

First evidence of sperm motility inhibition by the diatom aldehyde 2*E*,4*E*-decadienal

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ABSTRACT: Diatoms synthesise bioactive unsaturated short-chain aldehydes (SCAs) in response to wound-activation. The defensive SCAs are toxic to developmental stages of a range of invertebrate species including copepods, sea urchins, polychaetes and ascidians. Embryotoxicity is dose-dependent, with inhibition of cleavage, a reduction in hatching success, and teratogenic effects in neonates symptomatic. Bioactivity is directed at cellular and molecular targets. Diatom extracts and the SCA 2*E*,4*E*-decadienal (DD) affect microtubule and microfilament stability. Bioactivity is not restricted to embryogenesis, but has also been demonstrated to affect fertilization success in broadcast-spawning macroinvertebrates. This study has sought to further identify the responses of marine invertebrate gametes and the process of fertilization to DD exposure with particular reference to sperm motility. Incubation of gametes with the saturated aldehydes decanal and undecanal and the fatty acid 5,8,11,14,17-eicosapentaenoic acid (EPA) did not affect fertilization success. Exposure of gametes to DD inhibits fertilization success in a dose-dependent manner. Fertilization rate declined to 50% of control values at concentrations of 1.55 $\mu\text{g ml}^{-1}$ for *Arenicola marina*, 3.98 $\mu\text{g ml}^{-1}$ for *Nereis virens*, 7.94 $\mu\text{g ml}^{-1}$ for *Psammechinus miliaris* and 10 $\mu\text{g ml}^{-1}$ for *Asterias rubens*. Pre-incubation of oocytes in DD affected fertilization success to a limited degree; however pre-incubation of sperm in DD caused a pronounced dose- and time-dependent decline. DD exposure inhibits sperm migration rates and flagellar beating at concentrations as low as 0.05 $\mu\text{g ml}^{-1}$. The sperm remained alive, as evidenced by oscillation of the sperm head; however, flagellar beating was inhibited. The negative effect of DD exposure was further enhanced as sperm density was reduced. Analysis by general linear model revealed a significant interaction between DD concentration, period of sperm exposure and fertilizing sperm density ($F = 6.7$, $p = 0.01$). We suggest that diatom-derived SCAs can significantly impact fertilization success in broadcast-spawning invertebrates. The identification of sperm motility inhibition as a symptom of DD exposure offers a potentially useful cellular model to further study the effects of novel diatom-derived bioactive molecules.

KEY WORDS: Sperm motility · Diatom toxin · Aldehyde · Decadienal · Fertilisation · Sperm

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INTRODUCTION

The fundamental importance of diatom primary productivity in supporting higher trophic level production has been a principal tenet of marine ecology (Legendre 1990, Mann 1993). However, our understanding of the role of diatoms in facilitating ecosystem functioning, specifically the reproductive fitness and recruitment success of their principal grazers, is rapidly changing. Laboratory and field studies have shown that diatom feeding can initiate developmental arrest in copepod

embryos and induce significant anatomical deformities in hatched larvae, with inevitable survival and fitness consequences (Poulet et al. 1994, Uye 1996, Miralto et al. 1999, Carotenuto et al. 2002). In contrast, dinoflagellate diets that were previously regarded as nutritionally insufficient to support substantial larval recruitment can promote high levels of secondary production (Chaudron et al. 1996, Runge & de Lafontaine 1996, Miralto et al. 1999). The bioactive compounds responsible are derived from diatoms and are formed as part of an oxylipin chemical defence system and have been

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identified as a family of unsaturated short chain aldehydes (SCAs) including 2,4-decadienal (DD) (Miralto et al. 1999, d'Ippolito et al. 2002a,b, 2003, Pohnert et al. 2002). Aldehyde production is a wound-activated process under enzymatic control. A phospholipase, A_2 , liberates free mono- and polyunsaturated fatty acids including 5,8,11,14,17-eicosapentaenoic acid (EPA) and arachidonic acid immediately following cell damage, which are converted by lipoxygenases to lipid hydroperoxides. A hydroperoxide lyase then transforms these to defensive aldehydes (Pohnert 2000, 2002, Pohnert & Boland 2002). Formation is rapid, requiring less than 3 min for high localised concentrations in excess of $1.5 \mu\text{g ml}^{-1}$ to be achieved (Pohnert 2000).

