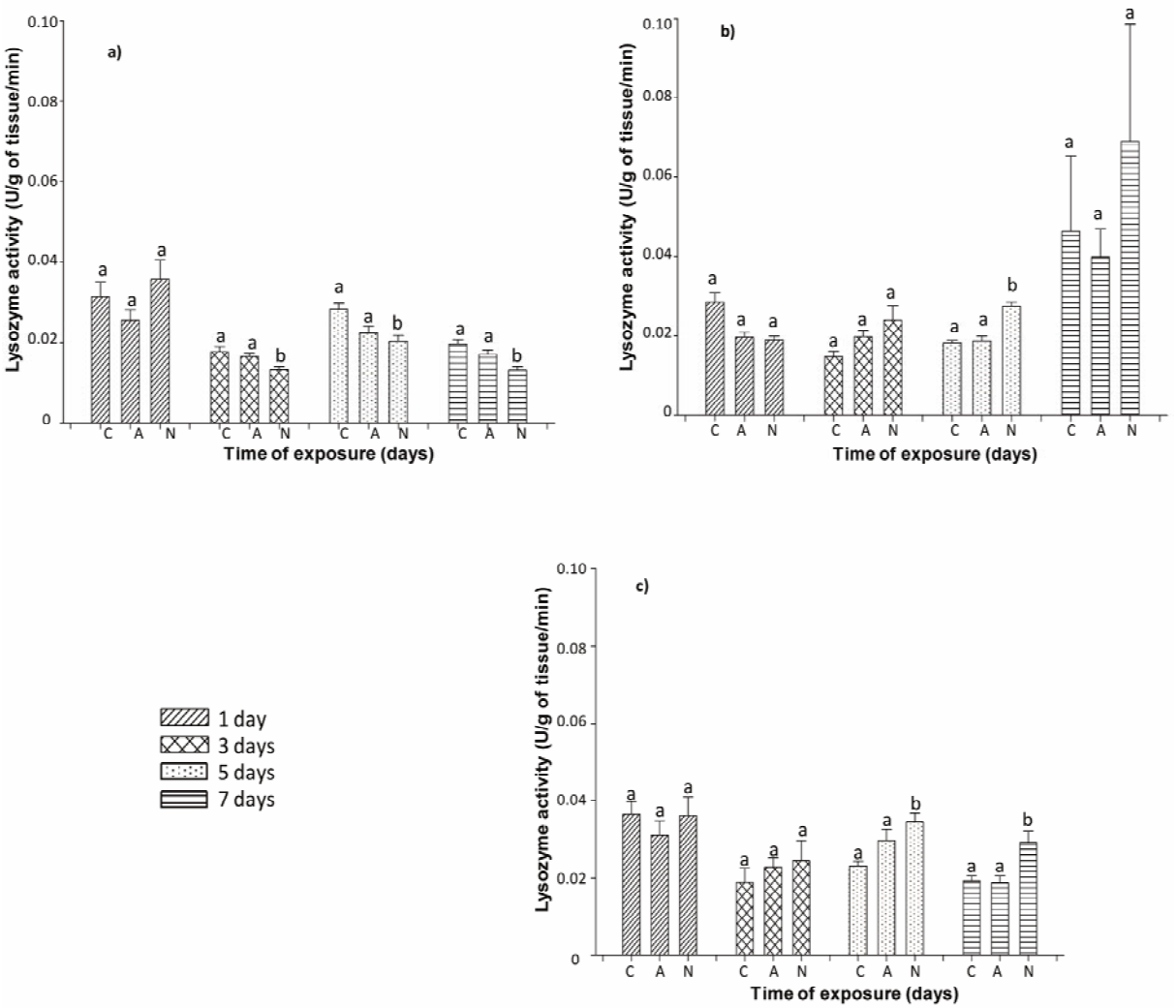

Fig. 1 Activity of lysozyme in digestive gland of oyster (n = 10) exposed to sublethal concentrations a) 1 µg/l, b) 20 µg/l, and c) 50 µg/l of Naphthalene (N) during 1, 3, 5, and 7 days, and control oysters: Acetone (A), and seawater (C). The data are represented as the $\overline{X}$ ± Standard error of the mean. Different letters between the bars of the same group indicate a significant difference (p <0.05).

compounds with high oxidant activity (Glinski and Jarosz, 1997; Gopalakrishnan *et al.*, 2011).

Notwithstanding this, the effectiveness of the defense mechanisms of oysters can be altered by contaminants, which causes greater susceptibility in organisms to infectious diseases (Gagnaire *et al.*, 2007). Studies on the immunotoxicity of PAH in molluscs have demonstrated drastic changes in the immunocompetence of this type of organism (Sauve *et al.*, 2002; Gagnaire *et al.*, 2004; Thiagarajan *et al*., 2007). However, the majority of studies have mainly centered on the cell's immune response (Pichaud *et al.*, 2008; Hannam *et al.*, 2010; Croxton *et al.*, 2012; Giannapas *et al.*, 2012) and, to date, there are no studies to our knowledge on the effects of naphthalene on the humoral immune response of the oyster *C. corteziensis*. Thus, the objective of the present work was to evaluate the effect of subacute exposure to sublethal concentrations of naphthalene on the parameters of the humoral immune response (the activity of lysozyme, phenoloxidase, and NO production) in the oyster *C. corteziensis*, a species native to the Eastern Tropical Pacific.

