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Impact of 20:4n-6 supplementation on the fatty acid composition and hemocyte parameters of the Pacific Oyster Crassostrea gigas.

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Abstract: Arachidonic acid (20:4n-6, ArA) and its eicosanoid metabolites have been demonstrated to be implicated in immune functions of vertebrates, fish, and insects. Thus, the aim of this study was to assess the impact of ArA supplementation on the FA composition and hemocyte parameters of oysters *Crassostrea gigas*. Oyster dietary conditioning consisted of direct addition of ArA solutions at a dose of 0, 0.25, or 0.41 mg ArA per mL of seawater into tanks in the presence or absence of T-Iso algae. Results showed significant incorporation of ArA into gill polar lipids when administered with algae (up to 19.7%) or without algae (up to 12.1%). ArA supplementation led to an increase in hemocyte numbers, phagocytosis, and production of reactive oxygen species by hemocytes from ArA-supplemented oysters. Moreover, the inhibitory effect of *Vibrio aestuarianus* extracellular products on the adhesive proprieties of hemocytes was lessened in oysters fed ArA-supplemented T-*Iso*. All changes in oyster hemocyte parameters reported in the present study suggest that ArA and/or eicosanoid metabolites affect oyster hemocyte functions

Keywords: Crassostrea gigas, Bivalve, Nutrition, Poly-unsaturated fatty acids, Arachidonic Acid, Oyster immunity, Hemocyte parameters

Introduction

The dietary impact of long chain n-3 and n-6 essential polyunsaturated fatty acids (PUFAs) have been extensively studied in human medical research, including the modulation of the human immune system. Thus, long chain n-3 PUFAs has been shown to have anti-inflammatory and immunomodulatory properties for a number of immune functions including phagocytosis, reactive oxygen species production, and lymphocyte proliferation [1, 2]. The impact of dietary long chain n-6 PUFAs on immunity however has been less well investigated and is still controversial with both pro-inflammatory and immunosuppressive properties having been associated with these molecules [2]. *In vitro* studies have focused on the mechanisms by which n-6 PUFAs especially arachidonic acid (20:4n-6, ArA) and its eicosanoid metabolites can affect immune functions [3-8]. These studies demonstrated that ArA, generally found in high amount in membranes of higher vertebrates (human, rat), likely via its eicosanoid metabolites can influence phagocytosis, NADPH oxidase activation, actin polymerisation and Ca^{+} release.
Similar studies on fish nutrition have evaluated the impact of n-3 and n-6 dietary PUFAs on immune functions [9-15]. Thus, Lin and Shiau [13] demonstrated that lipid supplemented diets resulted in increased numbers of white blood cells and enhances phagocytosis and the generation of reactive oxygen species in grouper *Epeniphelus malabarcius* when compared to fish fed lipid poor diets. Wu *et al.* [15] investigated more specifically the effect of long chain n-3 PUFAs (20:5n-3 (EPA) and 22:6n-3 (DHA)) on the immune functions of the grouper *Epinephelus malabarcius*. The authors reported that DHA enhanced phagocytic function and T-cell proliferation and was superior to EPA in influencing the cellular defence responses of the grouper. Moreover, eicosanoid metabolites derived from ArA PUFA are also significant in fish immunity. Tafalla *et al.* [16] demonstrated that LTB_4 in leukocyte-derived supernatants have an anti-viral activity against viral hemorrhagic septicemia virus in the turbot *Scophthalamus maximus*. However, the authors did not exclude the involvement of other factors as PGE_2.
In bivalves**,** for more than two decades, studies have focused on the determination of the nutritional values of various algal species to support and improve larval development, growth, and metamorphosis under hatchery conditions [17-23]. Fewer studies have investigated the impact of nutrition on immune responses of bivalves [24-26]. Thus recently, Delaporte *et al.* [26] have demonstrated that feeding Pacific oysters *Crassostrea gigas* and Manila clams *Ruditapes philippinarum* with cultures of the microalga *Chaetoceros calcitrans* (a diatom) led to increases in hemocyte numbers, phagocytosis and reactive oxygen species production. These authors suggested that the fatty acid composition of *Chaetoceros calcitrans*, characterised by high proportion of EPA (17.8%) and ArA (2.0%), may be responsible for the changes observed in immune responses of both species, as demonstrated for vertebrates and fishes fed on different oils. Similarly, Hégaret *et al.* [24] showed that immune parameters of the Eastern oyster *Crassostrea virginica* were modulated by the quality of the algal diet. Nonetheless, no specific PUFA could be clearly identified as responsible for those changes. Lastly, dietary conditioning using lipid emulsion was used to assess the impact of EPA on hemocyte parameters of the oyster *C. gigas* [25]. In this study, a decrease of phagocytosis and reactive oxygen species production was temporarily observed for oyster fed the highest dose of EPA. These results suggested that this PUFA may have an impact on hemocyte function, but unfortunately without excluding a concomitant impact of ArA since the lipid emulsion also contained a significant amount of ArA.
The aim of this current study was to specifically determine the impact of dietary ArA on hemocyte parameters of *C. gigas*. To do this, a new supplementation method developed by

Séguineau *et al.* [27] based on the direct addition of individual PUFA along with T-*Iso* algae (deficient in ArA) was used.

Materials and Methods

Oyster dietary treatments

One year old oysters, *C. gigas* (Thunberg 1793), produced in 2003 at the IFREMER hatchery in La Tremblade (Charente, France), were conditioned in May 2004 at the IFREMER shellfish laboratory in Argenton (Finistère, France) in 50-L tanks. Oysters were acclimated to the experimental temperature of 17°C for two weeks prior to the dietary experiment. After the acclimation period, oysters were divided randomly and distributed into 9 tanks (70 oysters per tanks, 3 replicates per treatment) filled with 45 L of 20µm-filtered seawater, mixed and aerated using an immersed pump. Thereafter, oysters were fed *Isochrysis* sp., clone T-*Iso,* supplemented with ArA solution for 4 weeks. T-*Iso* was chosen for this experiment because of its low concentration in arachidonic acid (0.1%, [26]). The daily algal ration was established at 4% algal dry weight per oyster dry weight. A peristaltic pump was adjusted to distribute the algae ration over a 18 hours period per day. ArA (Sigma A-9376) was dissolved in ethanol at the concentration of 10 mg mL^{-1} according to the procedure of Séguineau *et al.* [27]. This ArA solution was further diluted in ethanol to allow addition of a same volume of ethanol per tank and to obtain the following final concentrations: 0 µg (control ethanol solution), 0.25µg, or 0.41 µg of ArA per mL of sea water. ArA solutions were added twice a day (half dose in the morning and half dose in the evening in order to avoid ArA oxidation). Tanks and oysters were daily cleaned. Every two weeks, 15 oysters were sampled for biochemical analyses and 20 oysters for immune analyses. After the first sampling, volume of seawater per tank was adjusted in order to maintain a stable effective volume per oyster during the experiment. No mortalities were recorded over the whole experiment.