The inhibitory activities of SCAs to invertebrates have been demonstrated in copepod, polychaete, echinoderm and ascidian embryos (Caldwell et al. 2002, d'Ippolito et al. 2002b, Tosti et al. 2003). Effects include the arrest of embryogenesis, induction of teratogenic effects in larvae and inhibition of fertilisation success. Anti-mitotic activity attributable to diatom extracts and SCAs has also been demonstrated in human carcinoma cell lines (Nappep et al. 1996, Bergé et al. 1997, Miralto et al. 1999). DD has also been demonstrated to possess allelochemical and bactericidal properties (Bisignano et al. 2001, Casotti et al. 2001).

In contrast to the growing body of evidence describing the detrimental effects of diatom-derived SCAs on embryogenesis, there is a paucity of information regarding the effects of SCAs on fertilisation biology. The process of fertilisation is highly sensitive to interference from xenobiotics, of both natural and anthropogenic origin (Johnson & Epel 1983, Pesando et al. 1991). Fertilisation inhibition due to algal metabolites has been described previously (Wicklund 1954, Granmo et al. 1988, Pesando et al. 1991). Caldwell et al. (2002) have demonstrated that both crude diatom extracts and DD can, in a dose-dependent manner, inhibit secondary fertilisation processes in marine invertebrates. Also, Buttino et al. (1999) have observed that sea urchin embryos incubated with extracts of *Thalassiosira rotula* shortly after sperm/egg binding were unable to complete pronuclear fusion due to depolymerisation of microtubule assemblies. Recently, it has been demonstrated that DD and decatrienal inhibit actin reorganisation, the fertilisation current and voltage-gated Ca^{2+} activity of the plasma membrane in the ascidian *Ciona intestinalis* (Tosti et al. 2003). Fertilisation is a cytoskeletal-dependent process. Both microtubule and microfilament components are pivotal for the completion of a number of salient stages during fertilisation, including sperm/egg binding, sperm aster formation, pronuclear migration and fusion, and cytoplasmic reorganisation. As such, inter-

ference by SCAs with cytoskeletal stability and functioning during fertilisation will significantly affect fertilisation success.

The precise nature of SCA-induced fertilisation inhibition needs to be examined. To better understand the relationship between SCA toxicity and fertilisation inhibition, it is necessary to determine the effects of diatom aldehydes on germ-cell functionality. Fertilisation requires the participation of both male and female gametes. This study focuses specifically on the inhibitory effect of DD on sperm motility. In particular, using the broadcast-spawning echinoderms *Asterias rubens* and *Psammechinus miliaris* and the polychaetes *Arenicola marina* and *Nereis virens* as model species, we aim to address the question of whether DD can limit fertilisation success by affecting sperm motility.

MATERIALS AND METHODS

Animal collection and maintenance. Sexually mature *Asterias rubens* were collected from the coast of Northumberland, NE England, during May and June 2001 at low water of spring tides. Individuals were sexed by gonadal biopsy. The sexes were maintained separately in aerated plastic tanks containing seawater at 5°C and held in constant darkness.

The Scottish Association of Marine Science, Oban, UK, supplied sexually mature *Psammechinus miliaris* during August 2001. The echinoderms were held at 10°C and a photoperiod of 12L:12D in a seawater recirculation system.

Sexually mature *Arenicola marina* were collected by digging from Hauxley Nature Reserve, Northumberland, in October and November 2001 during low water of spring tides. Individuals were sexed by microscopical examination of a sample of coelomic fluid. The polychaetes were kept individually in plastic containers with approximately 200 ml of seawater held at 10°C and a photoperiod of 12L:12D. *Nereis virens* were maintained at Seabait Ltd, Lynemouth, UK (see next subsection).

Gamete collection. Gamete maturation and spawning was induced in *Asterias rubens* by injection of 1-methyladenine (Sigma) to give an intracoelomic concentration of $1 \mu\text{M}$. *Psammechinus miliaris* were induced to spawn by injection of 0.5 ml of 0.5 M isotonic KCl. Sperm activation and spawning was induced in *A. marina* by injection of 8,11,14-eicosatrienoic acid (Sigma) to give a concentration of $13 \mu\text{g g}^{-1}$ body mass (Pacey & Bentley 1992). Female spawning was induced by injection of a prostomial homogenate from other mature females at a concentration of 1 prostomium per individual. Gametes were collected in Eppendorf tubes and stored on ice until required. Gametes of *Nereis*

virens were collected from mature individuals maintained at Seabait Ltd and transported on ice immediately to the laboratory.