Materials and Methods

Animals

Crassostrea corteziensis oysters of commercial length and weight (8 ± 2 cm and 70 ± 30 g, respectively, and approximately 7 months old) were acquired at a local market in the State of Nayarit, Mexico and were immediately transported to the laboratory for their depuration during a 30 day period prior to experimentation (Adamo *et al.*, 1997). For this purpose, the oysters were maintained in a 50 l recirculation system with filtered seawater (salinity, 26 ± 2 %, pH 8.7 ± 0.2, and temperature, 26 ± 2 °C, 12 h:12 h dark-light cycles) and constant

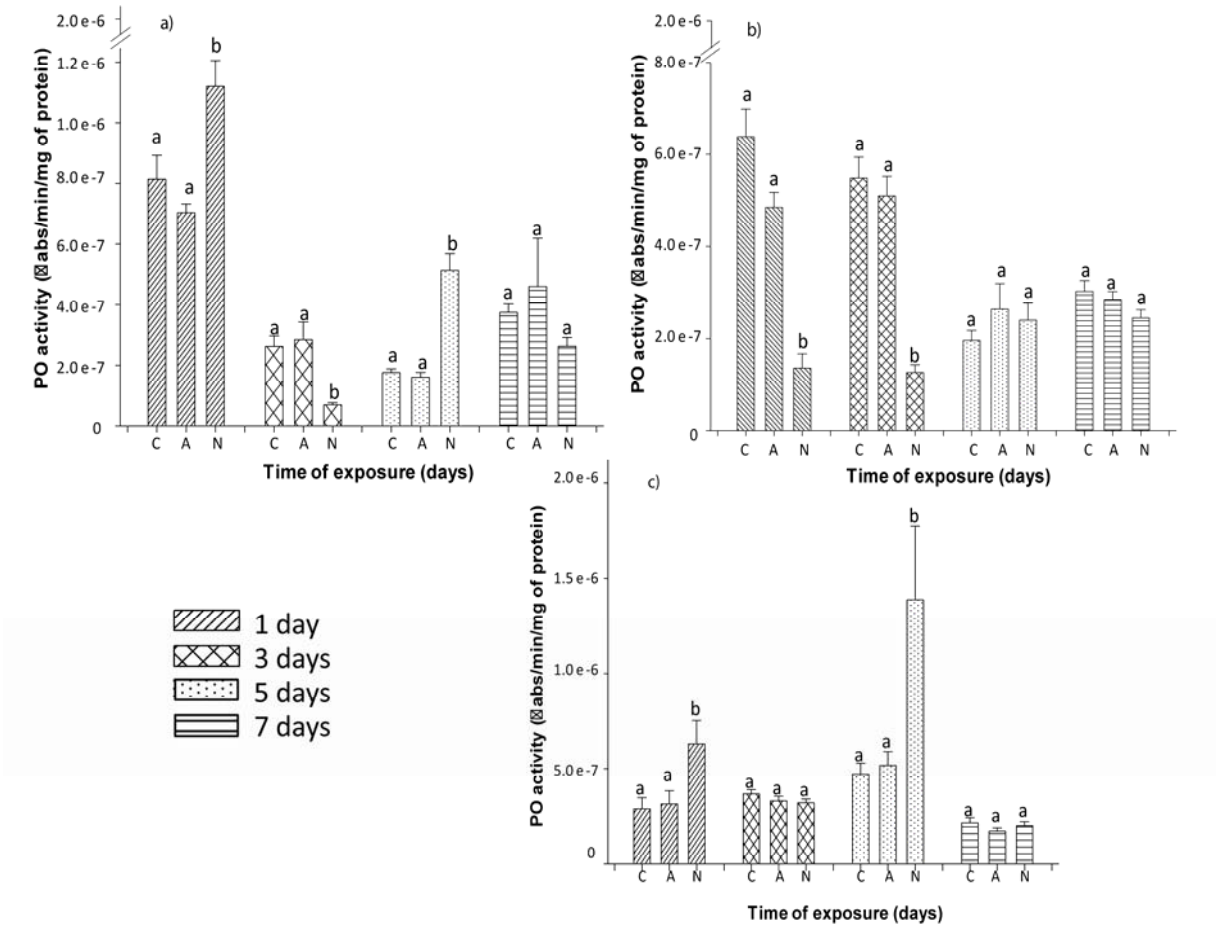

Fig. 2 Activity of phenoloxidase in digestive gland of oyster (n = 10) exposed to sublethal concentrations a) 1 µg/l, b) 20 µg/l, and c) 50 µg/l of Naphthalene (N) during 1, 3, 5, and 7 days, and control oysters: Acetone (A), and seawater (C). The data are represented as the $\overline{X}$ ± Standard error of the mean. Different letters between the bars of the same group indicate a significant difference (p <0.05).

aeration. They were fed daily with dehydrated alga spirulina dissolved in filtered seawater (Castillo-Rodríguez and García-Cubas, 1984).