One month later, a separate control experiment was conducted for a period of 2 weeks with the same experimental protocol to assess the assimilation of dissolved ArA without algal supply in oysters from the same origin.

Condition index and biochemical composition

At each sampling date, whole oyster, shell, and wet flesh weights were measured on 15 oysters. The condition index of oysters was calculated, as described by Walne and Mann [28], following the formula: dry flesh weight / dry shell weight X 1000. Thereafter, three pools constituted by the wet flesh tissues of five animals were generated. Pools were frozen in liquid nitrogen (-196°C) and treated as described in Delaporte *et al.* [29]. Total lipid content was estimated according to Bligh and Dyer [30] and carbohydrate and protein contents were measured colorimetrically following the procedures of Dubois *et al.* [31] and Lowry *et al.* [32], respectively. Results are expressed as mg of carbohydrate, lipids and protein per mg of oyster dry flesh weight.

Fatty acid analysis of oyster gills

Sampling and extraction

Gills were dissected from the same animals utilised for the hemocyte-parameter analyses. Three pools of gills from 5 animals were constituted and frozen in liquid nitrogen at -196°C. Pooled gill samples were ground with a Dangoumeau homogeniser, and 300 mg of the ground tissue was transferred to a tube containing 6 ml of chloroform-methanol mixture (2:1, by vol). After centrifugation, the lipid extract was transferred to a clean tube, sealed under nitrogen, and stored at -20°C.

Separation of polar and neutral lipid

Neutral and polar lipids of gill lipid extracts were purified on a Silica gel micro-column according to Marty *et al.* [33] and analysed as described in Delaporte *et al.* [29]. Briefly, an aliquot of the lipid extract in chloroform/methanol was evaporated to dryness. Then, lipids were re-dissolved in a chloroform-methanol mixture (98:2, v:v) and placed on a silica gel microcolumn [30x5 mm i.d. Kieselgel, 70-230 mesh (Merck) previously heated to 450°C and deactivated with 5 wt % water]. Neutral lipids were eluted with a chloroform-methanol mixture (98:2, v:v), and the polar lipids were eluted separately with methanol. After trans-esterification of the neutral and polar lipid extracts from gills by 10% w:w boron trifluoride/methanol (Metcalfe and Schmitz, 1961), fatty acid methyl esters (FAME) were analysed in a gas chromatograph equipped with an on-column injector, a DB-Wax (30 m x 0.25 mm, 0.25 µm film thickness) capillary column, and a flame ionization detector. Hydrogen was used as the carrier gas. The fatty acids were identified by comparing their retention times with those of standards and confirmed by gas liquid chromatograph-mass spectrometry (GC-MS).

Fatty acid composition of gills was expressed as weight percentage of the total fatty acids of each lipid fraction. Total fatty acid content (neutral + polar lipids) per gill sample was expressed as µg of FAs per mg of gill wet weight.

As polar lipids generally provide a good approximation of cell membrane lipids and are the dominant lipids in hemocyte and gill tissues [26, 29, 34], only fatty acid composition values of the polar lipid fraction of gill lipid extracts were presented in this study to simplify the presentation of fatty acid data.

Measurements of immunological parameters by flow cytometry

Measurements of hemocyte types, numbers, and functions were performed on a FACScalibur flow cytometer (B-D Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser. Hemolymph sampling and hemocyte parameter measurements are described below. For each assay, samples were filtered through a 80µm mesh prior to flow cytometer analysis in order to eliminate large debris (> 80 µm)**.**

Hemolymph sampling

Hemolymph was withdrawn from individual oysters using a 1 mL plastic syringe fitted with a 25-gauge needle via a notch adjacent to the adductor muscle created just prior the bleeding. All hemolymph samples were examined microscopically for contamination (e.g., oocytes, sperm, algae) and stored individually in micro-tubes at 0 °C. Three pools of five individual samples were used for the following immunological assays.

Hemocyte viability, total hemocyte and sub-population concentrations

An aliquot of 100 µL of pooled hemolymph was transferred into a tube containing a mixture of 200 µL anti-aggregant solution for hemocytes (AASH) and 100 µL filtered sterile seawater (FSSW). AASH was prepared according to Auffret and Oubella [35]. Hemocyte DNA was stained with two fluorescent DNA/RNA specific dyes, SYBR Green I (final concentration 10X) and propidium iodide (PI, final concentration of 20 $\mu g.mL^{-1}$), in darkness at room temperature for 60 minutes before flow-cytometric analysis. SYBR Green I permeates both dead and live cells, while PI permeates only through membranes of dead cells. SYBR Green fluorescence was measured at 500-530 nm (green) by flow cytometry while PI fluorescence was detected at 550-600 nm (red). Thus, by counting the cells stained by PI and cells stained by SYBR Green, it was possible to estimate the percentage of viable cells in each sample.

All SYBR Green I stained cells were plotted on a FSC (size) - SSC (granularity) cytogram allowing identification of hemocyte sub-populations. Three sub-populations were distinguished according to their size and granularity and termed granulocytes (high FSC and high SSC), hyalinocytes (high FSC and low SSC) and small agranulocytes (low FSC and low SSC). Total and differential hemocyte concentrations are expressed as number of cells per mL.

Phagocytosis assay

An aliquot of 100 µL pooled hemolymph, diluted with 100 µl of FSSW, was mixed with 30 µL of YG 2.0 µm fluoresbrite microspheres, diluted to 2% in FSSW (Polysciences, Eppelheim, Germany). After 120 minutes of incubation at 18°C, hemocytes were fixed with 230 µL of a 6% formalin solution and analysed at 500-530 nm by flow cytometry to detect cells containing fluorescent beads. The percentage of phagocytic cells was estimated by the percentage of hemocytes that had engulfed three beads and more according to Delaporte *et al.* [26].

