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The influence of dietary supplementation of arachidonic acid on prostaglandin production and oxidative stress in the Pacific oyster *Crassostrea gigas*

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Abstract :

In a previous study, dietary supplementation with arachidonic acid (ARA) to oysters *Crassostrea gigas* increased haemocyte numbers, phagocytosis, and production of reactive oxygen species level (ROS) by haemocytes (Delaporte et al., 2006). To assess if the observed stimulation of these cellular responses resulted from changes of ARA-related prostaglandin (PG) production, we analysed prostaglandin E2 metabolite (PGEM) content on the same oysters fed three levels of ARA. Dietary supply of polyunsaturated fatty acids (PUFA) could also induce an oxidative stress that could similarly increase cellular responses; therefore, two indicators of oxidative stress were analysed: peroxidation level and antioxidant defence status. Together the observed positive correlation between ARA and PGEM levels and the absence of lipid peroxidation and antioxidant activity changes supports the hypothesis of an immune stimulation via PG synthesis. Although ARA proportion in oyster tissues increased by up to 7-fold in response to ARA dietary supplementation, peroxidation index did not change because of a compensatory decrease in n-3 fatty acid proportion, mainly 22:6n-3. To further confirm the involvement of PG in the changes of haemocyte count, phagocytosis and ROS production upon ARA supplementation, it would be interesting to test cyclooxygenase and lipooxygenase inhibitors in similar experiments.

Keywords : Prostaglandins; n-6 unsaturated fatty acids; Lipid peroxidation; Antioxidants; ROS production

1. Introduction

Altering the composition of phospholipids in immune cells by changing the fatty acids (FA) supplied in the diet can modulate cell immune function and immune response through membrane alterations (raft, order, trafficking), signal transduction pathways, and lipid mediators (e.g. eicosanoids) (Calder, 2008). Eicosanoids are important lipid mediators in inflammation and healing in vertebrates. Eicosanoids include prostaglandins (PG), leukotrienes (LT), thromboxanes (TX), and lipoxins; they are derived from FA, principally ARA, which is released from membrane phospholipids (Pompéia et al., 2000). In mammals, prostaglandin PGE2 and leukotriene LTB4 are two potent immunomodulatory agents produced from ARA by the enzymes cycloxygenase (COX) and 5-lipoxygenase (LOX), respectively (Kelley et al., 2005). In insects, eicosanoids are also important mediators of cellular immune reactions such as phagocytosis, microaggregation, cell spreading, and nodulation reactions (Stanley and Miller, 2006; Merchant et al., 2008). Canesi et al. (2002) demonstrated that eicosanoids are involved in bacterial killing by mussel (*Mytilus edulis*) hemocytes by using specific inhibitors of COX and LOX.

The relationship between eicosanoids' synthesis and antibacterial response of hemocytes has been demonstrated in bivalve molluscs (Canesi et al., 2002). More recently, the ARA incorporation from diet in phospholipids, as well as their relation to an increase in immune responses: hemocyte number, phagocytic activity, reactive oxygen species (ROS) generation by hemocytes, desensibilization to pathogens (Delaporte et al., 2006) and prostaglandin E2 synthesis (Hurtado et al., 2009) has also been shown. Nevertheless, effects of FA on immune cell activity can also be independent of the changes in the secretion of eicosanoids (Kelley and Ruldoph, 2000). ARA can directly modulate phagocytosis, cytokine production, surface molecule expression, leukocyte migration, and antigen pre 69 sentation through stimulation of superoxide anion generation (Pompéia et al., 2000). Cellular enrichment with PUFA such as ARA has also been demonstrated to elicit *in vitro* an increase of ROS production and lipid peroxidation (Mazière et al., 1999). The susceptibility of membranes to oxidative damage depends on the degree of polyunsaturation level of FA, measured as peroxidation index (Hulbert et al., 2007). ARA can easily be peroxidized, generating free radicals such as the superoxide anion. Lipid peroxidation is a self propagating process and produces a broad range of reactive intermediates often measured as malondialdehyde (MDA), which has a longer half life than free radicals (Janero, 1990). However, ROS production and lipid peroxidation, generally referred to as "oxidative stress", may also be linked to prostaglandin synthesis from ARA, as it has been demonstrated in rat pheochromocytoma PC12 cells (Jiang et al., 2004) and human neuroblastoma cells (Kondo et al., 2001). All of the above studies suggest that ARA-derived prostaglandins may play an important role in regulation of immune responses including those in bivalve molluscs, such as increased hemocyte count, phagocytosis and ROS production previously reported (Delaporte et al., 2006).

From this perspective, antioxidant status of an organism is of critical significance in determining the effect of PUFA on immune and inflammatory responses (Kelley, 2001). To protect themselves against free radicals and ROS, bivalves like other organisms, possess an array of free radical scavenging antioxidant molecules and antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase (Manduzio et al., 2004; Monari et al., 2005; Box et al 2007; Cipak et al., 2008), which can be expected to increase upon oxidative stress and/or lipid peroxidation, potentially induced by ARA incorporation into tissues. In this work, we analyzed PGE2 metabolite (PGEM) levels, antioxidant capacity, enzymatic activities of catalase and SOD and lipid peroxidation in relation to the ARA supplementation of diet in oysters. It aimed to test the hypothesis that changes of hemocyte counts, phagocytosis
and ROS production upon ARA supplementation, as reported by Delaporte et al. (2006) involved prostaglandins.