Experimental groups

After the depuration period, the oysters (n = 10) were placed in glass fish tanks with 5 l of filtered seawater (without food, under the previously mentioned conditions) during a 24 h period for their acclimatization. After this time, the organisms were exposed to sublethal concentrations of naphthalene (1, 20, or 50 µg l^{-1}). The naphthalene (Sigma-Aldrich, 99 %) was taken from a stock solution (0.5 g/l), utilizing acetone as solvent at a 1:2 proportion (hydrocarbon:acetone). Three experimental groups were utilized (n = 30) per treatment: Experimental group I: oysters in seawater; Experimental group II: oysters in seawater with acetone, and Experimental group III: oysters in seawater with naphthalene. The oysters were exposed to the hydrocarbon during 1, 3, 5, or 7 days, with daily exchanges of water for each fish tank. During the experiment, the oysters were fed daily with alga spirulina.

Extraction of hemolymph and digestive gland

The hemolymph was obtained from the adductor muscle using a 1 ml syringe (0.7 mm×30 mm). The hemolymph of three oysters was grouped together (~600 µl) and mixed for the determination of NO production. For extraction of the digestive gland, we utilized tweezers and dissection scissors, immediately determined their weight, and processed these for determination of lysozyme and PO.

Lysozyme activity

Lysozyme activity was determined by means of the turbidimetric method described by Demers and Bayne (1997). The samples of the digestive gland were homogenized in KH_2PO_4 buffer 100 mM at 20 % (p/v) pH 5.9 and were centrifuged (1,000×*g* for 10 min at 25 °C). The supernatant (25 µl) was placed in a 96-well plate together with 175 µl of lyophilized

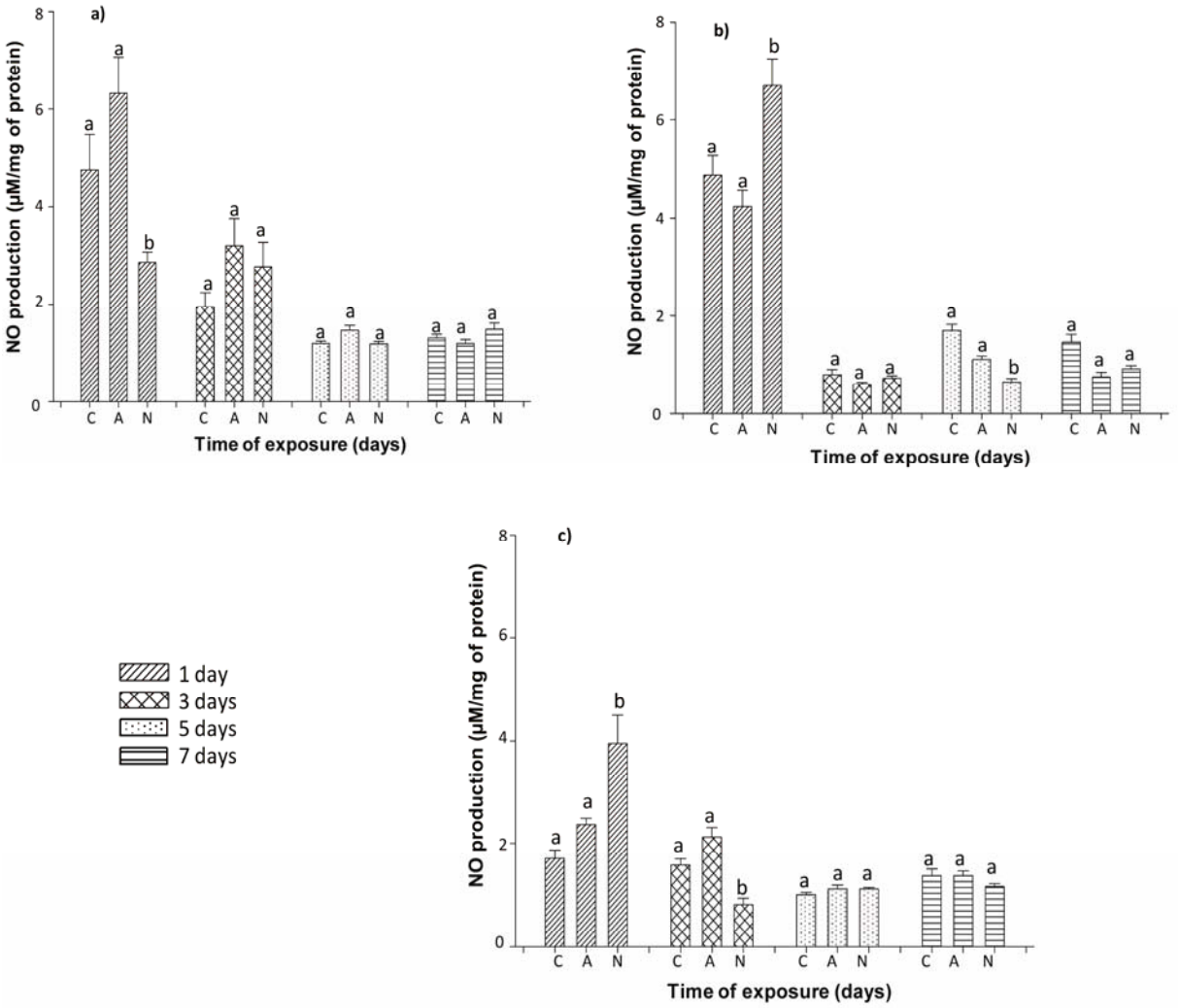

Fig. 3 Nitric oxide (NO) production in hemolymph of oyster (n = 10) exposed to sublethal concentration a) 1 µg/l, b) 20 µg/l, and c) 50 µg/l of Naphthalene (N) during 1, 3, 5, and 7 days and control oysters: Acetone (A), and seawater (C). The data are represented the $\overline{X}$ ± Standard error of the mean. Different letters between the bars of the same group indicate a significant difference (p <0.05).