Adhesion capacity

The adhesive capacity of *C. gigas* hemocytes was assessed by modifying the procedure of Choquet *et al.* [36]. In the present study, live pathogenic bacteria were replaced by the extra-cellular products (ECP) of the *Vibrio aestuarianus*, previously demonstrated to inhibit adhesive capacity of *C. gigas* hemocytes [37]. Two sub-samples of each hemolymph pool were distributed (100 µL per well) in 24-well microplates. An aliquot of 100 µL FSSW was added to the first sub-sample as a control and 100 µL ECP suspension to the second (30µg protein mL^{-1} final concentration). After three hours of incubation, the supernatant containing cells not adhering was transferred into a flow cytometer tube and fixed by addition of 200 µL of a 6% formalin solution. After 30 minutes of incubation with SYBR Green I, cell concentration was then evaluated as described above. Since the hemocyte adhesive capacity was measured with or without addition of ECP of *Vibrio aestuarianus*, results are expressed as the percentage of adhering hemocytes relatively to the initial total hemocyte concentration of the tested pool prior to incubation with or without ECP.

Reactive oxygen species (ROS) production

Measurement of the ROS production of hemocytes was assayed by modifying the procedure of Lambert *et al.* [38] using 2'7'-dichlorofluorescein diacetate (DCFH-DA). For this assay, two aliquots of 100 µL of hemolymph were diluted with 300 µL of FSSW in two flow cytometer tubes. In one of the tubes, 4µL of diphenylene iodonium chloride (DPI, 5µM final concentration), an NADPH-oxidase and NO-synthase inhibitor, was added.

Subsequently, DCFH-DA (final concentration of 0.01 mM) was added to each tube previously maintained on ice, and tubes were incubated at 18°C for 120 minutes. After the incubation period, DCF fluorescence, quantitatively related to intra-cellular ROS production by hemocytes, was measured at 500-530 nm by flow cytometry. Results are expressed as the mean fluorescence (in arbitrary units) detected in hemocyte sub-populations maintained in FSSW (basal ROS production) and after addition of DPI (inhibited ROS production).

Statistical analysis

One or two way analysis of variance (1-way or 2-way ANOVA) were performed for each biochemical and hemocyte parameter using STATGRAPHICS Plus 5.1 statistical software (Manugistics, Inc., Rockville, MD, USA), to test the arachidonic acid dose effect and the time effect after 2 and 4 weeks of conditioning. Percentage data were transformed (arcsin of the square root) before ANOVA or MANOVA, but are presented in figures and tables as untransformed percentage values.

Results

Condition index and Biochemical composition

No significant differences between dietary treatments were observed for oyster condition index, oyster tissue dry weight (DW) and carbohydrate, protein and lipid contents over the whole experiment (Table 1, 2-way ANOVA, $p>0.05$). However it can be noted that oyster tissue DW and carbohydrate content were lower at the end of the experiment than initially. Meanwhile, total lipid contents of oysters increased from 0.087 mg/mg DW initially to an average of 0.110 mg/mg DW at the end of the experiment (2-way ANOVA, $p<0.001$). Protein content of oysters was stable during the whole experiment.

Fatty acid composition of gill polar lipids

Impact of the dietary conditioning with with ArA-supplemented T-Iso

Feeding oysters with T-*Iso* alone (without ArA supplementation) resulted in an enrichment of gill polar lipids with 18:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 22:6n-3, and a decrease in 20:5n-3 (Table 2). Similar changes were observed in gill polar lipids of oysters fed ArA-supplemented T-*Iso* (Table 2), but ArA supplementation greatly increased the 20:4n-6 percentage of gill polar lipids as early as 2 weeks into the dietary conditioning period (Table 2, Figure 1). At the end of the experiment, percentages of 20:4n-6 reached 15.3% and 19.7% in gill polar lipid of oysters fed, respectively, T-*Iso* supplemented with 0.25 and 0.41 µg mL^{-1} of ArA solution while it stayed at 6.2% in those fed T-*Iso* alone. This high increase in 20:4n-6 also led to a significant decrease in 20:5n-3 and 22:6n-3, a significant increase of total n-6 fatty acid contents in gill polar lipids, and consequently a decrease in the n-3/n-6 ratio and 20:5n-3/20:4n-6 ratio. However, the proportions of the different fatty acid classes (SAFA, MUFA, PUFA) were not affected by the dietary conditioning.

Impact of conditioning with ArA solution and without algae (control experiment)
The fatty acid composition of gill polar lipids was also greatly affected by 2 weeks of ArA dietary conditioning with 0.25 and 0.41 µg mL^{-1} of ArA without algae supplementation (Table 3). The percentage of 20:4n-6 of gill polar lipids increased two fold after 2 weeks with ArA supplementation and reached 11.3 and 12.1% in oysters fed 0.25 and 0.41 µg mL^{-1}, respectively. However, no dose effect was recorded. Similarly but at a lesser extent than in the experiment with algae, feeding oysters with 0.25 and 0.41 µg mL^{-1} also resulted in a significant decrease of 22:6n-3, a significant increase of total n-6 fatty acids (from 9.8 to 14.5 and 15.5% respectively) in gill polar lipids, and a significant decrease in n-3/n-6 ratio (from 4.0 to 2.2 and 2.4 respectively) and 20:5n-3/20:4n-6 ratio (from 1.7 to 0.9 and 1.0).

Hemocyte parameters

Hemocyte viability and concentrations
Hemocyte viability was high and similar for oysters fed T-*Iso* supplemented with 0.0, 0.25 and 0.41 ArA µg mL^{-1} (Table 4).
During the experiment, the total hemocyte (including granulocytes, hyalinocytes and agranulocytes) tended to increase in oysters fed T-*Iso* supplemented with ArA solutions (1-way ANOVA, $p>0.05$). When hemocyte subpopulations were evaluated, it was apparent that granulocyte and hyalinocyte concentrations of oysters fed T-*Iso* supplemented with 0.25 and 0.41 µg mL^{-1} of ArA solutions tended to be higher than those of oysters fed T-*Iso* alone (Table 4).

Percentage of phagocytic hemocytes
After 2 weeks of experimental feeding, the percentage of phagocytic hemocytes from oysters fed T-*Iso* supplemented with ArA solutions was significantly higher than for oysters fed T-*Iso* alone (Figure 2, 1-way ANOVA, $p<0.05$); however, was not different anymore from control animals after 4 weeks of dietary conditioning (1-way ANOVA, $p>0.05$).