2. Materials and methods

2.1 Oyster dietary treatments, sample collection and processing

One year old oyster *Crassostrea gigas* were collected from the field (Aber-Benoît, Finistère, Brittany) and fed *Isochrysis galbana* clone Tahitian (T.*iso*), during an acclimation period of 10 days. ARA was supplemented twice a day directly into the water. The daily algal ration was established at 4% algal dry weight per oyster dry weight and supplied continuously into the

tanks holding the oysters. Water in the tanks was renewed twice a day with 1µm filtered seawater using a flow through system. As described by Delaporte et al. (2006), three levels of supplementation with ARA solution (Sigma, A-9376) were tested during 4 weeks: a control with 0 µg ARA, and two concentrations of ARA: 0.25 and 0.41 µg/mL of sea water. Oysters were sampled after 15 (n=15) and 30 days (n=15) of supplementation with ARA. At each sampling time, three pools of 5 animals were constituted. Whole tissue of oysters was frozen and stored in liquid nitrogen at -196 °C. The samples were ground with a Dangoumeau homogeniser, and the powder was used for all the parameters analysed here.

2.2 Fatty acid analysis

Ground tissue (300 mg) was transferred to a tube containing 6 mL of chloroform-methanol mixture (2:1, v:v). After centrifugation, the lipid extract was transferred to a clean tube, sealed under nitrogen, and stored at -20 °C. Neutral lipids (lipid reserves) and polar lipids (membrane lipids) were separated on a Silica gel micro-column as described by Marty et al. (1992), and fatty acids in each fraction were analysed as described by Delaporte et al. (2006). Fatty acid composition was expressed as percentage of total FA in each lipid fraction and as µg of FA per mg of oyster wet mass. The unsaturation index (PUI) and peroxidation index was calculated as described by Hulbert (2007):

Unsaturation index (PUI) = 1×(% monoenoics) + 2×(% dienoics) + 3×(% trienoics) + 4×(% tetraenoics) + 5×(% pentaenoics) + 6×(% hexaenoics)

Peroxidation index = 0.025×(% monoenoics) + 1×(% dienoics) + 2×(% trienoics) + 4×(% tetraenoics) + 6×(% pentaenoics) + 8×(% hexaenoics)

2.3 Prostaglandin analysis

Prostaglandin E_2 metabolite (PGEM) was assayed by the PG screening EIA Kit (Cayman Chemical No. 514531, Ann Arbor, MI, USA). As indicated in the Cayman Kit, PGE_2 is rapidly converted *in vivo* to PGEM and so measurement of this metabolite is more reliable for estimation of actual PGE_2 production in biological samples. According to the recommendations of the manufacturer, a purification step was performed on C-18 SPE cartridges (Waters, Milford, MA, USA). An aliquot of tissue (20 mg) was mixed in 500 µL water and incubated at 4 °C for 5 min with 2 mL ethanol, and then centrifuged at 1000 g for 10 min to precipitate proteins. The assay was performed on supernatant according to the protocol described in the kit. Samples and standards were transferred to microplates coated with mouse monoclonal antibodies against rabbit antibodies. Specific rabbit antibody against PGEM and tracer consisting of acetylcholinesterase (AChE) coupled to PGEM were added, and plates were

incubated for 18 h. After several washings, Ellman's reagent (substrate and chromogen) was added to measure AChE activity which is determined spectrophotometrically at 415 nm with a microplate reader (Bio-Tek Synergy HT, Bio-Tek Instrument, USA). Absorbance is proportional to the amount of PGEM from the tracer bound to the well which is inversely proportional to free PGEM from sample. Results are expressed in pg of PGEM per mg of tissue wet mass. Due to the high levels of ARA in tissues in this experiment compared to measured levels of PG, the cross reactivity was tested. The tests showed that 71% of free ARA and 64% of free EPA are lost during the purification step, and cross-reactivity of ARA in the tissue at the concentration used here with the EIA Kit was around 0.002% (Reza, 2009), in accordance with kit specifications of less than 0.01%.

2.4 Assay of lipid peroxidation

Lipid peroxides derived from polyunsaturated fatty acids are unstable and decompose to other complex compounds of which malondialdehyde (MDA) is the most abundant. The MDA was measured by a spectrophotometric assay using a commercial kit (MDA-586 BIOXYTECH method, Oxis Research, Portland, OR, USA). The method is based on the reaction of a chromogenic reagent, N methyl-2-phenylindole with MDA at 45 °C. Oyster powder (100 mg) was homogenized by sonication in 1 mL of Tris buffer 20mM pH=7.5 containing 5 mM butylated hydrotoluene (BHT) to avoid intra-assay oxidation. The homogenate was centrifuged at 15000 *g* (15 min, 4 °C) and the supernatant was added to a mixture of probucol N-methyl-2-phenylindole, hydrochloric acid as indicated in the kit. The reaction was performed at 45 °C for 60 min. After centrifugation at 10000 g (10 min), the clear supernatant was transferred into a microtitration plate and the absorbance was measured at 586 nm. A standard curve was established with a solution of tetramethoxypropane (TMP) hydrolysed during the acid incubation step generating MDA. The result is expressed in µmol MDA per g of wet mass.

2.5 Total antioxidant radical scavenging capacity (TARSC)

TARSC was analyzed by the method described by Fukumoto and Mazza (2000), which uses 2,2 diphenyl-1-picrylhydrazyl (DPPH) as a free radical. This method is based on the analysis of absorbance decrease of DPPH in presence of any antioxidant compound. Antioxidant compounds were extracted from 150 mg oyster powder with 1 mL mixture of methanol and water (50/50). After sonication, the homogenate was centrifuged at 3500 g (15 min, 4 °C) and the supernatant was added to 150 µM DPPH solution in a microtitration plate. The plate was covered and incubated in the dark at room temperature and the absorbance was measured at 520 nm after 30 and 60 min. A mixture of methanol:water was used as blank. As a positive control,

butylated hydroxyanisole (BHA) standard was used. The total antioxidant radical scavenging activity (TARSC) was expressed as DPPH inhibition percentage: (Blank OD - Sample OD)/Blank OD×100 during a 30 min incubation (Moncheva et al., 2004).

