The diatom-derived aldehyde decadienal affects life cycle transition in the ascidian *Ciona intestinalis* through nitric oxide/ERK signalling

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1. Summary

Polyunsaturated aldehydes (PUAs) are fatty-acid-derived metabolites produced by some microalgae, including different diatom species. PUAs are mainly produced as a wound-activated defence mechanism against microalgal predators or released from senescent cells at the end of a bloom. PUAs, including 2,4-*trans*-decadienal (DD), induce deleterious effects on embryonic and larval development of several planktonic and benthic organisms. Here, we report on the effects of DD on larval development and metamorphosis of the ascidian *Ciona intestinalis*. *Ciona* larval development is regulated by the cross-talking of different molecular events, including nitric oxide (NO) production, ERK activation and caspase 3-dependent apoptosis. We report that treatment with DD at the competence larval stage results in a delay in metamorphosis. DD affects redox balance by reducing total glutathione and NO levels. By biochemical and quantitative gene expression analysis, we identify the NO-signalling network affected by DD, including the upregulation of ERK phosphatase *mkp1* and consequent reduction of ERK phosphorylation, with final changes in the expression of downstream ERK target genes. Overall, these results give new insights into the molecular pathways induced in marine organisms after exposure to PUAs during larval development, demonstrating that this aldehyde affects key checkpoints of larval transition from the vegetative to the reproductive life stage.

2. Introduction

Diatoms are photosynthetic unicellular microalgae contributing up to 45% of total oceanic primary production and playing key roles in the transfer of energy through marine food chains [1]. The reason for their ecological success still remains a matter of discussion, and different mechanisms have been proposed to explain their widespread occurrence in the oceans. One main reason may be because they have evolved a chemical defence system which can potentially affect both predators and competitors. Indeed, different diatom species are able to produce a wide range of secondary metabolites belonging to the oxylipin family including polyunsaturated aldehydes (PUAs) with toxic effects on reproductive processes in grazing organisms such as crustacean copepods [2–4], echinoderms [5,6] and filter-feeding organisms such as ascidians [7]. These metabolites are the end-products of a lipoxygenase/hydroperoxide lyase metabolic pathway, initiated by damage of algal cells as would occur through grazing [8]. Briefly, cell damage activates lipase enzymes, causing the release of polyunsaturated fatty acids from cell membranes, which are then immediately oxidized and cleaved to form PUAs, including 2,4-*trans*-decadienal (DD). This aldehyde, in particular, is produced by some diatoms (e.g. *Thalassiosira rotula*,
3. Material and methods

3.1. Chemicals

All reagents were purchased from Sigma-Aldrich, Milan, Italy, unless otherwise stated.

3.2. Ciona intestinalis incubation experiments

Adult C. intestinalis were collected at Fusaro Lake in the district of Naples (40°49’10.6” north latitude, 14°0.3’ 32” east longitude). No specific permissions were required for this location that is not privately owned or protected in any way. The study did not involve endangered or protected species, and was carried out in strict accordance with European (Directive 2010/63) and Italian (Decreto Legislativo no. 116/1992) legislation for the care and use of animals for scientific purposes. Animals were transported to the laboratory and maintained at 18°C in tanks with circulating seawater and under constant light to allow gamete accumulation. Animal handling and fertilization were carried out as previously described [29,30]. In brief, eggs from single animals were fertilized with a mixture of sperm obtained from different individuals. Embryos were cultured at 18°C in 0.2 μm filtered seawater. Hatched larvae were obtained at 18–20 hpf at 18°C. Development was followed on live specimens with a Stemi 2000 (ZEISS) stereomicroscope. Samples at appropriate stages were identified using the morphological criteria previously reported by Chiba et al. [31] and were selected on the basis of at least 95% homogeneity. Larvae (about 100 larvae m⁻¹) were treated in tissue culture dishes in 50 ml of seawater at 18°C with DD at final concentrations ranging from 0.125 (0.8 μM) to 1.35 μg ml⁻¹ (8.9 μM) or with MKP inhibitor dusp1/6 I (Calbiochem, Merck Millipore, Milan, Italy) at 0.25 μM. Treated larvae were allowed to develop to the desired stage and were collected by low-speed centrifugation (500g) after washing in PBS buffer. Pellets were stored at ~80°C until use and subsequently used for protein extraction, NO detection and RNA preparation.