Adhesive capacity
After 2 and 4 weeks of dietary conditioning, no change in adhesive capacity of hemocytes incubated with FSSW could be seen among the three treatment groups (average of 93% of adhering cells during the whole experiment, data not shown). However, hemocytes of oysters fed T-*Iso* alone showed a greater reduction in adhesion in response to ECP than did oysters fed T-*Iso* supplemented with ArA solutions, but this was not statistically significant (Figure 3).

Reactive oxygen species (ROS) production
ROS production by granulocytes and hyalinocytes are presented in Figure 4. A significant dietary effect was observed for the basal ROS production of each hemocyte sub-population after 4 weeks of dietary conditioning (1-way ANOVA, $p<0.05$). Granulocytes and hyalinocytes of oysters fed T-*Iso* supplemented with ArA at 0.25 and 0.41 µg mL^{-1} generated the highest basal ROS production (average of 360 A.U.). An increase of 160 A.U. for granulocytes and hyalinocytes was observed, when compared to the values obtained with oysters fed T-*Iso* alone (average of 198 A.U.). Moreover, the basal ROS production increased continuously between the initial sampling and the end of the experiment for hemocytes of oysters fed T-*Iso* supplemented with ArA solutions, while those of oysters fed T-*Iso* alone remained quite stable.

Regarding ROS production after DPI incubation, an inhibition of ROS production was observed for both hemocyte sub-populations with an average of 64.5 and 56 A.U. of DCF fluorescence measured during the whole experiment in granulocytes and hyalinocytes, respectively. No dietary treatment effect was detected for each hemocyte sub-population when incubated with DPI (2-way ANOVA, $p>0.05$).

Discussion

Impact of the dietary conditioning on gill fatty acid composition

Changes measured in the fatty acid composition of hemocyte membranes were shown to be closely related to those in gill membranes in dietary experiments [25]; therefore, in the present study fatty acid analyses were performed on gills rather than on hemocyte samples, which are more difficult to collect in significant amounts.

The observed changes in fatty acid composition of gill polar lipids are in agreement with other studies using T-*Iso* as a mono-specific diet [26, 39-46]. More noteworthy was the increased incorporation of ArA in gill polar lipids from 4.5% to 19.7% for oysters fed 4 weeks T-*Iso* supplemented with ArA solutions. Such ArA enrichment of polar lipids was also reported by Séguineau *et al.* [27] using the same technique of supplementation in *C. gigas* oyster spat for 5 weeks. High concentrations of ArA in bivalve phospholipids observed in the present report and in the study of Séguineau *et al.* [27] have not been previously reported in the literature, whatever the tissue, the development stages and species considered. The highest percentage of ArA in polar lipids lastly found in the literature reached 7.5% in juveniles of *C. gigas* and 10% in *Ruditapes philippinarum* [26]. Also, our results confirmed that this supplementation technique greatly increased the membrane contents of polyunsaturated fatty acids, such as ArA. Interestingly, direct ArA supplementation without algae performed in this study showed that algal feeding was not necessary to obtain good incorporation of ArA supplied in solution directly into the tanks. Indeed, increases of 40% and 44% in the ArA content in gill polar lipids were observed after 2 weeks of conditioning (from 6.8% to 11.3% and 12.2%). Thus, we can suggest, as proposed by Bunde and Fried [47], that the ArA may be directly incorporated through mantle and gill membranes without passing through the digestive system. However, it must be stressed that ArA solutions were 2-fold more efficiently incorporated when combined with the microalgae than when provided alone. The free ArA added to the rearing tank could be coated to or be incorporated by the algae favouring thus its high incorporation and assimilation in the oyster lipids.

Concomitantly to ArA incorporation, a decrease in n-3 PUFAs, especially EPA content in gills was reported during the dietary experiment resulting in a decrease in the n-3/n-6 and EPA/ArA ratio. Although effect of ArA supplementation on oyster immune parameters may primarily reflect the ArA content increase, the decrease of EPA content and n-3/n-6 ratio may also affect these parameters. Indeed, in vertebrates, changes in n-3 and n-6 composition and their ratio are well known to affect immune parameters. For example, EPA by competing with ArA for the eicosanoid production can modulate immune functions [1,2].

Impact of the arachidonic acid supplementation on immune parameters

The immune system of bivalves relies on innate defense mechanisms for its response to pathogens. The cellular component of this innate immunity is mediated by hemocytes, which are responsible for recognition, phagocytosis, and elimination of non-self particles [48-50]. For this purpose, they generate a variety of microbicidal products including reactive oxygen species.
In the present study, total and sub-population hemocyte concentrations tended to be higher for oysters fed T-*Iso* supplemented with ArA. This is in agreement with increased hemocyte concentration observed in the study of Delaporte *et al.* [26] in oysters and clams fed *Chaetoceros calcitrans*, which carried the highest amounts of ArA compared to two others algae T-*Iso* and *Tetraselmis suecica*. However, in bivalves, it is not clear whether the increase in hemocyte concentration results from production of new cells due to an ArA riched diet effect or migration of cells from deeper tissue into the circulating system. Moreover, comparison of our results with vertebrate immune cell proliferation must be done with caution since in vertebrates this process occurs through the binding of a mitogen to a receptor (adaptive response) which lead to a complex cascade of biochemical events. However, Peres *et al.* [51] demonstrated that ArA can modulate lymphocyte proliferation of rats according to the concentration of ArA used for loading macrophages and the percentage of macrophage in the co-culture of cells. Concomitantly, in a review, Peters-Golden *et al.* [52] underlined that leukotrienes influence innate immune response by direct effect on leukocyte accumulation, microbial phagocytosis and killing and indirect effects mediated by elaboration of other inflammatory molecules. Consequently, although scarce references concerning eicosanoid production in bivalves exist [53-55], we proposed that changes in fatty acid composition, especially in ArA, may have affected eicosanoid metabolite production which may be implicated in the increased hemocyte concentration observed in the present study in oysters.