2.6 Antioxidant enzymes

For enzymatic assays, an aliquot of ground powder was homogenized by sonication in phosphate buffer 0.01 M, pH =7.4 with 0.1% triton and 1 mM EDTA and centrifuged at 15000 g (15 min, 4 °C). The supernatant was used for enzymatic assays, catalase and superoxide dismutase (SOD).

Catalase activity

Catalase (EC 1.11.16) activity was measured with a commercial kit (Amplex Red Catalase assay kit, Molecular probes Invitrogen, Oregon USA) in which catalase first reacts (30min) with H_2O_2 to produce water and oxygen. Then, the Amplex Red reagent provided in the kit reacts with an unreacted H_2O_2 in the presence of horseradish peroxidase to produce a highly fluorescent oxidation product, resorufin, with a maximum absorption at 540 nm. A control without catalase was used. Results were expressed in U/mg wet mass from a standard curve of catalase activity between 0 to 2.5 U/mL.

Superoxide dismutase (SOD) activity

The total SOD (EC 1.15.1.1) activity was determined with the xanthine oxidase–cytochrome C method according to Marie et al. (2006) with some modifications. The assay is based on competition between SOD and cytochrome C for superoxide anions generated by the reaction of hypoxanthine with xanthine oxidase. The reduction rate of cytochrome C was measured at 550 nm in phosphate buffer 50mM with 0.18 mM EDTA, 100 mM hypoxanthine, 30µM cytochrome C and 20µl of diluted sample. The reaction was initiated by addition of 20µl of xanthine oxidase (0.05U/mL). The results were expressed as U SOD per mg wet mass.

2.7 Statistical analysis

Measured parameters were checked for normality and homogeneity (Sokal & Rohlf, 1995). Percentages were transformed to arcsin (square root) before statistical analysis but are shown as untransformed data. One-way analyses of variance (ANOVA) were applied for all variables to detect significant effects of different levels of ARA supplementation during 15 and 30 days. An additional one way ANOVA was also used to analyse the differences as a result of time of conditioning (15 and 30 days *vs.* initial values) for the treatment with T.*iso* + ARA 0 µg/mL.

Differences between means for each group were determined by a post-hoc Tukey test. Pearson's correlation analysis was done to define the association between prostaglandin and ARA concentrations in whole oyster. Analyses were performed using STATISTICA™ version 5.5 and differences were reported as significant if $P \leq 0.05$. Data are reported as mean ± standard error (SE).

3. Results

3.1 Fatty acid composition in phospholipids and lipid reserves

The fatty acids composition of oysters after 30 days treatment with three ARA concentrations is shown in Table 1. The proportion of 20:4n-6 in phospholipids and lipid reserves increased with increasing levels of this fatty acid in oyster diet. The proportion of 20:4n-6 was higher in phospholipids (membrane lipids) than neutral lipids (lipid reserves), reaching final concentration of 9.2 % and 7.5 %, respectively, for the highest level of ARA in the diet. The increase of 20:4n-6 resulted in significant decreases ($P<0.05$) in other PUFA such as 18:3n-3 and 22:6n-3. These relative changes in individual fatty acids following ARA supplementation led to a significant increase ($P<0.05$) of total n-6 fatty acids and a decrease of total n-3 fatty acids, as well as the n-3/n-6 and 20:5n-3/20:4n-6 ratio. A slight, but significant increase ($P<0.05$) of total PUFA and PUI in lipid reserves was observed for the highest ARA (0.41 μg/mL) treatment, whereas in phospholipids this effect was observed for both levels of ARA supplementation. However, no differences were observed in lipid peroxidation index. When expressed as absolute concentration (μg/mg wet mass), 20:4n-6 increased 4 and 7 fold in lipid reserves and 2.8 and 4.4 fold in phospholipids for the 0.25 and 0.41 μg/mL treatments, respectively (Fig. 1).

3.2 Prostaglandin levels and correlation with 20:4n-6

After 15 days, levels of prostaglandin E_2 metabolite (PGEM) increased, though not significantly, with increasing ARA levels in the diet (Fig. 2). Levels of PGEM continued to increase after 30 days in both groups supplemented with ARA, with significant differences ($P<0.05$) observed between both supplemented groups and the control group. In addition, PGEM levels in control oysters were significantly lower ($P<0.05$) at both 15 and 30 days, as compared to the beginning of the experiment. A significant correlation ($R^2 = 0.67$, $P < 0.01$) between PGEM and ARA concentrations in total lipids (combining lipid reserves and phospholipids) in whole oysters was observed after 30 days (Fig. 3).

3.3 Lipid peroxidation and antioxidant capacity.

No difference in lipid peroxidation, expressed as MDA concentration, was observed following ARA supplementation at different doses and times (Table 2). Similar results were obtained with the more common method measuring TBARS (results not shown). The total antioxidant radical scavenging capacity (TARSC), inferred from the DPPH inhibition percentage, was not influenced by the time of conditioning, but there was a significant increase of the TARSC for the oysters supplemented with the highest dose of ARA (0.41 µg/mL) at day 15 after conditioning, although this effect was no longer observed after 30 days.

The antioxidant capacity was also inferred from the activity of the antioxidant enzymes, SOD and catalase (Table 2). The catalase activity was not influenced by ARA supplementation, as no effect of ARA dose and time of conditioning was observed (Table 2). The SOD activity was not influenced by ARA concentration; however, a significant increase with time of conditioning was observed for the control group (Table 2).