Micrococcus lysodeikticus dissolved in KH_2PO_4 (0.15 %). We immediately determined absorbance (450 nm) at time 0 and afterward at 15 min. The difference of absorbance (Δabs) was interpolated on a Standard curve (0.041 - 0.25 U g^{-1}) of Chicken egg lysozyme (CEL).

PO activity

Detection of PO activity in digestive gland was performed through the measurement of the transformation of L-Dihydroxyphenylalanine (L-DOPA) into dopachrome, as described previously by Luna-Acosta *et al.* (2010). Prior to the determination of PO, the digestive-gland samples were homogenized in PBS solution 0.1 M, pH 7.3, and centrifuged (1,400×*g* for 15 min at 25 °C). An aliquot of the supernatant was placed in a 96-well plate with 100 µl of L-DOPA (4 mg ml^{-1}) as substrate. Absorbance was determined immediately (Time 0) and after 5 h. Δabs was determined at 450 nm in a plate reader. The difference of absorbance was expressed as PO activity and was corrected by the concentration of protein. As target, we utilized PBS solution mixed with 100 µl of L-DOPA.

Nitric oxide production

Nitric oxide (NO) production was evaluated as described previously by Tafalla *et al.* (2002) by means of the Griess reaction, which quantifies the nitrites (NO_2^-) present in the hemolymph. Fifty-µl aliquots of hemolymph, together with 50 µl of sulfanilamide at 1 % and 50 µl of 0.1 % N-(1-naphthyl) ethylenediamine, were incubated for 10 min at ambient temperature in a 96-well plate. The absorbance of the samples was measured at 545 nm in a plate reader. The molar concentration of the nitrites of each sample was determined by a standard $NaNO_2$ reference curve (10 - 100 mM)

and, as target we utilized PBS solution together with sulfanilamide and N-(1-naphthyl) ethylenediamine. NO production was corrected by the concentration of protein.

Determination of proteins

Total concentration of proteins was measured by means of the Bradford method (1976) with modification (Girón-Pérez *et al*., 2013). The sample (50 µl) was added into a 96-well plate together with 250 µl of Bradford reagent. The plate was incubated at ambient temperature for 10 min and absorbance was determined at 545 nm in a plate reader. A known concentration of the albumin (0.25 - 5.0 mg ml^{-1}) was utilized to perform a standard reference curve ($r^2 = 0.983$).

Statistical analysis

For statistical analysis, we employed SigmaStat® ver. 3.5. statistical software. We determined the normality and homogeneity of the data variances with the Kolmogorov-Smirnov test and the Levene F test. For normal data distribution, we utilized Analysis of variance (ANOVA) followed by the Bonferroni subtest. For non-parametric data, we used the Kruskal-Wallis test followed by a multiple Tukey-type comparison. The statistical difference was determined with a level of $p < 0.05$.

Results

Effect of Naphthalene on Lysozyme Activity

The exposure of 1 µg l^{-1} of naphthalene during 3, 5, and 7 days significantly diminished lysozyme activity in oysters with respect to controls (Fig. 1a), while the 20-µg l^{-1} concentration of the hydrocarbon induced an increase in the activity of the enzyme on day 5 (Fig. 1b). On exposing the oysters to 50 µg l^{-1} of this hydrocarbon, we observed an increase in the activity of this parameter on days 5 and 7 (Fig. 1c).

Effect of Naphthalene on Phenoloxidase (PO) Activity

The activity of PO in *C. corteziensis* with 1- and 50 µg l^{-1} of naphthalene during days 1 - 5 showed a significant increase with respect to that of the controls (Figs 2a, c). On the other hand, we observed a diminution in this parameter after exposure to 20 µg l^{-1} of naphthalene on days 1 and 3 (Fig. 2b), as well as exposure to 1 µg l^{-1} on day 3 (Fig. 2a).

Effect of Naphthalene on the Nitric Oxide (NO) production

The results indicate that the NO production in organisms exposed to 1 µg l^{-1} of naphthalene during 1 day diminishes significantly compared with that of the control; notwithstanding this, the concentration of this molecule did not change significantly with respect to the control in oysters exposed during 3, 5, and 7 days to this concentration (Fig. 3a). With regard to the effect of exposure to 20 µg l^{-1} of naphthalene, we observed that the NO production increased in the exposed oysters during 1 day at this concentration, while organisms exposed during 5 days presented a diminution of this molecule (Fig. 3b). On the other hand, oysters exposed to 50 µg L^{-1} presented an increase and a decrease of NO at days 1 and 3 post-exposure, respectively (Fig. 3c).

On the other hand, on comparing the response time of each parameter evaluated in the oysters exposed to naphthalene, the results indicate that NO is the most sensitive biomarker with respect to time, followed by PO and lysozyme (Fig. 4), because it is clear that NO production is affected in the first 24 h post-exposure to the hydrocarbon.