With regard to hemocyte functional activities, phagocytosis by hemocytes was significantly affected by the dietary treatments after 2 weeks of conditioning. At this time, the percentage of phagocytic cells of oysters fed T-*Iso* supplemented with 0.25 and 0.41 µg mL^{-1} of ArA was higher than that of oysters fed T-*Iso* alone. The highest percentage of phagocytic hemocytes was associated with the highest ArA content of gill polar lipids. The relationship between ArA and phagocytosis observed in this study may be also related to ArA derived eicosanoid metabolites. Indeed, Canesi *et al.* [56] demonstrated that bacterial killing of hemocytes of mussel *Mytilus edulis* was reduced by inhibitors of PLA_2 and cycloxygenase activities, indicating that eicosanoid production is involved in mediating the response to bacterial challenge. Moreover, ArA metabolites (i.e. prostaglandins) were demonstrated to be involved in the nodulation process of insect hemocytes, which is the predominant response to large bacterial infections and results in the formation of an overlapping sheath of hemocytes around an infection target [57-62]. Several *in vitro* studies in vertebrates also demonstrated a modulating effect of ArA in the phagocytosis process [3-8]. Meanwhile, Brock *et al.* [63] demonstrated that LTB_4 and PGE_2 are successively expressed in macrophages during a time exposition to LPS affecting the subsequent bacterial activity of macrophage when challenged with *Klebseilla pneumonia.* Therefore, changes in the percentage of phagocytic hemocytes observed over the ArA dietary experiment may be related to subtle changes in the ratio of ArA derived eicosanoids (i.e. LTB4 / PGE2) produced by hemocytes.

Concomitantly with the increase of phagocytosis, an increase of adhesive capacity of ECP challenged hemocytes from oysters fed T-*Iso* supplemented with 0.25 and 0.41 µg mL^{-1} of ArA was observed. This result is similar to the study of Mandato *et al.* [58] and Miller *et al.* [64], who demonstrated that ArA metabolites are involved in the hemocyte spreading process (similar to the process involved in our adhesive assay) in insects and suggests that eicosanoid products may also be implicated in the adhesive capacity of oyster hemocytes. Interestingly, although the inhibitory effect of ECP on hemocyte adhesive capacity demonstrated by Labreuche *et al.* [37] was confirmed in this study, ArA supplementation appeared to counteract its effect.

An increase of the basal ROS production by hemocytes was observed at the end of the experiment for oysters fed T-*Iso* supplemented with ArA solutions. It is important to note that addition of diphenylene iodonium chloride (DPI), an inhibitor of the NADPH-oxidase and of the NO-synthase pathways, strongly decreased the basal ROS production from hemocytes. This allowed us to confirm that the basal ROS production was associated with NADPH-oxidase and /or NO-synthase-like activities. Also, the highest incorporation of ArA content in gill polar lipids of those oysters seemed to be associated with basal ROS production as previously observed with the *C. calcitrans* dietary conditioning [26]. In an *in vitro* study, Mazière *et al.* [65] demonstrated that human fibroblasts incubated with ArA presented higher ROS production without stimulation than control fibroblasts, but also reported an higher content of lipid peroxidation products. Also, in a review, Calder [1] noted that LTB_4 (produced from ArA) enhanced ROS production of vertebrate leukocytes. Thus, in our study, the enormous change in ArA content in gill polar lipids may have lead to a an overproduction of ROS by NADPH-oxidase (with or without the production of LTB_4) and/or an increase of lipid peroxide products as in vertebrates.

Conclusion

In conclusion, this study demonstrated that supplementation of ArA appeared to enhance hemocyte concentration, phagocytosis activity and basal ROS production, and to reduce susceptibility of hemocyte adhesive capacity to *Vibrio aestuarianus*. Based on established knowledge on the role of ArA and its metabolites on immune functions in vertebrates and insects, we propose that ArA and/or its metabolites may be also important in immune responses of oysters. However, additional studies are needed and the use of inhibitors of eicosanoid synthesis should help to establish the involvement of these ArA metabolites in oyster immune parameters. Finally, as the level of ArA reported in gill polar lipids at the end of the dietary conditioning attained the highest level ever reported in literature, we wondered if such level of ArA was not above physiologically-normal levels in oysters. New experiments with lower doses of ArA should be done, perhaps in combination with a supply of antioxidants such as vitamin E and /or C.

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Table 1: Condition index (n=15, Mean ± SD), oyster tissue dry weight in grams (n=15, Mean ± SD) and carbohydrate, lipid and protein contents expressed in mg per mg oyster dry weight (n=3 pools of 5 oysters, Mean ± SD) of oysters fed T-*Iso* supplemented with 0, 0.25 and 0.41 µg mL^{-1} of ArA. Asterisks indicate significant difference with initial composition (T-test, $p<0.05$).

	Initial	**After 4 weeks of conditioning**		
		0 µg ArA mL^{-1}	**0.25 µg ArA mL^{-1}**	**0.41 µg ArA mL^{-1}**
Condition index	4.3 ± 0.9	3.6 ± 0.7	3.9 ± 0.5	3.5 ± 0.6
Oyster dry weight	0.476 ± 0.17	0.341 ± 0.07*	0.341 ± 0.15*	0.302 ± 0.09*
Carbohydrate content	0.205 ± 0.02	0.160 ± 0.02*	0.155 ± 0.01*	0.151 ± 0.02*
Lipid content	0.087 ± 0.00	0.115 ± 0.01*	0.109 ± 0.01*	0.107 ± 0.00*
Protein content	0.359 ± 0.03	0.403 ± 0.01	0.380 ± 0.02	0.393 ± 0.02

Table 2: Fatty acid composition of the gill polar lipids, expressed as weight percentage of total fatty acids in the fraction, and total FAs, expressed as µg of FAs per mg of gill wet weight of *C. gigas* fed T-*Iso* supplemented 0, 0.25 and 0.41 µg mL^{-1} of ArA. Different lower-case letters indicate significant differences between dietary treatments (1-way ANOVA, $p<0.05$). Asterisks indicate significant difference with initial composition (T-test, $p<0.05$).