4. Discussion

Supplementing ARA directly into the water as free FA has been previously reported to increase concentration of this FA in lipid reserves and phospholipids of oyster spat (Seguineau et al., 2005). Supplementation with esterified forms of ARA seems to influence only the ARA accumulation in lipid reserves of oysters (Hurtado et al., 2009). Supply of dissolved ARA directly into the water where oyster are grown appeared thus to be more efficient for ARA incorporation in polar lipids (Delaporte et al., 2006). This could reflect a difference of absolute amount of ARA supplied/ingested, or a better digestibility and absorption of free ARA compared to esterified ARA (Kinsella, 1991). Additionally, differences in oyster species, frequency of ARA feeding and microalgae diet used in the above studies could also explain such differences in ARA incorporation. ARA accumulation seems to be tissue specific as a much stronger incorporation of ARA in gill phospholipids (19.7%) (Delaporte et al., 2006) than in the whole animal phospholipids (9.3%, present study, same experiment as Delaporte et al. (2006) was observed. Gills might be incorporating the dissolved form of ARA more readily, as was proposed by Delaporte et al. (2006). However, a higher proportion of ARA in gill phospholipids (6.2% of total fatty acids, Delaporte et al., 2006) compared to whole organism (2.3% of total fatty acids, present study) was observed even in control oysters not fed dissolved ARA. Therefore, the incorporation of ARA into membrane phospholipids of gills could be associated with a tissue-specific function. In other aquatic organisms, ARA is selectively incorporated (reaching up to 20-30% of total fatty acids) in gill phospholipids of fish (German and Hu, 1990;

Harel et al., 2000; Fountoulaki et al., 2003) and crabs (Lucu et al., 2008), where it modulates Na/K-ATPase activity during osmoregulation (Van Anholt et al., 2004).

Oyster fed ARA decreased their levels of n-3 PUFA in tissues. The reduction of n-3 was mainly attributable to docosahexaenoic acid (DHA) (22:6n-3), while the proportion of eicosapentaenoic acid (EPA) (20:5n-3) remained relatively constant. In contrast, EPA was the main n-3 FA to decrease when given diets rich in n-6 to fish (Bell et al., 1992; 1993). The importance of EPA over other n-3 fatty acids in oyster has been previously proposed for maturation (Soudant et al., 1999) and immune response (Delaporte et al., 2007). This relative compensation of total PUFA by a decrease in DHA rather than that of EPA suggest a different role for EPA in molluscs as compared to vertebrates.

In our previous study (Delaporte et al., 2006), supplementation with ARA to adult oysters at 0.25 and 0.41 µg/mL directly in seawater tended to increase total hemocyte counts after 15 and 30 days of dietary conditioning. Phagocytosis was significantly impacted by ARA supplementation after 15 days of feeding while the strongest impact of ARA supplementation (both doses as compared to control) was observed for ROS production (a two-fold increase) after 4 weeks of ARA supplementation. Furthermore, hemocytes of oysters fed ARA were less sensitive to extracellular products of a pathogenic *Vibrio aestuarianus*. Relationships between ARA supplementation and cellular immune responses in *C. gigas* could be explained by different mechanisms: 1) a specific immune response mediated by PG or other eicosanoids such as leukotriene LTB_4 and/or 2) a direct action of ARA through changes of membrane composition and/or 3) lipid peroxidation of ARA generating free radicals and modifying thus, immune cell functioning.

We found a positive correlation between ARA levels in *C. gigas* tissues and PGEM concentration after feeding ARA for 30 days. Accordingly, in *C. corteziensis* ARA-rich emulsions supplemented in the diet increased PGEM levels in oyster tissues in a dose dependent manner (Hurtado et al., 2009). Also, the effects of membrane fatty acid composition, diets rich in 18:2n-6 and ARA or the ARA content of immune cell phospholipids on the production and type of PG and other eicosanoids has been reported in fish and mammals (Bell et al., 1992, 1993; Tocher et al., 1997; Peterson et al.,
1998; Lund et al., 2008; Villalta et al., 2008). Therefore, it is likely that ARA supply to oysters could promote the increase in other eicosanoids such as leukotrienes (LTB_4) in addition to PG. In vertebrates, it has been shown that LTB_4 increase together with PGE_2 as a direct result of ARA proportion in immune cell membrane phospholipids, and both are modulated in parallel by ARA dietary supply (Kelley, 2001; Calder, 2008).

The proinflammatory influence of ARA in humans is well known; it can significantly increase

the number of circulating neutrophils, the *in vitro* production of PGE_2 and LTB_4 by the stimulated peripheral blood mononuclear cells, and the secondary response to influenza vaccine (Kelley, 2001). PGE_2 and PGI_2 are the predominant pro-inflammatory prostanoids, although they can also exert immuno-suppressive effects and can be involved in the resolution of the inflammation (Stables and Gilroy, 2011). LTB_4 is a powerful pro-inflammatory agent that stimulates phagocytosis, chemotaxis and aggregation in neutrophils as well as the release of lysozyme and the generation of oxygen radicals (Toda et al., 2002) and is important in the host response against infection by enhancing microbicidal activities (Stables and Gilroy, 2011).

A link between PG and immune responses in molluscs was given by Canesi et al. (2002), who provided indirect information through inhibition of phospholipase A2 and COX on eicosanoid involvement in bacterial killing by mussel (*M. edulis*) hemocytes. In contrast, no relationship was established between eicosanoids produced through COX and LOX pathways in hemocytes and the immune capacities of the starfish *Asterias rubens* (Pope et al., 2007). In addition to different responses among species, effects of eicosanoids may vary according to their concentration; small doses of PGE_2 and LTB_4 stimulate some of the immune cells, whereas higher concentrations inhibit the same cells (Kelley, 2001). This may explain the differences of hemocyte time responses according to parameters; ARA supplementation resulted in a temporary increase in phagocytosis after 2 weeks, but no longer observed after 4 weeks. However, ROS production only increased after 4 weeks of ARA supplementation.