Standard preparation. Commercial standards of DD (Acros Organics, UK), EPA, decanal (Sigma, UK) and undecanal (Aldrich, UK) were obtained for toxicity-testing. Due to their low solubility in water, they were dissolved initially in 0.5 ml ethanol and then transferred to 0.45 µm-filtered seawater (FSW) to give stock solutions of 100 µg ml⁻¹ concentration, from which serial dilutions were performed to give the required experimental concentrations. All compounds were soluble at the concentrations assayed. Ethanol concentrations did not exceed 1 µl ml⁻¹ in any incubation experiment. A solvent blank was included in all purified toxin trials.

Effects of toxin exposure on *in vitro* fertilisation success. Unfertilised oocytes were stocked in FSW at 15°C in polystyrene 24-well microplates containing 1 ml of oxygen-saturated incubation medium either, FSW, solvent blank, DD, EPA, decanal or undecanal at set concentrations of 0.5, 1, 5, 10 and 20 µg ml⁻¹. Approximately 200 oocytes were stocked per well, and 20 µl of sperm solution in FSW were added to give a final concentration of 2.5 × 10⁶ ml⁻¹. The plates were sealed and gently agitated for 30 s to increase sperm/egg encounters, and stored at 15°C for 4 h. Fertilisation success was determined using a Zeiss inverted microscope and was defined as complete elevation of the fertilisation membrane. The use of first cleavage as an indicator of fertilisation success is unsuitable in this instance as DD is an inhibitor of embryonic mitosis.

Effects of oocyte pre-incubation with DD on fertilisation success. Unfertilised oocytes were incubated in set concentrations (0.05, 0.5, 2.5, 5, 12.5, 25 and 50 µg ml⁻¹) of DD or FSW for fixed times ranging from 1 to 30 min. Oocytes were then washed 3 times in FSW before being stocked in 1 ml of oxygen-saturated FSW equilibrated to 15°C. Oocytes were stocked at a density of between 200 and 250 oocytes ml⁻¹ in sterile polystyrene 24-well microplates. We used 4 replicate incubations per treatment for *Psammechinus miliaris*, 7 for *Arenicola marina* and 3 for *Asterias rubens* and *Nereis virens*. Sperm that had been allowed to activate in FSW for 10 min was then added to give a final concentration of 1 × 10⁶ ml⁻¹. The plates were sealed and stored at 15°C for 4 h. Fertilisation success was determined using a Zeiss inverted microscope.

Effects of sperm pre-incubation with DD on fertilisation success. Unfertilised oocytes that had not been exposed to DD were stocked as above. Sperm at a concentration of 5 × 10⁶ sperm ml⁻¹ were incubated in set concentrations of DD or FSW at 15°C for fixed times ranging from 1 to 30 min. After each set time, 250 µl of

sperm was added to unfertilised oocytes to give a final concentration of 1 × 10⁶ ml⁻¹. To study the effects of sperm motility inhibition combined with reduced sperm concentration, the above sperm incubation assay was conducted with additional final sperm concentrations of 10⁵ and 10⁴ sperm ml⁻¹ using *Psammechinus miliaris*. Treatments were incubated and scored as above.

Sperm motility. In this study, 2 motility assays were used. The capillary motility assay is an adaptation of that described by Morton & Sagadraca (1981). We completely filled 50 µl Blaubrand glass capillary tubes with either DD of set concentrations or FSW, and sealed one end with paraffin wax. Sperm at a concentration of 8 × 10⁶ ml⁻¹ were drawn in to fill 5 µl capillary tubes, one end of which was then sealed using paraffin wax. The capillary tube containing the sperm was then inserted into the larger tube in such a way that the wax sealed both tubes together without trapping air bubbles or wax residue at the interface. The capillaries were then placed under a compound microscope fitted with an eyepiece graticule and the progress of the sperm front was monitored after every min for the first 10 min, then every subsequent 5 min until 30 min had elapsed. The microscope was switched off between readings to avoid overheating the sperm. The traces were converted to sperm front migration rates (µm s⁻¹), based on measurements of curve height after 15 min. Observations were made at room temperature, which varied between 18 and 24°C.

The second assay is adapted from Morton et al. (1982). We placed 1 ml of DD of set concentrations or FSW in Eppendorf tubes to which sperm was added to give a final concentration of 2 × 10⁶ sperm ml⁻¹. Motility estimates were made by examining droplets of swirled sperm suspensions placed on microscope slides at room temperature. Motility was scored as the percentage of sperm motile (0 to 100%, estimated to the nearest 10%) and 'rate' (0 to 10), where 0 represents no movement whereas 10 is equivalent to the rate of movement for sperm incubated in FSW at time, $t = 0$.