	initial	**After 2 weeks of conditioning**			**After 4 weeks of conditioning**		
		0 µg ArA mL^{-1}	**0.25 µg ArA mL^{-1}**	**0.41 µg ArA mL^{-1}**	**0 µg ArA mL^{-1}**	**0.25 µg ArA mL^{-1}**	**0.41 µg ArA mL^{-1}**
Fatty acids	(N=2)	(N=2)	(N=3)	(N=3)	(N=3)	(N=3)	(N=3)
14:0	0.9 ± 0.3	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.3	1.0 ± 0.2
16:0	10.5 ± 0.5	9.6 ± 0.2^{a}*	9.4 ± 0.3^{b}*	8.9 ± 0.2^{b}*	9.5 ± 0.6^{a}*	8.4 ± 0.2^{b}*	8.4 ± 0.2^{b}*
18:0	5.9 ± 0.0	5.0 ± 0.2^{a}*	4.4 ± 0.2^{b}*	4.8 ± 0.1^{a}*	4.8 ± 0.4*	4.9 ± 0.3*	4.9 ± 0.2*
16:1n-7	2.2 ± 0.3	2.0 ± 0.1^{a}	1.9 ± 0.0^{b}	1.7 ± 0.0^{c}*	1.8 ± 0.1^{a}	1.3 ± 0.3^{b}*	1.1 ± 0.1^{b}*
18:1n-9	1.1 ± 0.2	2.3 ± 0.0^{a}*	1.9 ± 0.2^{b}*	1.9 ± 0.1^{b}*	2.6 ± 0.1^{a}*	2.1 ± 0.2^{b}*	2.0 ± 0.2^{b}*
18:1n-7	4.1 ± 0.2	4.9 ± 0.1*	5.3 ± 0.3*	5.4 ± 0.2*	5.2 ± 0.2*	5.3 ± 0.7*	5.5 ± 0.1*
20:1n-11	3.1 ± 0.1	2.6 ± 0.4	2.2 ± 0.3*	2.1 ±0.1*	2.0 ± 0.3*	2.2 ± 0.5*	1.9 ± 0.2*
20:1n-7	6.4 ± 0.5	5.2 ± 0.1*	5.0 ± 0.2*	5.0 ± 0.2*	5.0 ± 0.1*	5.4 ± 0.4*	5.2 ± 0.1*
18:2n-6	1.0 ± 0.3	3.5 ± 0.1^{a}*	2.7 ± 0.2^{b}*	2.7 ± 0.1^{b}*	4.0 ± 0.4^{a}*	2.8 ± 0.1^{b}*	2.6 ± 0.1^{b}*
18:3n-3	0.4 ± 0.0	0.9 ± 0.0^{a}*	0.8 ± 0.1^{ab}*	0.7 ± 0.0^{b}*	1.1 ± 0.0^{a}*	0.7 ± 0.0^{b}*	0.6 ± 0.1^{b}*
18:4n-3	0.7 ± 0.0	1.3 ± 0.0^{a}*	1.0 ±0.0^{b}*	1.0 ± 0.0^{b}*	1.4 ± 0.0^{a}*	1.1 ± 0.1^{b}*	1.0 ± 0.1^{b}*
20:2i	0.6 ± 0.0	0.6 ± 0.0^{a}	0.4 ±0.1^{b}*	0.3 ± 0.0^{b}*	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}*	0.3 ± 0.0^{b}*
20:2j	0.6 ± 0.1	0.6 ± 0.0^{a}	0.6 ± 0.1^{a}	0.4 ± 0.1^{b}	0.6 ± 0.1^{a}	0.3 ± 0.1^{b}*	0.3 ± 0.1^{b}*
20:2n-6	0.3 ± 0.2	0.7 ± 0.0*	0.8 ± 0.1*	0.7 ± 0.1*	1.1 ± 0.2*	1.2 ± 0.1*	1.1 ± 0.1*
20:3n-6	0.1 ± 0.0	0.2 ±0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0*	0.3 ± 0.0*	0.3 ± 0.0*
20:4n-6	3.7 ± 0.1	3.7 ± 0.1^{a}	12.2 ± 1.4^{b}*	14.2 ± 0.3^{c}*	6.2 ± 1.5^{a}*	15.3 ± 1.0^{b}*	19.7 ± 0.5^{c}*
20:5n-3	17.1 ± 0.7	13.5 ± 0.1^{a}*	11.6 ± 0.4^{b}*	11.7 ± 0.2^{b}*	12.0 ± 1.4^{a}*	9.7 ± 0.9^{b}*	9.0 ± 0.6^{b}*
22:2i	1.7 ± 0.2	1.9 ± 0.0^{a}	1.6 ± 0.1^{b}	1.4 ± 0.0^{c}*	2.1 ± 0.4	1.7 ± 0.3	1.5 ± 0.1
22:2j	11.2 ± 0.4	8.9 ± 0.1^{a}*	7.3 ± 0.2^{b}*	7.6 ± 0.1^{c}*	7.4 ± 0.1*	7.1 ± 0.7*	6.5 ± 0.3*
22:5n-6	0.9 ± 0.2	0.8 ± 0.1^{a}	1.7 ± 0.0^{b}*	1.7 ± 0.1^{b}*	1.0 ± 0.2^{a}	2.1 ± 0.2^{b}*	2.7 ± 0.1^{c}*
22:5n-3	1.7 ± 0.2	1.4 ± 0.1	1.2 ± 0.1*	1.2 ± 0.1*	1.2 ± 0.1^{a}*	1.1 ± 0.1^{ab}*	1.0 ± 0.0^{b}*

22:6n-3	17.5 ± 0.5	21.2 ± 0.5^{a}*	18.4 ± 0.7^{b}	17.2 ± 0.4^{c}	21.3 ± 0.3^{a}*	17.2 ± 1.0^{b}	15.5 ± 0.8^{c}*
Total SAFA	19.8 ± 0.8	18.2 ± 0.4^{a}*	16.9 ± 0.4^{b}*	17.2 ± 0.2^{b}*	17.4 ± 1.3*	16.1 ± 0.7*	16.4 ± 0.6*
Total MUFA	19.7 ± 0.4	19.9 ± 0.1^{a}	19.0 ± 0.2^{b}	18.8 ± 0.3^{b}	19.6 ± 0.1^{a}	19.8 ± 0.5^{a}	18.7 ± 0.4^{b}
Total PUFA	60.5 ± 1.3	61.8 ± 0.4^{a}	63.7 ± 0.8^{b}*	64.2 ± 0.5^{b}*	62.9 ± 1.1*	64.0 ± 1.1*	64.8 ± 0.9*
Total n-6	7.1 ± 0.4	9.5 ± 0.2^{a}*	18.8 ± 1.4^{b}*	21.3 ± 1.0^{b}*	13.4 ± 2.3^{a}	23.0 ± 2.0^{b}*	27.1 ± 0.2^{c}*
Total n-3	38.9 ± 1.4	39.8 ± 0.2^{a}	34.4 ± 0.7^{b}*	33.0 ± 0.4^{c}*	38.6 ± 1.0^{a}	31.4 ± 2.0^{b}*	28.7 ± 1.2^{b}*
Total NMIs	14.1 ± 0.3	11.9 ± 0.1^{a}*	10.2 ± 0.1^{b}*	9.5 ± 0.3^{c}*	10.5 ± 0.1^{a}*	9.4 ± 1.0^{b}*	8.6 ± 0.3^{b}*
n-3/n-6	5.5 ± 0.5	4.2 ± 0.1^{a}*	1.8 ± 0.2^{b}*	1.6 ± 0.1^{b}*	2.9 ± 0.6^{a}*	1.4 ± 0.2^{b}	1.1 ± 0.0^{b}
20:5n-3/20:4n-6	4.6 ± 0.1	3.7 ± 0.1^{a}*	1.0 ± 0.1^{b}*	0.8 ± 0.0^{c}*	2.4 ± 0.8^{a}*	0.6 ± 0.1^{b}	0.5 ± 0.0^{c}
Total FAs (µg/g gill WW)	6.3 ± 0.3	6.3 ± 0.8	7.1 ± 0.4	7.6 ± 0.2	7.5 ± 0.2	7.2 ± 0.7	7.3 ± 0.5