A number of studies using COX and LOX inhibitors in vertebrates indicate that the effects of FA on immune cell activity can be independent of the changes in the secretion of eicosanoids (Kelley and Rudolph, 2000; Pompéia et al., 2000). Changes of the FA composition of immune cells can directly affect phagocytosis, ROS production, cytokine production, leukocyte migration, T-cell signalling and antigen presentation capability (Pompéia et al., 2000; Calder, 2008). A dose-dependent effect of ARA on superoxide anion production through NADPH-oxidase system but independent of eicosanoid production was observed in human and rat leucocytes (Pompéia et al., 2003). Such direct effects of ARA on oyster hemocyte functions deserve to be explored by using COX and LOX inhibitors on hemocytes collected from oysters fed different levels of ARA.

As mentioned before, the effect of ARA supplementation on hemocyte activities was observed on ROS production after 4 weeks; it can be hypothesized that lipid peroxidation of ARA generated free radicals which in turn leads to superoxide anion production. Peroxidation and autoxidation of PUFA are general mechanisms for ROS production (Peck, 1994b). PUFA in membranes are more prone to lipotoxicity producing structural changes that may lead to cell damage (Gonzalez-Flecha et al., 1992), decreased membrane fluidity, oxidation of thiol groups

of enzymes in the membranes, and liberation of breakdown products which produce damage elsewhere (Peck, 1994a). By supplementing high quantities of ARA in the oyster's diet, we expected to increase the quantity of double bonds, and thus the unsaturation (PUI) and peroxidation indices (Table 1). PUI of reserve and membrane lipids increased significantly upon ARA supplementation at 0.4 µg/mL while peroxidation index was not affected. This may indicate a tight regulation of double bonds in both reserve and membrane lipids, possibly to limit exposure to lipid peroxidation when oysters are exposed to PUFA enriched diet. The increase of ARA in both membrane and reserve lipids was mainly compensated by decreases of DHA which has two more double bonds than ARA and of 18:3n-3 which has one less double bond.

Nevertheless, this does not exclude that ARA supplementation may have resulted in lipid peroxidation that should be inferred through the production of by-products such as malondialdehyde (MDA). MDA has been reported to occur at rates 200-times higher than the rates of PGE_2 synthesis in vertebrate kidneys; so ARA, together with other PUFA would be much more likely to be peroxidized by oxygen free radicals than to be used for PG synthesis (Gonzalez-Flecha et al., 1992). However, in good agreement with the absence of significant changes in peroxidation index of reserve and membrane lipids, no significant differences in MDA content was found according to ARA supplementation levels.

To further test if ARA supplementation changed the oxidative status of the whole oysters, it was important to assess the antioxidant capacities and activities of oysters exposed to increasing dietary supply of ARA. Box et al. (2007) stated that an adaptation response of enzymes activities and no significant differences in MDA concentration indicated that the antioxidant response was able to avoid lipid peroxidation. Environmental stressors such as dissolved oxygen influence antioxidant enzyme activities (SOD, catalase, and glutathione peroxidase GPX) in *Mytilus galloprovincialis* (Santovito et al., 2005), clams *Chamelea gallina* (Monari et al., 2005) and blue mussels *Mytilus edulis* (Manduzio et al., 2004). Moncheva et al. (2004) demonstrated that the antioxidant capacity of whole tissue of mussels is higher in polluted sites. Moreover, antioxidant status is of critical significance in determining the effects of PUFA on immune and inflammatory responses (Kelley et al., 2001). In the present study, no significant differences in catalase or SOD activities in relation to ARA supplementation were observed, suggesting the general antioxidant enzyme pathway was not activated. Only a temporary but significant increase of antioxidant capacity in terms of percent inhibition of DPPH was found after 15 days of ARA enrichment at 0.41 $\mu g.mL^{-1}$. Such transitory response remains unexplained. However, from lipid peroxidation status and the overall antioxidant capacity evaluated in the present study, it appears that ARA supplementation unlikely resulted in a substantial and sustained

oxidative stress.

5 Conclusions

The effect of supplementing ARA in the diet on oyster hemocyte parameters could be mediated through synthesis of PG and involves several cellular immune responses (number of circulating hemocytes, phagocytosis, ROS production, susceptibility to bacterial virulence factors). The lack of lipid peroxidation and activation of antioxidant responses upon increased ARA levels in diet indicate that immune responses could not be attributed to a general increase in oxidative stress. To further establish the involvement of PG in the observed cellular changes (hemocyte count, phagocytosis and ROS production), it would be interesting to test COX and LOX inhibitors in future experiments.

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549

550 **Figure captions**

551

552 Fig.1. ARA (μg/mg wet mass) in phospholipids and lipid reserves of *Crassostrea gigas* after 30
553 days of the dietary conditioning with T.*iso* supplemented with 0, 0.25, and 0.41 μg/mL of ARA
554 (n=3 pools of 5 animals each). Results are reported as mean ± SE. Means not sharing the same
555 letter within each lipid class are significantly different ($P<0.05$).

556

Fig. 2. Prostaglandin E metabolite (PGEM) in *Crassostrea gigas* before conditioning (T0) and after 15 and 30 days of conditioning with T.*iso* supplemented with 0, 0.25 and 0.41 µg/mL of ARA. Results are reported as mean ± SE. Means not sharing the same letter within each time sampling are significantly different ($P<0.05$) by ANOVA followed by Tukey test. An additional ANOVA was done to analyze the differences as a result of time of conditioning with T.*iso* + ARA 0 µg/mL; significant differences ($P<0.05$) compared to T0 values are marked by an asterisk (*).

Fig. 3. Relation between PGEM and ARA levels (lipid reserves + phospholipids) in *Crassostrea gigas* after 30 days of conditioning with T.*iso* supplemented with 0, 0.25 and 0.41 µg/mL of ARA. $Y = 1.1 + 39.2X$, $R^2=0.67$, $P<0.05$

569 Table 1: Fatty acid composition (percent of total fatty acids) in lipid reserves and phospholipids
570 of *Crassostrea gigas* (whole oyster) after 30 days of the dietary conditioning with T.*iso*
571 supplemented with 0, 0.25, and 0.41 µg/mL of ARA (n=3 pools of 5 animals each). Results are
572 reported as Mean ± SE. Fatty acids in phospholipids and lipid reserves were analyzed
573 separately. Different lower-case letters indicate significant difference between dietary treatments
574 (1-way ANOVA, *P<0.05*).