3.3. Protein extraction

Larval pellets were homogenized in two volumes of RIPA lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.6, 5 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1 mM PMSF and Complete Protease Inhibitor Cocktail Tablets, Roche, Monza, Italy) and phosphatase inhibitors (PhosSTOP Cocktail Tablets, Roche). After centrifugation, protein concentration was determined using a Bio-Rad protein assay reagent (Bio-Rad, Milan, Italy) with bovine serum albumin as a standard.
**3.4. Fluorimetric determination of nitric oxide concentration**

Larval pellets (50–60 mg) were homogenized in 0.5 ml of PBS and sonicated three times for 1 min at 30% amplitude. The samples were centrifuged at 13500g for 5 min at 4°C, and the supernatants were collected for NO analysis using 2,3-diaminonaphthalene (DAN) to form the fluorescent product 1-(H)-naphthotriazole. Briefly, 80 μl of samples were incubated at 26°C with 0.06 U ml⁻¹ nitrate reductase, 2.5 μM FAD and 100 μM NADPH for 1 h. Then, 10 μl of DAN (0.05 mg ml⁻¹ in 0.62 M HCl) was added, and the sample was incubated for 20 min in the dark. After stabilization with 1N sodium hydroxide, formation of 1-(H)-naphthotriazole was measured using a spectrofluorimeter (Shimadzu RF-5301 PC) with excitation and emission at 365 and 425 nm, respectively. Calibration curves were measured daily with sodium nitrite (1–10 μM) in PBS. The experiments were carried out on three biological replicates.

**3.5. Glutathione assay**

Total GSH was determined by using the GSH assay kit (Sigma). Briefly, larval pellets (50 mg) were homogenized in 1 volume of PBS. An aliquot was used for protein determination, and another aliquot was added to 3 volumes of 5% 5-sulfosalicylic acid and mixed. Samples were then frozen (−80°C) and thawed at 37°C twice, left for 5 min at 4°C and finally centrifuged at 10 000g for 10 min. The supernatant was used for glutathione determination by performing a kinetic assay in which catalytic amounts (nmoles) of GSH caused a continuous reduction of 5,5′-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid measured spectrophotometrically at 405 nm with a Thermo Scientific™ Multiskan™ FC Microplate Photometer.

**3.6. Immunoblot analysis**

Larval lysates were prepared as previously described [29] and examined using 10% SDS–PAGE. The gel transferred to the nitrocellulose membrane was analysed with antibodies against p44/42 MAP kinase (ERK 1/2; ERK; 1 : 1000; Cell Signaling, EuroClone, Milan, Italy) and Phospho-p44/42 MAPK (P-ERK; 1 : 500; Cell Signaling). After washing in PBS with 0.1% Tween, labelled proteins were detected by ECL PLUS (GE Healthcare, EuroClone, Milan, Italy).

**3.7. Bioinformatic analysis**

*Ciona* transcripts were retrieved from Aniseed (http://www.aniseed.cnrs.fr/). The identification of transcripts was performed using the TBLASTX program.

**3.8. RNA extraction and cDNA synthesis**

Total RNA was extracted at different developmental stages using TRIZOL (Life Technologies, Milan, Italy) according to the manufacturer’s instructions. Briefly, extraction with chloroform/isooamyl alcohol (24 : 1) was performed following RNA precipitation by addition of glycogen and isopropyl alcohol. Contaminating DNA was degraded by treating each sample with DNase (Roche) and then removing the enzyme with RNeasy MinElute™ Cleanup Kit (Qiagen, Milan, Italy). The amount of total RNA was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, by Nanodrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies). The integrity of RNA was checked in agarose gel electrophoresis by visualizing intact rRNA subunits (28S and 18S). For each sample, 1 μg of total RNA extracted was retrotranscribed with iScript™ cDNA synthesis kit (Bio-Rad), following the manufacturer’s instructions. cDNA was diluted 1 : 10 with H₂O prior to use in real-time qPCR experiments.