Note: NMIs= Non Methylene Interrupted Fatty Acids; 20:2i=20:2Δ5,11 ; 22:2i=22:2Δ7,13 ; 20:2i=20:2Δ5,13; 22:2j=22:2Δ7,15 ; WW: wet weight

Table 3: 20:4n-6 content and main FAs classes of the gill polar lipids, expressed as weight percentage of total fatty acids of the fraction, and total FAs, expressed as µg of FAs per mg of gill wet weight of *C. gigas* fed different doses of ArA without algae. Different lower-case letters indicate significant differences between dietary treatments (1-way ANOVA, $p<0.05$). Asterisks indicate significant difference with initial composition (T-test, $p<0.05$).

	Initial	**After 2 weeks of conditioning**		
		0 µg ArA mL^{-1}	**0.25 µg ArA mL^{-1}**	**0.41 µg ArA mL^{-1}**
Fatty acids	(N=3)	(N=3)	(N=3)	(N=2)
20:4n-6	6.8 ± 0.3	6.7 ± 0.1^{a}	11.3 ± 0.8^{b}*	12.1 ± 1.2^{b}*
20:5n-3	11.2 ± 0.2	11.5 ± 0.2	10.9 ± 1.0	10.4 ± 0.5
22:6n-3	24.0 ± 0.4	22.2 ± 0.6^{a}*	19.9 ± 0.2^{b}*	19.9 ± 0.3^{b}*
Total SAFA	16.7 ± 0.5	17.1 ± 0.5	17.1 ± 0.2	17.5 ± 1.1
Total MUFA	21.9 ± 0.6	21.9 ± 0.1	20.9 ± 0.2*	20.5 ± 0.1*
Total PUFA	61.3 ± 0.2	61.0 ± 0.4	62.0 ± 0.0*	62.0 ± 1.2
Total n-6	9.8 ± 0.3	9.7 ± 0.0^{a}	14.5 ± 0.9^{b}*	15.5 ± 1.4^{b}*
Total n-3	38.9 ± 0.3	37.2 ± 0.4^{a}*	34.4 ± 0.9^{b}*	33.7 ± 0.3^{b}*
n-3/n-6	4.0 ± 0.1	3.8 ± 0.0^{a}	2.4 ± 0.2^{b}*	2.2 ± 0.2^{b}*
20:5n-3/20:4n-6	1.7 ± 0.1	1.7 ± 0.1^{a}	1.0 ± 0.2^{b}*	0.9 ± 0.1^{b}*
Total FAs (µg/g gill WW)	5.5 ± 0.1	4.8 ± 0.8^{a}	6.1 ± 0.5^{b}	6.0 ± 0.3^{b}

Note: WW=wet weight

Table 4: Total and differential hemocyte concentrations and percentage of viable cells in oysters fed T-*Iso* supplemented with 0, 0.25 and 0.41 µg mL^{-1} of ArA. (n=3, Mean ± SD). Different lower-case letters indicate significant difference between dietary treatments (1-way ANOVA, $p<0.05$). Asterisks indicate significant difference with initial composition (T-test, $p<0.05$).

	Initial	**After 2 weeks of conditioning**			**After 4 weeks of conditioning**		
		0 µg ArA mL^{-1}	**0.25 µg ArA mL^{-1}**	**0.41 µg ArA mL^{-1}**	**0 µg ArA mL^{-1}**	**0.25 µg ArA mL^{-1}**	**0.41 µg ArA mL^{-1}**
Total hemocyte counts : ($X10^5$ cell mL^{-1})	4.3 ± 0.6	4.3 ± 0.7	5.7 ± 1.1	6.1 ± 0.6 *	4.4 ± 1.5	5.8 ± 1.5	6.0 ± 1.1
Granulocytes	0.3 ± 0.1	0.5 ± 0.2	0.7 ± 0.2*	1.3 ± 0.0*	0.6 ± 0.2	1.0 ± 0.3*	1.1 ± 0.2*
Hyalinocytes	2.2 ± 0.3	2.6 ± 0.4	3.8 ± 0.8*	3.6 ± 0.8*	3.1 ± 1.1	3.7 ± 0.8*	3.9 ± 1.2*
Agranulocytes	1.7 ± 0.4	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.2*	0.7 ± 0.1*	1.1 ± 0.7	1.0 ± 0.2*
Viability percentage (%)	91.6 ± 1.5	90.9 ± 2.0	90.1 ± 1.7	91.7 ± 1.3	88.7 ± 2.3	91.2 ± 1.3	89.9 ± 2.5

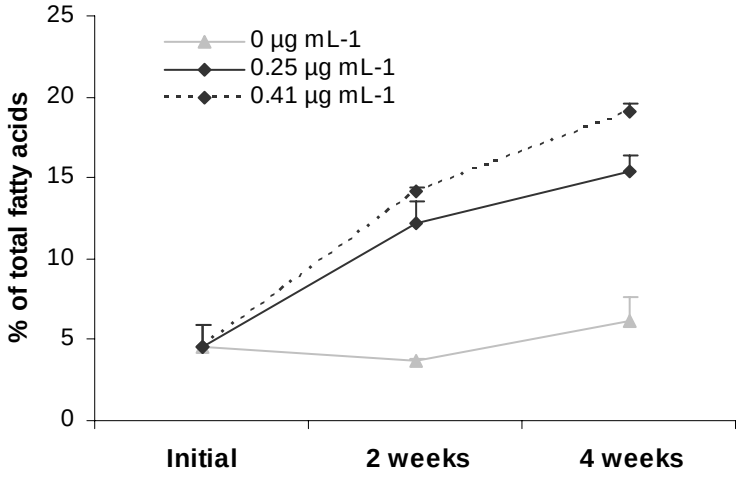


Figure 1: ArA weight percentage of *C. gigas* gill polar lipids during the dietary conditioning (T-*Iso* supplemented with 0, 0.25 µg mL^{-1} and 0.41 µg mL^{-1} of ArA).