Discussion

In bivalve molluscs, an effective immune response is essential for the maintenance of the health of the organism; any alteration in the mechanisms of immunity can be accompanied by consequences in the development of infections and can even have repercussions on growth, reproduction, and survival (Blaise *et al.*, 2002). In this regard, there are studies that show that PAH cause effects on the immune system in molluscs, which could induce stimulation or immunosuppression, thus causing diminution of resistance to diseases (Gagnaire *et al.*, 2006; Matozzo *et al*., 2009; Hannam *et al*., 2010). The majority of studies on bivalve molluscs have focused on the cellular immune response, and data are scarce in which the effect is reported of this type of compound on the humoral immune response. In particular, there are, to our knowledge, no reports in which the immunotoxic effect of naphthalene is evaluated in humoral defense mechanisms (PO, NO, and lysozyme) in *C. corteziensis*. To our knowledge, this is the first study that evaluated the parameters of the humoral immune response in this species native to the coasts of the Eastern Tropical Pacific that, in addition, has economic importance in the Mexican Pacific.

In the immune system of bivalves, lysozyme is one of the most important bacteriolytic agents against various species of bacteria (Gopalakrishnan *et al*., 2011). This enzyme is found in mucosal secretions and digestive gland, and additionally is released by hemocytes during phagocytosis and participates in the inactivation of pathogens. Notwithstanding this, the activity of lysozyme can be affected by the presence of contaminants such as PAH (Ordás *et al.*, 2007; Gopalakrishnan *et al*., 2011). In the present study, lysozyme activity in the digestive gland of the *C. corteziensis* oyster was modified by exposure to naphthalene, because the enzyme's activity diminished significantly after exposure to 1 µg l^{-1} (on days 3, 5, and 7 post-exposure) and increased significantly at concentrations of 20 µg l^{-1} (on day 5) and of 50 µg l^{-1} (on days 5 and 7) of the hydrocarbon. Studies referring the effect of PAH on this parameter indicate that benzo[a]pyrene inhibits lysozyme activity in the hemolymph of bivalves (Gopalakrishnan *et al.*, 2009; Matozzo *et al*., 2009; Gopalakrishnan *et al.*, 2011). However, it has also been demonstrated that exposure to mixtures of PAH, such as crude oil, increases the activity of this enzyme (Oliver *et al*., 2003; Ordás *et al*., 2007).

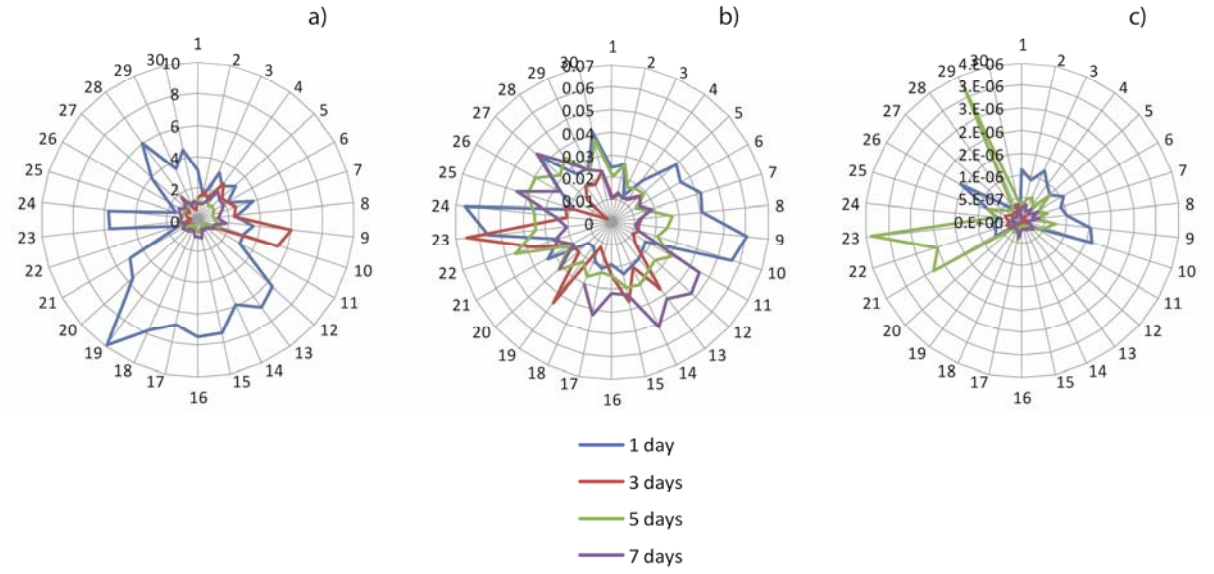

Fig. 4 Time of global response of the immunological parameters evaluated in oysters exposed to Naphthalene (N). Each line represents one oyster exposed to the hydrocarbons (oyster 1 - 10: 1 µg/l; 11 - 20: 20 µg/l; 21 - 30: 50 µg/l) at different times. a) Nitric oxide (NO) production (uM/mg of protein); b) Phenoloxidase activity (Δabs/min/mg of protein), and c) Lysozyme activity (U/min/g of tissue).