**3.9. Gene expression by real-time qPCR**

For real-time qPCR experiments, the data from each cDNA sample were normalized using ribosomal protein R27a as a reference gene, the levels of which remained relatively constant in all the developmental stages examined and during treatment with DD. For each target gene, specific primers were designed on the basis of nucleotide sequences with the help of PRIMER3WEB v.4.0.0 (table 1). For some genes, we used previously reported primers [28]. The amplified fragments using Taq high fidelity PCR system (Roche) were purified from agarose gel using QIAquick gel extraction kit (Qiagen) and specificity of PCR products was checked by DNA sequencing (Molecular Biology Service, SZN, Naples). Specificity of amplification reactions was verified by melting curve analysis. The efficiency of each primer pair was calculated according to standard method curves using the equation $E = 10^{-1/\text{slope}}$. Five serial dilutions were set up to determine $C_t$ values and reaction efficiencies for all primer pairs. Standard curves were generated for each oligonucleotide pair using the $C_t$ values versus the logarithm of each dilution factor. PCR efficiencies were calculated for control and target genes and were found to be 2 in most cases, with the exception of *gclm* and *glrx* with values of 1.92 and 1.97, respectively. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.3 μM for each primer and 16 FastStart SYBR Green master mix (total volume of 10 μl). PCR amplifications were performed in a ViiaTM 7 real-time PCR system (Applied Biosystems) thermal cycler using the following thermal profile: 95°C for 10 min, one cycle for cDNA denaturation; 95°C for 15 s and 60°C for 1 min, 40 cycles for amplification; 72°C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60°C to 95°C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all real-time qPCR reactions were carried out in triplicate. Fluorescence was measured using the ViiaTM 7 software (Applied Biosystems). The expression of each gene was analysed and internally normalized against R27a using relative expression software tool (REST) software based on the Pfaffl method [34,35]. Relative expression ratios above twofold were considered significant. Experiments were repeated at least three times.

**3.10. Statistical analysis**

Statistical analysis was performed using GraphPad PRISM v. 4.00 for Windows (GraphPad Software, San Diego, CA). The results were reported as means ± s.e.m. and analysed by unpaired *t*-test for comparison between the groups; $p < 0.05$ was considered statistically significant. For real-time
qPCR analysis, significance was tested using the ‘pairwise fixed reallocation randomization test’, developed by REST software [35]. The number of experiments is reported in the figure legends.

4. Results

4.1. Decadienal affects metamorphosis of Ciona intestinalis

To investigate the effect of DD on C. intestinalis larval settlement and subsequent metamorphosis, larvae were treated at different times from hatching, corresponding to early, middle and late larval stages. Larvae were morphologically analysed after 24 h treatment. DD did not significantly affect the rate of metamorphosis when added at the early stage, whereas middle and late larvae treated with 0.5 μM DD encountered a significant delay in metamorphosis with respect to controls in which the majority of larvae had completely metamorphosed into juveniles. Indeed, one day after exposure, late larvae treated with DD did not completely metamorphose, as shown by an increase in the percentage of late larvae or larvae during tail regression to 72% with respect to 20% in controls and a concomitant decrease of juveniles to 22% with respect to 78% in controls (figure 1a).

Table 1. Features of transcripts used for gene expression analysis: gene acronyms, accession numbers, primer sequences and information on gene regulation in Ciona.