	Lipid Reserves			Phospholipids		
supplied ARA µg/mL	0	0.25	0.41	0	0.25	0.41
14:0	5.31 ± 0.15	4.94 ± 0.26	4.93±0.31	1.76 ± 0.1	1.63 ± 0.06	1.62 ± 0.12
16:0	14.0 ± 0.61	14.4 ± 0.34	13.5 ± 0.41	9.81 ± 0.47	9.40 ± 00.23	9.04 ± 0.11
18:0	2.73 ± 0.02	3.11 ± 0.08	2.76±0.06	4.7 ± 0.12	5.05 ± 0.11	4.81 ± 0.08
16:1n-7	3.51 ± 0.27	3.39 ± 0.24	3.22± 0.17	1.48 ± 0.09	1.2 ±0.04	1.37 ± 0.06
18:1n-9	5.52 ± 0.48	4.67 ± 0.24	4.72± 0.24	2.17 ±6	2.09 ± 0.04	2 ± 0.13
18:1n-7	4.3 ± 0.03	4.77 ± 0.08	4.51± 0.13	2.79 ± 0.22	3.29 ± 0.09	3.52 ± 0.08
18:2n-6	6.54 ± 0.55	4.9 ± 0.36	5.43±0.31	3.08 ± 0.31	2.54 ± 0.13	2.66 ± 0.2
18:3n-3	3.43 ± 0.23 [a]	2.58 ± 0.19 b	2.81± 0.14 [ab]	1.35 ± 0.1 a	1.17 ± 0.05 [ab]	1.12 ± 0.07 [b]
18:4n-3	7.33 ± 0.23	6.23 ± 0.3	6.56 ± 0.18	1.89 ± 0.03	1.73 ± 0.08	1.51 ± 0.1
20:4n-6	0.96 ± 0.01 [a]	4.12 ± 0.16 [b]	7.46 ± 0.88 [c]	2.33 ± 0.06 [a]	6.89 ± 0.19 [b]	9.25 ± 0.31 [c]
20:5n-3	13.3 ± 0.55	14.8 ± 0.44	13.6 ± 0.45	10.9 ± 0.3	10.8 ± 0.18	10.3 ± 0.32
22:5n-6	1.02 ± 0.1	0.80 ± 0.06	0.85 ±0.04	1.62 ± 0.01 [a]	1.47 ± 0.01 [b]	1.32 ± 0.07 [c]
22:5n-3	0.58 ± 0.02	0.61 ± 0.01	0.59 ± 0.01	1.20 ± 0.02	1.13 ± 0.03	1.32 ± 0.07
22:6n-3	12.8 ± 0.35 [a]	11.4 ± 0.21 [b]	11.2 ± .0.5 [b]	16.4 ± 0.14 [a]	15.3 ± 0.19 [b]	14.3 ± 0.14 [c]
Total PUFA	53.8 ± 1.2[ab]	52.5 ± 0.6 [a]	55.4 ± 0.8 [b]	49.2 ± 0.6 [a]	51.3 ± 0.4 [b]	51.2 ±0.2 [b]
Total n-6	10.2 ± 0.65 [a]	11.4 ± 0.37 [a]	15.4 ± 0.72 [b]	8.8 ± 0.37 [a]	13.2 ± 0.43 [b]	15.6 ± 0.51 [c]
Total n-3	39.2 ± 0.62 [a]	36.4 ± 0.52 [b]	37.3 ±0.57 [ab]	33.4 ± 0.43 [a]	31.4 ± 0.18 [b]	29.4 ± 0.24 [c]
n-3/n-6	3.86 ± 0.22 [a]	3.27 ± 0.08 [b]	2.37± 0.13 [b]	3.82 ± 0.2 [a]	2.39 ± 0.08 [b]	1.89 ± 0.07 [c]
EPA /ARA	13.8 ± 0.41 a	3.59 ± 0.04 b	1.87 ± 0.2 c	4.68 ± 0.18 a	1.56 ± 0.07 b	1.11 ± 0.06 c
Unsaturated index (PUI)	227 ± 3.5 [a]	227 ± 1.4 [a]	236 ± 2.9 [b]	204 ± 1.6 [a]	224 ± 1.5 [b]	224 ± 1.1 [b]
Peroxidation index	243 ± 3.5 [a]	243 ± 1.4 [a]	251 ± 4 [a]	239 ± 2.2 [a]	244 ± 1.4 [a]	240 ± 0.8 [a]

575 Table 2: Lipid peroxidation (measured as malondialdehyde concentration in µmol/mg wet mass), total antioxidant radical scavenging capacity
576 (measured as DPPH inhibition percentage), catalase and superoxide dismutase activities in *Crassostrea gigas* before conditioning (T0) and after 15
577 and 30 days of conditioning with T.*iso* supplemented with 0, 0.25 and 0.41µg/mL of ARA. Results are reported as Mean ± SE. Significant effect of
578 treatment evaluated by ANOVA was only observed for DPPH inhibition percentage; means not sharing the same letter within each time sampling
579 are significantly different by Tukey test. An additional ANOVA was done to analyze the differences as a result of time of conditioning with T.*iso* +
580 ARA 0 µg/mL; significant differences (P<0.05) compared to T0 values are marked by *.
581