PO is one of the enzymes that participate in the humoral immune response in bivalves. This enzyme is key in the process of melanization and participates in the recovery of antigens through a capsule of melanin, or in the direct elimination of microorganisms by toxic quinones produced during the production of melanin (Soderhall and Cerenius, 1998). There are studies that show that PO activity is a sensitive immune parameter to exposure to PAH, because this parameter is observed to be affected on increasing or decreasing its activity due to exposure to these contaminants. For example, in the mussel *Mytilus edulis*, in the oyster *Crassostrea gigas*, and in the abulone *Haliotis diversicolor* exposed to fluoranthene, benzo[a]fluoranthene, and benzo[a]pyrene, respectively, PO activity increases significantly (Coles and Pipe, 1994; Bado-Nilles *et al*., 2008; Gopalakrishnan *et al.*, 2011), while in *C. gigas* oysters exposed to the soluble fraction of Heavy fuel oil (HFO) (Bado-Nilles *et al.*, 2009) and to the soluble fraction of Light crude oil (LCO) (Bado-Nilles *et al*., 2010), the activity of this enzyme diminished. In this study, the PO activity of this enzyme decreased. In addition, in this study, the PO activity in the digestive gland of the oyster *C. corteziensis* was modified by exposure to the three concentrations of naphthalene evaluated and showed dysregulation in this parameter, because the activity of the enzyme diminished to 1 µg l^{-1} on day 3 and to 20 µg l^{-1} on days 1 and 3 post-exposure and increased significantly with respect to the control after days 1 and 5 at 1- and 50 µg l^{-1}.

NO is an ubiquitous signalling molecule with immunoregulatory and antimicrobial effects (MacMicking *et al*., 1997; Rivero, 2006); due to its non-polar nature, it can pass easily through the membranes of pathogens, causing damage to the DNA, proteins, and lipids; in addition, NO also reacts with the Superoxide anion (O_2^-) generated during the respiratory burst (Fang, 2004). The hemocytes of various marine molluscs, such as the mussel *M. edulis, Mytilus galloprovincialis*, the oyster *C. gigas*, and the clam *Ruditapes decussatus*, are capable of producing NO in response to immunological stimuli; notwithstanding this, their production can be altered by exposure to PAH (Franchini *et al*., 1995; Nakayama and Maruyama, 1998; Arumugan *et al*., 2000; Torreilles and Romestand, 2001; Stefano *et al*., 2002; Tafalla *et al*., 2002; Novas *et al.*, 2004). Gopalakrishnan *et al*. (2011) reported, in abulone (*Haliotis diversicolor*) exposed to 0.05 mg l^{-1} de benzo[a]pyrene during 14 - 21 days, an increase in the production of NO; however, in exposure periods of 3 - 7 days, no effect was observed. Our results indicate that the production of NO in naphthalene-exposed *C. corteziensis* oysters diminished significantly after exposure to 20 and 50 µg l^{-1} during 1 day, while after exposure to 1-, 20-, and 50 µg l^{-1} during 1, 5, and 3 days, respectively, an increase was observed in this parameter.

Evaluation of the mechanisms of defense, on the one hand, can provide early warning signals due to exposure to contaminants (biomarkers) and, on the other hand, can indicate the degree of resistance to infections (Hannam *et al.*, 2009), because an increase in the activity of an enzyme (lysozyme and PO) or of some molecule (NO) implicated in immunity can be interpreted as a response of the organism to protecting itself against

antigens, a response that can be modulated by extrinsic factors such as xenobiotics. However, excessive activation of the mechanisms of defense can be harmful for the organism, while the inhibition of these mechanisms can be interpreted as immunosuppression (Huggett *et al.*, 1992; Thiagarajan *et al.*, 2006). In this manner, changes in the activity of enzymes or in the concentration of molecules can affect the survival of organisms when these are exposed to antigens.

The results obtained evidence the immunotoxic effect of naphthalene on the oyster *C. corteziensis*. However, the data reported in the literature suggest that this hydrocarbon possesses an immunotoxic potential that is less than other PAH, such as pyrene, benzo(a)pyrene, fluoranthene, among others. Notwithstanding this, it is necessary to perform more studies in this field, in that this investigative work is, to our knowledge, the first approximation reported of the effect of naphthalene on this native species of the Tropical Pacific.

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References

Adamo R, Pelosi S, Trotta P, Sansone G. Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. Mar. Chem. 56: 45-49, 1997.

Arumugan M, Romestand B, Torreilles J. Nitrite released in haemocytes from *Mytilus galloprovincialis, Crassostrea gigas* and *Ruditapes decussatus* upon stimulation with phorbol myristate acetate. Aquat. Living Resour. 13: 173-177, 2000.

Bado-Nilles A, Gagnaire B, Thomas-Guyon H, Le Floch S, Renault T. Effects of 16 hydrocarbons and two oils on haemocyte and haemolymphatic parameters in the Pacific oyster, *Crassostrea gigas* (Thunberg). Toxicol. *in Vitro* 22: 1610-1617, 2008.

Bado-Nilles A, Quentel C, Auffret M, Le Floch S, Gagnaire B, Renault T, *et al.* Immune effects of HFO on European sea bass, *Dicentrarchus labrax*, and Pacific oyster, *Crassostrea gigas.* Ecotox. Environ. Safe. 72: 1446-1454, 2009.

Bado-Nilles A, Renault T, Faury N, Le Floch S, Quentel C, Auffrete M, *et al*. *In vivo* effects of LCO soluble fraction on immune-related functions and gene transcription in the Pacific oyster, *Crassostrea gigas* (Thunberg). Aquat. Toxicol. 97: 196-203, 2010.