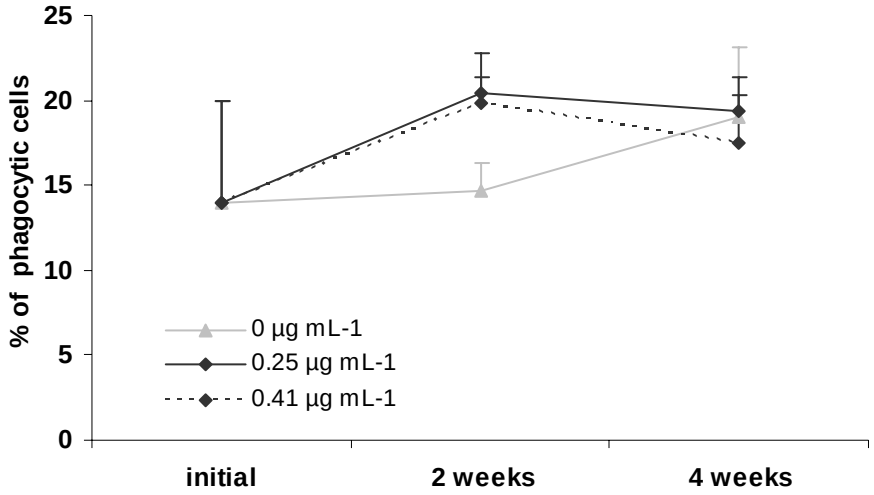


Figure 2: Percentage of phagocytic hemocytes of *C. gigas* fed T-*Iso* supplemented with 0, 0.25 and 0.41 µg mL^{-1} of ArA.
Figure 2

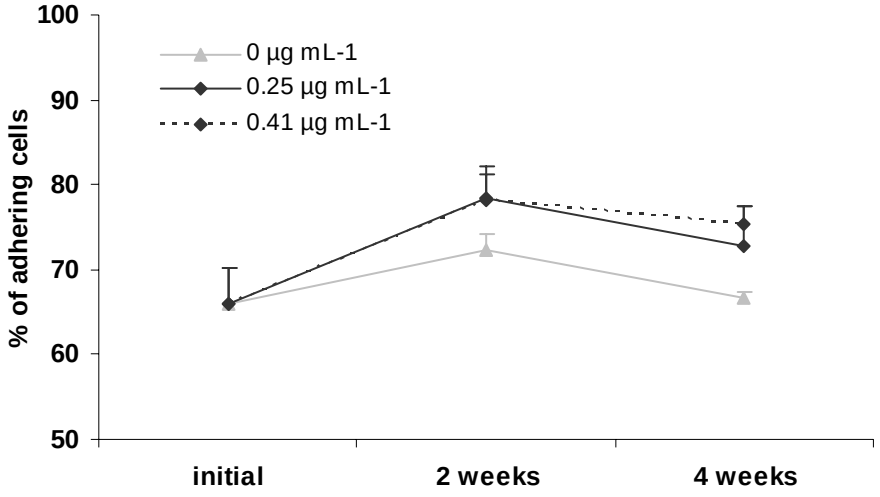
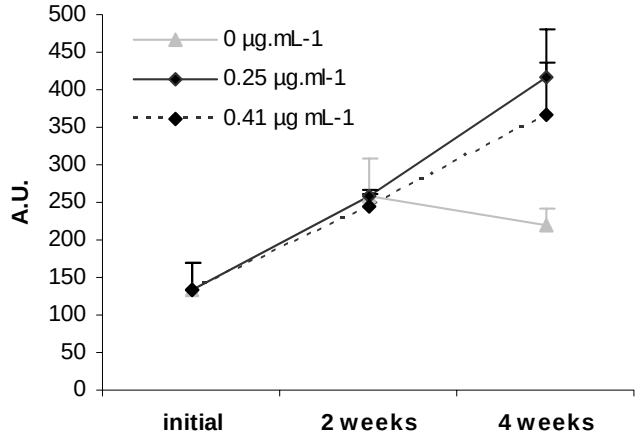


Figure 3: Percentage of adhering cells after three hours of incubation with 30 µg mL^{-1} of *Vibrio aestuarianus* extra cellular products (ECP) prepared in sea water.

A



B

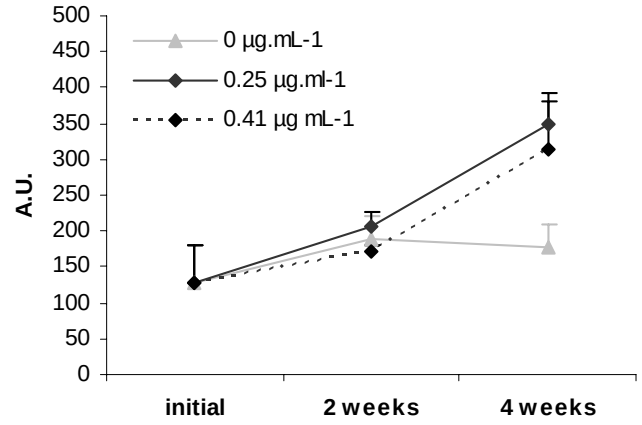


Figure 4 : Reactive oxygen species production of granulocytes (A) and hyalinocytes (B) of oysters fed T-*Iso* supplemented with 0, 0.25 and 0.41 µg mL^{-1} of ArA. Hemocytes were incubated 2 hours with DCFH-DA in sterile sea water. Results are expressed in Arbitrary Units (A.U.).