	T0	T15 (after 2 weeks of feeding ARA)			T30 (after 4 weeks of feeding ARA)		
supplied ARA µg/mL		0	0.25	0.41	0	0.25	0.41
Malondialdehyde (MDA) (µmol/mg wet mass)	0.057 ± 0.004	0.044 ± 0.003	0.044 ± 0.005	0.052 ± 0.005	0.056 ± 0.008	0.046 ± 0.002	0.059 ± 0.004
DPPH inhibition percentage	24.48 ± 6.89	20.73 ± 0.76 [a]	14.9 ± 2.75 [a]	29.84 ± 0.47 [b]	16.94 ± 0.68 [a]	12.11 ± 0.64 [a]	17.27 ± 2.52 [a]
Catalase (U/ mg wet mass)	2.5 ± 0.16	2.5 ± 0.39	2.27 ±0.12	2.31 ± 0.04	2.55 ± 0.17	2.27 ± 0.27	2.4 ± 0.13
Superoxide dismutase (SOD) (U/mg wet mass)	1.76 ± 0.14	2.89 ± 0.16*	1.86 ± 0.36	2.91 ± 0.92	3.16 ± 0.25*	2.37 ± 0.38	2.78 ± 0.13

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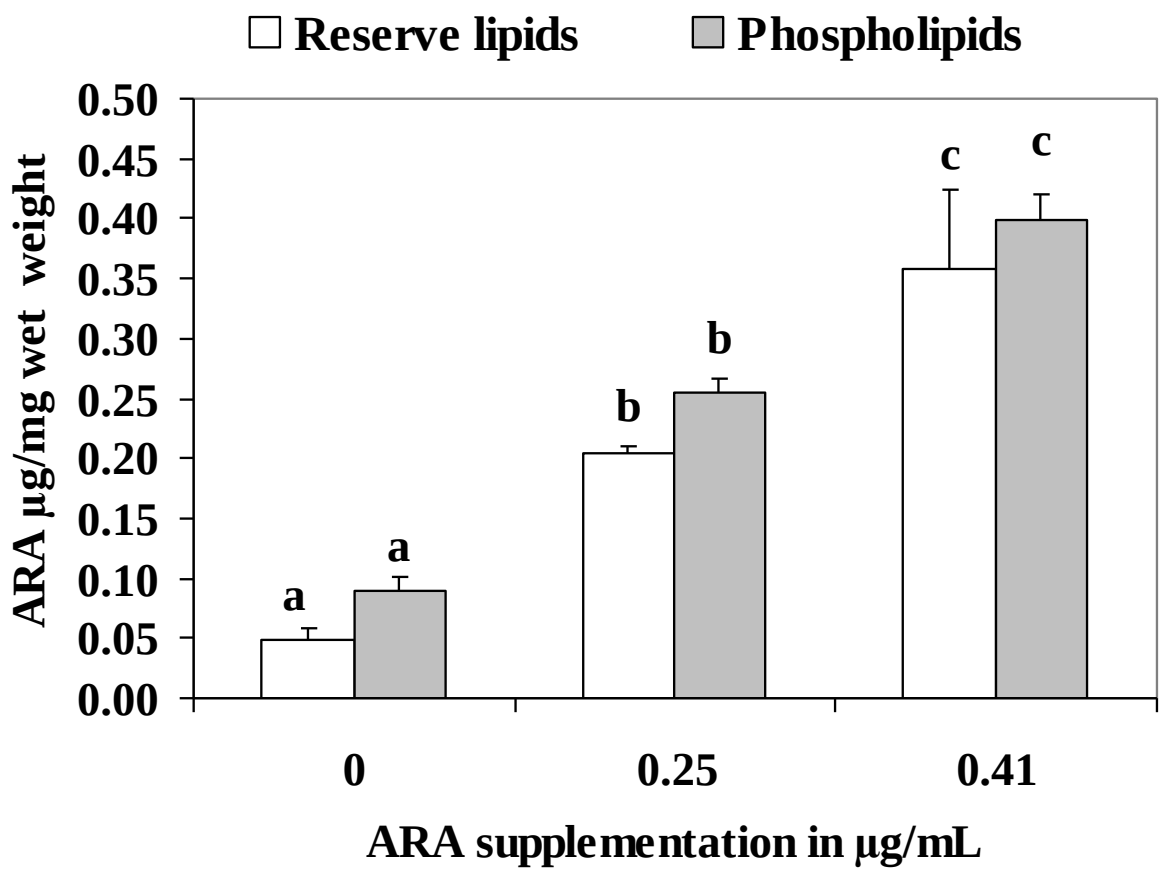