Blaise C, Trottier S, Gagné F, Lallement C, Hansen P. Immunocompetence of bivalve haemocytes as evaluated by a miniaturized phagocytosis assay. Environ. Toxicol. 17: 160-169, 2002.

Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Cáceres-Martínez J, Vásquez-Yeomans R, Padilla-Lardizábal G, Del Río-Portilla MA. *Perkinsus marinus* in pleasure oyster *Crassostrea corteziensis* from Nayarit, Pacific coast of Mexico. J. Invertebr. Pathol. 99: 66-73, 2008.

Castillo-Rodríguez Z, García-Cubas A. Taxonomía y Anatomía Comparada de las ostras en las costas de México. Ann. Ins. Cienc. Mar Limnol. 13: 294-314, 1984.

Coles J, Pipe R. Phenoloxidase activity in the haemolymph and haemocytes of the marine mussel *Mytilus edulis*. Fish Shellfish Immunol. 4: 337-352, 1994.

CONAPESCA. National Commission of Aquaculture and Fishing of Mexico Comisión Nacional de Acuacultura y Pesca. 2013. http://www.conapesca.sagarpa.gob.mx/wb/cona/consulta_especifica_por_produccion

Croxton A, Wikfors G, Schulterbrandt-Gragg RD. Immunomodulation in eastern oysters, *Crassostrea virginica*, exposed to a PAH-contaminated microphytobenthic diatom. Aquat. Toxicol. 118-119: 27-36, 2012.

Demers N, Bayne C. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Dev. Comp. Immunol. 21: 363-36, 1997.

Fang, F. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. 2: 820-832, 2004.

FAO. Food and Agriculture Organization of the United Nations. 2010. http://www.fao.org/docrep/013/i1890t/i1890t.pdf

Franchini A, Conte A, Ottaviani E. Nitric oxide: an ancestral immunocyte effector molecule. Adv. Neuroimmunol. 5: 463-478. 1995.

Gagnaire B, Thomas-Guyon H, Renault T. *In vitro* effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunol. 16: 501-512, 2004.

Gagnaire B, Thomas-Guyon H, Burgeot T, Renault T. Pollutant effects on Pacific oyster, *Crassostrea gigas* (Thunberg), hemocytes: screening of 23 molecules using flow cytometry. Cell Biol. Toxicol. 22: 1-14, 2006.

Gagnaire B, Gay M, Huvet A, Jean-Yves D, Saulnier D, Renault T. Combination of a pesticide exposure and a bacterial challenge: *in vivo* effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg). Aquat. Toxicol. 84: 92-102, 2007.

Giannapas M, Karnis L, Dailianis S. Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects. Comp. Biochem. Physiol. 155C: 182-189, 2012.

Girón-Pérez MI, Romero-Bañuelos CA, Toledo-Ibarra GA, Rojas-García AZ, Medina-Diaz IM, Robledo-Marenco ML, *et al*. Evaluation of pollution in Camichin estuary (Mexico): Pro-oxidant and antioxidant response in oyster

(*Crassostrea corteziensis*). Comp. Biochem. Physiol. 165A: 476-482, 2013.

Glinski Z, Jarosz J. Molluscan immune defenses. Arch. Immunol. Ther. Exp. 45: 149-155, 1997.

Gopalakrishnan S, Harikrishnan T, Wei-Bin H, Ke-Jian W. Immunomodulation in the marine gastropod *Haliotis diversicolor* exposed to benzo[a]pyrene. Chemosphere 75: 389-397, 2009.

Gopalakrishnan S, Wei-Bin H, Qiang-Wei W, Man-Li Wu, Jie-Liu, Ke-Jian W. Effects of tributyltin and benzo[a]pyrene on the immune-associated activities of hemocytes and recovery responses in the gastropod abalone, *Haliotis diversicolor*. Comp. Biochem. Physiol. 154C: 120-128, 2011.

Hannam M, Bamber S, Moody J, Galloway T, Jones M. Immune function in the Arctic scallop, *Chlamys islandica*, following dispersed oil exposure. Aquat. Toxicol. 92: 187-194, 2009.

Hannam M, Bamber S, Moody A, Galloway T, Jones M. Immunotoxicity and oxidative stress in the Arctic scallop *Chlamys islandica*: effects of acute oil exposure. Ecotox. Environ. Safe. 73: 1440-1448, 2010.

Hansen BH, Altin DH, Vang SH, Nordtug T, Olsen AJ. Effect of naphthalene o gene transcription in *Calanus finmarchinus* (Crustacea, Copepoda). Aquat. Toxicol. 86: 157-165, 2007.

Huggett R, Kimerle R, Mehrle O Jr. Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress, Ann Arbor, MI, USA: Lewis Publishers, University of Michigan, 1992.

Itoh N, Okada Y, Takahashi K, Osada M. Presence and characterization of multiple mantle lysozymes in the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol. 29: 126-135, 2010.

Luna-Acosta A, Rosenfeld E, Amari M, Fruitier-Arnaudin I, Bustamante P, Thomas-Guyon H. First evidence of laccase activity in the Pacific oyster *Crassostrea gigas*. Fish Shellfish Immunol. 28: 719-726, 2010.