| gene acronym | accession number | gene name | gene regulation | primer sequences (5’–3’) |
|--------------|------------------|-----------|----------------|------------------------|
| R27a         | KYOTOGRAIL2005.209.6.1 | ribosomal protein | reference | For-ATCCACCCCTACCTTGTG Rev-GGAGATCTCTGGCACGTTTCA |
| dhg          | ci0100130315      | dehydrogenase with different specificity | ERK-regulated [28] | For-GACGATCTCTTGTACGACG Rev-GCCCGGTGATATGTTGTTTCA |
| ets          | ci0100152617      | Ets-related transcription factor | ERK-regulated [28] | For-GACGATCTCTTGTACGACG Rev-GCCCGGTGATATGTTGTTTCA |
| gcit         | XM_002128788.2    | glutamate–cysteine ligase regulatory subunit | stress-regulated [32] | For-AAGCTAGGGGAGAGATGAGT Rev-TTCGAGGGTGTATATGCGGTC |
| glrx         | ENSCING00000014681 | glutaredoxin |                | For-GCTTACCCAGCTATCGCAAGA Rev-AGCCACTCCAATGCTTACC |
| ggt          | ci0100152935      | gamma-glutamyl transpeptidase |                | For-GCCGAGAAGAGAGTCTGTCG Rev-GGGGCTCTGTTGGAACAGC |
| gis          | ENSCING00000009385 | glutathione synthase | stress-regulated [32] | For-TCCGCACTTCAAACACCA Rev-TGGTCTACCAACTTCCTG |
| gtm          | ENSCING00000015785 | glutathione transferase |                | For-GGCAAGAAGAGAGTCTGTCG Rev-GGGGCTCTGTTGGAACAGC |
| mkp1         | ci0100138796      | dual-specific MAPK phosphatase 1 | NO-regulated [30] | For-CACTTTACGAGGACTCG CATT Rev-CCTCAGGTTGCGAGGACTCG |
| mkp3         | ci0100140262      | dual-specific MAPK phosphatase 3 | NO-regulated [30] | For-CACTTTACGAGGACTCG CATT Rev-CCTCAGGTTGCGAGGACTCG |
| MnSOD         | ci0100142427      | manganese superoxide dismutase |                | For-GGCAAGAAGAGAGTCTGTCG Rev-GGGGCTCTGTTGGAACAGC |
| mx           | ci0100149359      | matrix metalloprotease | ERK-regulated [28] | For-GATTCCGAGCTATGATC Rev-CGTTCTCTGCTGATATGTTG |
| NOS          | ci0100153759      | nitric oxide synthase |                | For-AGAGTAGAAAAGCTGCGCAATA Rev-AACCAATGGGATGTTGTTAG |
| sushi        | ci0100139289      | cell adhesion molecule | JNK-regulated [28] | For-CAACGATGGGAAGGAAATGAGGTC Rev-GGCAAGAAGAGAGTCTGTCG |

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had completely blocked metamorphosis, with 97% of late larvae and only 3% of juveniles with respect to the control. At higher concentrations (1.35 µg ml⁻¹), the effect was toxic for larvae, with DD hampering larval development and tail retraction (figure 1d).

4.2. Decadienal induces changes in nitric oxide levels and redox homeostasis

We have previously demonstrated that the endogenous modulation of NO levels in *Ciona* larvae affects the rate of metamorphosis [29]. In order to understand if NO mediates the response of the larvae to the toxin, we measured NO levels in *Ciona* larvae treated with the sublethal concentration of 0.5 µg ml⁻¹ DD. NO levels significantly decreased after different times of DD treatment (figure 2). To understand if the change in NO levels was related to the fine regulation of redox homeostasis in response to DD, we first analysed the expression patterns of some genes involved in redox homeostasis. These genes were identified by *in silico* analysis of the *Ciona* genome and are reported in table 1. These genes include those coding for manganese superoxide dismutase (*Mn-SOD*), a key enzyme involved in the scavenging of reactive oxygen species [36,37], NO synthase (NOS), responsible for NO synthesis [26,38], two enzymes responsible for GSH synthesis, the glutamate–cysteine ligase regulatory subunit (*gclm*) and GSH synthase (*gss*) [32,33], glutaredoxin-1 (*glrx*) involved in GSH reduction [32,33], gamma-glutamyl transpeptidase (*ggt*) involved in GSH metabolism and recycling, and GSH S-transferase (*gstm*), which catalyses the conjugation of GSH to xenobiotic substrates for detoxification [39,40]. The relative expression of all these genes was followed by real-time qPCR experiments, after treating middle larvae (20–21 hpf) with 0.5 µg ml⁻¹ DD (figure 3) and collecting them after 2, 4 and 6 h of treatment. The expression of *gclm* and *ggt* progressively increased at all hours tested, whereas the expression of the other genes, including NOS, was unaffected. These results prompted us to measure total GSH levels in larvae treated with DD. A significant reduction...
control
unpaired
t
dent experiments. Data are expressed as means + processed with the GSH assay kit. Results are representative of three indepen-
times of larval development. Histograms show the differences in expression levels of analysed genes, according to real-time qPCR. Middle
larvae incubated with 0.5 µg ml−1 DD were harvested after 2, 4 and 6 h. Data are reported as fold differences compared to the control (larvae in sea-
water without DD; mean ± s.d.). Fold differences equal to or greater than ±2 were considered significant.
in total GSH levels was observed after 2 h treatment (figure 4), whereas thereafter, GSH levels were comparable to the controls.