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588 **Figure 1**
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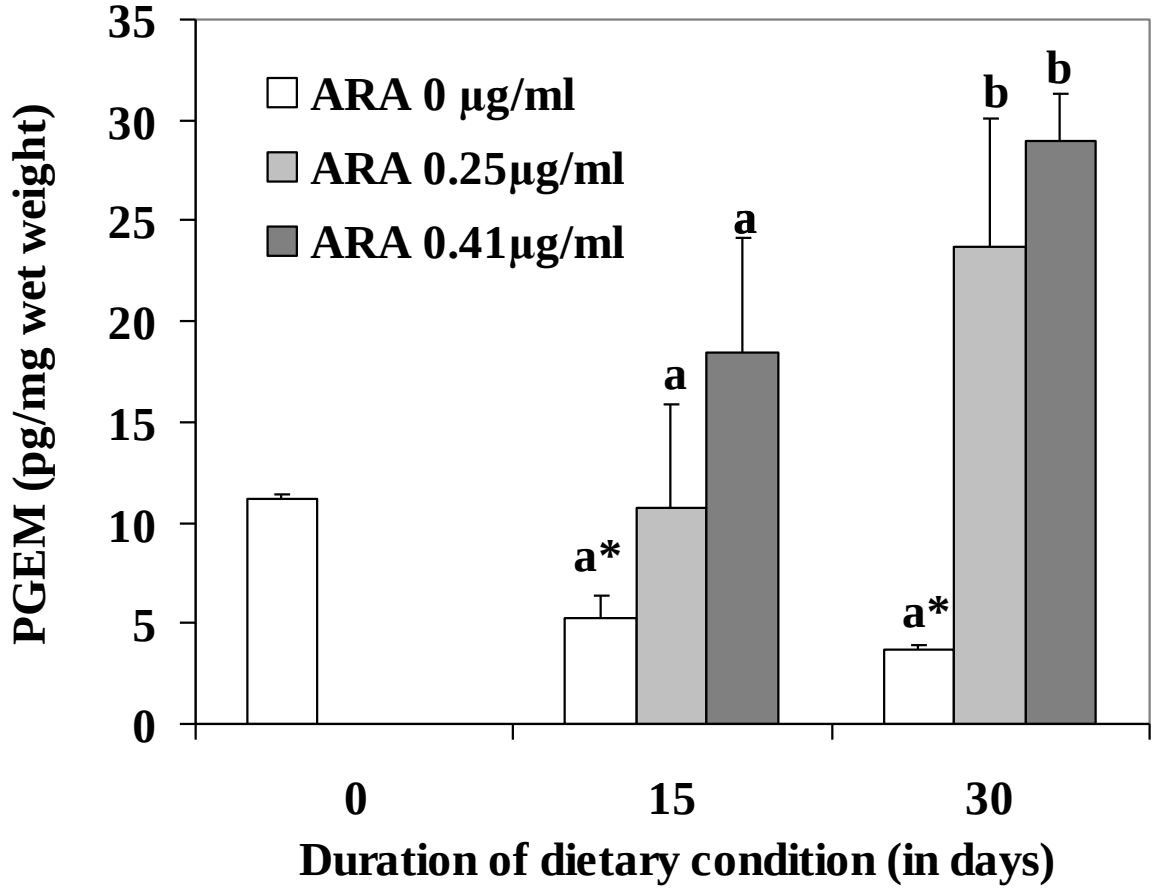
35
30
25
20
15
10
5
0
PGEM (pg/mg wet weight)
ARA 0 µg/ml
ARA 0.25µg/ml
ARA 0.41µg/ml
a*
a
a
a*
b
b
0
15
30
Duration of dietary condition (in days)

Figure 2

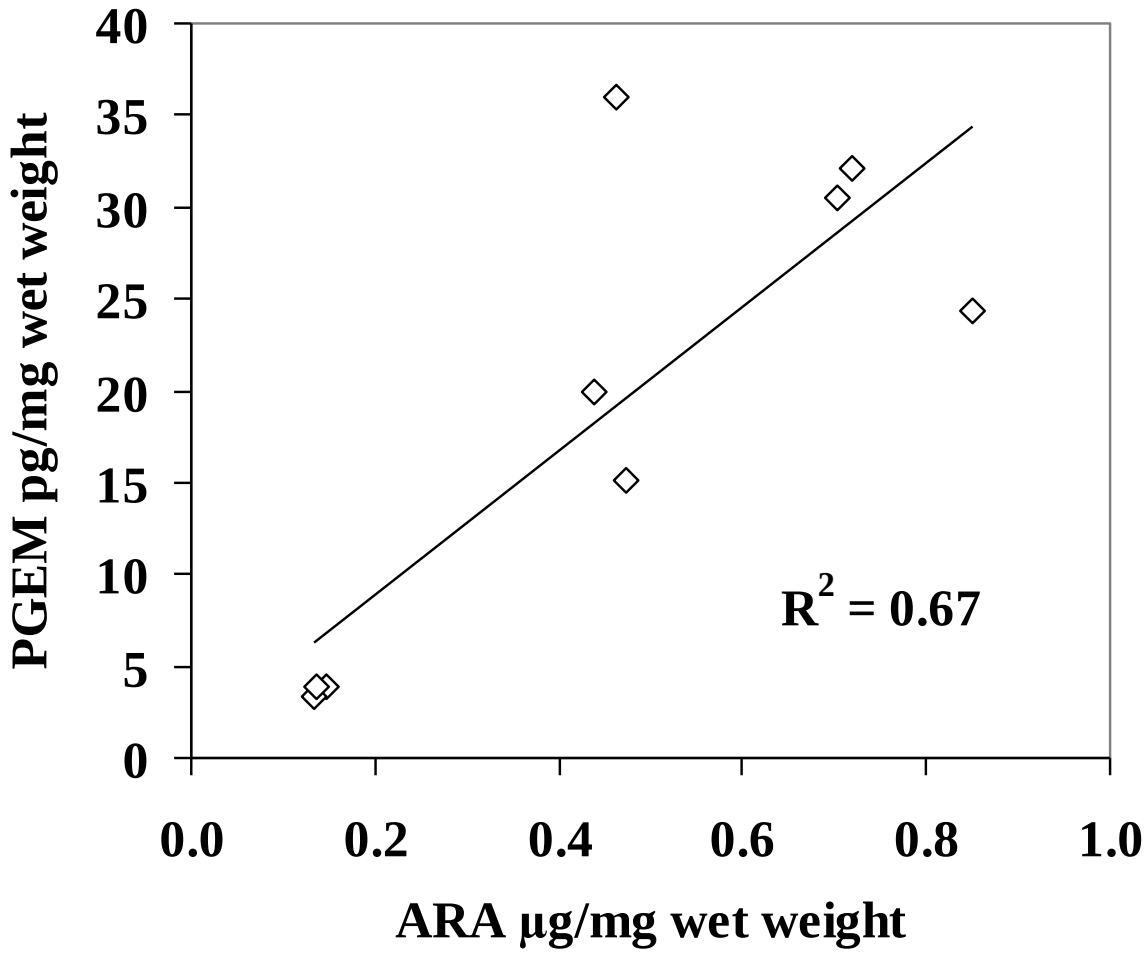
PGEM pg/mg wet weight
40
35
30
25
20
15
10
5
0
0.0
0.2
0.4
0.6
0.8
1.0
ARA µg/mg wet weight
R2 = 0.67

Figure 3