Luna-Acosta A, Saulnier D, Pommier M, Haffner P, Decker S, Renault T, *et al.* First evidence of a potential antibacterial activity involving a laccase-type enzyme of the phenoloxidase system in Pacific oyster *Crassostrea gigas* haemocytes. Fish Shellfish Immunol. 31: 795-800, 2011.

MacMicking J, Xie Q, Nathan C. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15: 323-350, 1997.

Matozzo V, Monari M, Foschi J, Cattani O, Serrazanetti G, Marin M. First evidence of altered immune response and resistance to air exposure in the clam *Chamelea gallina* exposed to benzo[a]pyrene. Arch. Environ. Contam. Toxicol. 56: 479-488, 2009.

McDade JE, Tripp MR. Lysozyme in the hemolymph of the oyster *Crassostrea virginica*. J. Invertebr. Pathol. 9: 531-535, 1967.

Nakayama K, Maruyama T. Differential production of active oxygen species in photo-symbiotic and non-symbiotic bivalves. Dev. Comp. Immunol. 22: 151-159, 1998.

Nappi A, Vass E. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune-reactions. Pigment Cell Res. 6: 117-126, 1993.

Novas A, Cao A, Barcia R, Ramos-Martínez JI. Nitric oxide release by hemocytes of the mussel *Mytilus galloprovincialis* Lmk was provoked by interleukin-2 but not by lipopolysaccharide. Int. J. Biochem. Cell Biol. 36: 390-394, 2004.

Oliver L, Fisher W, Volety A, Malaeb Z. Greater hemocyte bactericidal activity in oysters (*Crassostrea virginica*) from a relatively contaminated site in Pensacola Bay, Florida. Aquat. Toxicol. 64: 363-373, 2003.

Ordás M, Albaiges J, Bayona J, Ordas A, Figueras A. Assessment of *in vivo* effects of the Prestige fuel oil spill on the Mediterranean mussel immune system. Arch. Environ. Contam. Toxicol. 52: 200-206, 2007.

Pichaud N, Pellerin J, Fournier M, Gauthier-Clerc S, Rioux P, Pelletier E. Oxidative stress and immunologic responses following a dietary exposure to PAHs in *Mya arenaria*. Chem. Cent. J. 2: 23, 2008.

Ramdine G, Fichet D, Louis M, Lemoine S. Polycyclic aromatic hydrocarbons (PAHs) in surface sediment and oysters (*Crassostrea rhizophorae*) from mangrove of Guadeloupe: levels, bioavailability, and effects. Ecotox. Environ. Safe. 79: 80-89, 2012.

Rivero A. Nitric oxide: an antiparasitic molecule of invertebrates. Trends Parasitol. 22: 219-225, 2006.

Sauvé S, Hendawi M, Brousseau P, Fournier M. Phagocytic response of terrestrial and aquatic invertebrates following *in vitro* exposure to trace elements. Ecotox. Environ. Safe. 5: 21-29, 2002.

Shöderhäl K, Cerenius L. Role of the prophenoloxidase activating system in invertebrate immunity. Curr. Opin. Immunol. 10: 23-28, 1998.

Stefano G, Salzet M, Magazine H. Cyclic nitric oxide release by human granulocytes, and invertebrate ganglia and immunocytes: nano-technological enhancement of amperometric nitric oxide determination. Med. Sci. Monitor. 8: 199-204, 2002.

Tafalla C, Novoa B, Figueras A. Production of nitric oxide by mussel (*Mytilus galloprovincialis*) hemocytes and effect of exogenous nitric oxide on phagocytic functions. Comp. Biochem. Physiol. 132B: 423-431, 2002.

Thiagarajan R, Gopalakrishnan S, Thilagam H. Immunomodulation in the marine green mussel *Perna viridis* exposed to sub-lethal concentrations of Cu and Hg. Arch. Environ. Contam. Toxicol. 51: 392-399, 2006.

Torreilles J, Romestand B. *In vitro* production of peroxynitrite by haemocytes from marine bivalves: C-ELISA determination of 3-nitrotyrosine level in plasma proteins from *Mytilus galloprovincialis* and *Crassostrea gigas*. BMC Immunol. 2: 1, 2001.

Vargas Albores F, Barraco M. Mecanismo de defensa de los moluscos bivalvos, con énfasis en pectínidos. En: Maeda-Martínez A. (Editor), Los Moluscos Pectínidos en Latinoamérica: Ciencia y Acuacultura, Cap. 7, México, D.F., México: Editorial Limusa, pp 127-146, 2001.

Vijayavel K, Gomathi RD, Durgabhavani K, Balasubramanian MP. Sublethal effect of naphthalene on lipid peroxidation and antioxidant status in the edible marine crab *Scylla serrata*. Mar. Pollut. Bull. 48: 429-433, 2004.

Vijayavel K, Balasubramanian M. Changes in oxygen consumption and respiratory enzymes as stress indicators in an estuarine edible crab *Scylla serrata* exposed to naphthalene. Chemosphere 63: 1523-1531, 2006.

Zambrano M, Prada J, Arencibia G, Vidal A. Bioacumulación de naftaleno y fluoranteno en el molusco bivalvo *Anadara tuberculosa* (Sowerby, 1833). Rev. U.D.C.A. Act. & Div. Cient. 15: 283-290, 2012.

Zuykov M, Pelletier E, Harper D. Bivalve mollusks in metal pollution studies: from bioaccumulation to biomonitoring. Chemosphere 93: 201-208, 2013.