4.3. Decadienal affects ERK signalling

ERK activation represents a key event in Ciona life cycle transition. Its inhibition hampers the wave of apoptosis and subsequent metamorphosis of the ascidian [27,28]. We therefore decided to investigate the effect of DD on ERK phosphorylation. DD induced a significant decrease in P-ERK as early as 2 h post-treatment (figure 5a). The densitometric analysis of the P-ERK immunopositive bands with respect to the constitutive ERK band revealed a significant reduction of ERK phosphorylation at 2, 4 and 8 h after treatment (figure 5b).

To investigate if DD also affected gene transcription occurring downstream to ERK activation, we selected a series of ERK target genes mostly expressed in territories specialized for metamorphosis, such as palps and papillae, where NOS expression, NO production and ERK activation occur [24,25]. These genes include dehydrogenase with different specificities (dhg), ets-related transcription factor (ets) and matrix metalloprotease-24-precursor (mx). We also selected the specific map kinase phosphatases mkp1 and mkp3, which we previously demonstrated to be regulated by NO during Ciona larval development [30]. Finally, we used sushi, a JNK target, the expression of which is necessary to induce apoptosis of tail cells at the onset of metamorphosis in Ciona [28], as an unrelated gene (table 1).

The relative expression of these genes was followed by real-time qPCR experiments, after treating middle larvae with 0.5 µg ml−1 DD (figure 6). Larvae were collected at 2, 4 and 6 h of treatment. The expression of dhg started to progressively increase after 2 h and until 6 h of treatment, whereas ets and mx were downregulated after 2 h and 4–6 h treatment,
respectively. mkp1 was found to be upregulated after 2 h treatment, whereas the expression of the other genes, mkp3 and sushi, was unaffected.

To confirm the role of mkp1 in the regulation of ERK activity during larval development, we treated hatched larvae with 0.25 μM of the dual-specific phosphatase inhibitor (dusp 1/6 I). After 4 h of treatment, the inhibitor caused an increase in larval settlement, as assessed by the significant increase of larvae attached to the substrate and the concomitant decrease of swimming larvae with respect to the control (figure 7a). The specificity of the inhibitor for ERK activity was confirmed by the increase of ERK phosphorylation (figure 7b).

5. Discussion

Metamorphosis of marine organisms represents an evolutionarily advantageous process that allows life cycle transition from the vegetative to reproductive stage when environmental conditions are favourable for offspring survival. Briefly, the vegetative stage consists of swimming larvae that acquire the competence to receive specific environmental cues to induce the settlement and the initiation of metamorphosis into the reproductive adult. The binding of environmental cues to cell surface receptors of larvae activates molecular pathways that finally lead to an extensive transformation of the body plan through apoptotic processes. NO plays a key role in these transformations, by regulating the timing of life cycle transitions in several marine organisms, including ascidians [26,29,30,41–48]. During C. intestinalis larval development, NO is known to affect different molecular targets. Briefly, NO affects caspase-3 activity [26], induces ERK and P-ERK nitration [29], and decreases MAPK phosphatase levels with consequent ERK activation [30].

In this paper, we report that Ciona larvae respond to the diatom-derived toxic aldehyde 2,4-trans-decadienal (DD) when they become competent to sense environmental stimuli and can choose a favourable substrate to attach to and initiate metamorphosis. Larvae may come into contact with DD after the sinking and sedimentation of diatom blooms when concentrations of this aldehyde can be high enough to affect the initial stages of metamorphosis [10]. This is of considerable ecological relevance considering the importance of diatom blooms in nutrient-rich aquatic environments.

This study provides first insights into the molecular mechanisms that mediate the toxic effects of DD on Ciona larval development and initial stages of metamorphosis, by highlighting the involvement of the NO/ERK pathway.

First of all, we report that DD induces a redox unbalance in Ciona larvae, owing to changes in GSH homeostasis. GSH is the most abundant antioxidant molecule in cells, playing a key role in cellular defence mechanisms, owing to its ability to reduce oxidized compounds [49]. Therefore, GSH contributes to maintain integrity of cell membranes and organelles, and a correct rate of metabolism and growth. The early reduction of total GSH levels after DD treatment can be explained considering that DD is a hydroxylalkenal compound that can reduce intracellular GSH content, as already reported in human erythroleukaemia and in human lung carcinoma [50,51]. DD can rapidly react with thiol compounds such as GSH to form conjugated complexes with consequent inhibition of some enzymatic activities and final induction of cytotoxicity. In our system, GSH depletion could be responsible for a transient loss of cell membrane integrity leading to calcium unbalance, with consequent NOS inhibition and reduction of endogenous NO levels by treating larvae with NOS inhibitors or NO scavengers [29,30]. Indeed, also in this case, DD causes a reduction in NO levels, leading to
the upregulation of \textit{mkp1}, the gene coding for the specific MAPK phosphatase responsible for ERK dephosphorylation in \textit{Ciona} [30]. The fact that inhibition of this phosphatase induces ERK activation and larval settlement highlights the key role of ERK signalling in deciding the time of substrate attachment. The reduction in ERK phosphorylation upon DD exposure could represent a defence strategy to avoid the attachment of larvae to unfavourable substrates such as those closest to toxic diatom blooms.

The DD-induced decrease in ERK phosphorylation also affected the expression of ERK target genes, including the metabolic enzyme dehydrogenase \textit{dhg} and the developmental genes \textit{ets} and \textit{mx}. The progressive upregulation of \textit{dhg} may be also due to the activation of a detoxification mechanism by larvae against this aldehyde, as already occurs with genes involved in GSH homeostasis. On the other hand, the downregulation of \textit{ets} and \textit{mx} could explain the weaker attachment of papillae to the substrate and the delay in tail retraction in most of the larvae treated with DD. Indeed, \textit{ets} encodes a transcription factor that regulates the expression of genes involved in several developmental processes [53]. On the other hand, \textit{Mx} encodes a metalloprotease involved in the composition and remodelling of the extracellular matrix during embryonic development [54,55]. Its regulation has been shown to affect apoptosis during amphibian metamorphosis [56]. Moreover, our finding that DD regulates \textit{ets} expression levels via NO-mediated ERK activation is in agreement with a previous report in breast cancer [57].

The above results can be schematically summarized in a model in which NO and ERK signalling cross-talk together in response to DD exposure (figure 8). DD induces the reduction of endogenous NO levels likely owing to GSH depletion. A reduction in NO levels induces the early upregulation of \textit{mkp1} with consequent ERK inactivation and changes in downstream transcription of the metabolic enzyme dehydrogenase \textit{dhg} and the key developmental genes \textit{ets} and \textit{mx}, thereby causing a delay in metamorphosis.

6. Conclusion

\textit{Ciona} is a broadcast spawning marine invertebrate which usually invests in a single annual spawning event in early spring [58] and should synchronize reproduction with specific environmental conditions to enhance the probability of survival. Diatoms also have an annual cycle, with blooms prevalent during the spring and early summer. Therefore, if \textit{Ciona} gamete release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate. On the other hand, \textit{Ciona} could time egg release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate. On the other hand, \textit{Ciona} could time egg release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate. On the other hand, \textit{Ciona} could time egg release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate. On the other hand, \textit{Ciona} could time egg release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate. On the other hand, \textit{Ciona} could time egg release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate. On the other hand, \textit{Ciona} could time egg release coincides with a heavily grazed diatom bloom, PUAs may limit larval development and consequent metamorphosis, thereby affecting recruitment rates of future generations of this important benthic invertebrate.

Future studies will be devoted to validating the results obtained with the pure molecule, exposing mature \textit{Ciona} adults to natural decadienal-producing diatoms.

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