Statistical methods. Linear regression analysis on MINITAB 12 was used to determine EC₅₀, which was taken as the concentration required producing a reduction of 50% in fertilisation success of control experiments. Percentage fertilisation success was adjusted relative to the control percentage fertilisation success following Abbot's formula:

$$p = p_i C \frac{p_i - C}{1 - C}$$

where p_i denotes the observed non-zero dose response and C represents the natural fertilisation success of controls (Hubert 1984). We used 1-way analysis of variance followed by Fisher's least-significant difference post hoc analysis to test for the effect of DD concentration, and a

2-way analysis of variance to analyse the interaction of gamete exposure period and DD concentration. A general linear model was used to test for a significant interaction between incubation period, DD concentration and sperm concentration. Percentage data was arcsine(square root)-transformed prior to analysis.

RESULTS

Effects of toxin exposure on fertilisation success

Incubation of gametes with DD resulted in reduced fertilisation success in all 4 species (Fig. 1); however fertilisation was still evident even at a concentration of

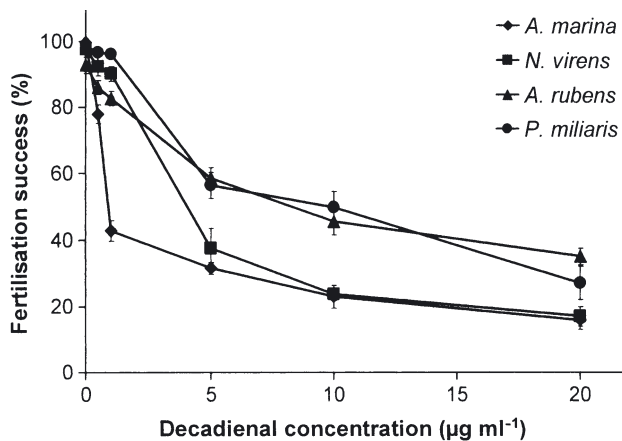


Fig. 1. *Asterias rubens*, *Psammechinus miliaris*, *Arenicola marina* and *Nereis virens*. Percentage fertilisation success of embryos incubated with increasing concentrations of 2E,4E-decadial (DD). Mean (\pm SE) of 4 replicates for each concentration

20 $\mu\text{g ml}^{-1}$. Fertilisation success declined rapidly in *Arenicola marina*, dropping from a mean of $99.75 \pm 0.25\%$ for controls to $16 \pm 2.9\%$ at the maximum concentration of $20 \mu\text{g ml}^{-1}$ ($F = 83.61$, $p < 0.001$). A minimum inhibitory concentration of $0.5 \mu\text{g ml}^{-1}$ was determined. A similar fertilisation failure rate was recorded for *Nereis virens*, with a mean fertilisation success of $17 \pm 2.9\%$ at $20 \mu\text{g ml}^{-1}$ ($F = 90.01$, $p < 0.001$). However, the EC_{50} value determined for *N. virens* ($3.98 \mu\text{g ml}^{-1}$) was higher than for *A. marina* ($1.55 \mu\text{g ml}^{-1}$). Both *Asterias rubens* ($F = 64.23$, $p < 0.001$) and *Psammechinus miliaris* ($F = 53.31$, $p < 0.001$) were able to maintain higher fertilisation success relative to the polychaetes, with means of 35.1 ± 2.5 and $27 \pm 5\%$ respectively at $20 \mu\text{g ml}^{-1}$. A higher EC_{50} value was determined for *A. rubens* ($10 \mu\text{g ml}^{-1}$ compared with $7.94 \mu\text{g ml}^{-1}$ for *P. miliaris*). Microscopical examination revealed that fertilisation-cone formation was inhibited at DD concentrations above $1 \mu\text{g ml}^{-1}$; however cone formation occurred normally at $< 1 \mu\text{g ml}^{-1}$ concentrations. Incubation with decanal, undecanal or EPA did not affect fertilisation success at any concentration assayed and no interference with fertilisation cone-formation was observed (data not shown).

Pre-incubation of oocytes with DD

Conducting fertilisation success experiments whereby both oocytes and sperm are simultaneously exposed to a xenobiotic agent reveals little about the sensitivity of either germ cell to the toxin. Therefore, oocytes and sperm were incubated independently in DD and fertilised with germ cells that were not toxin-exposed. Data for *Asterias rubens* are presented in Fig. 2. A 2-way ANOVA for *A. rubens* DD-exposed oocytes re-

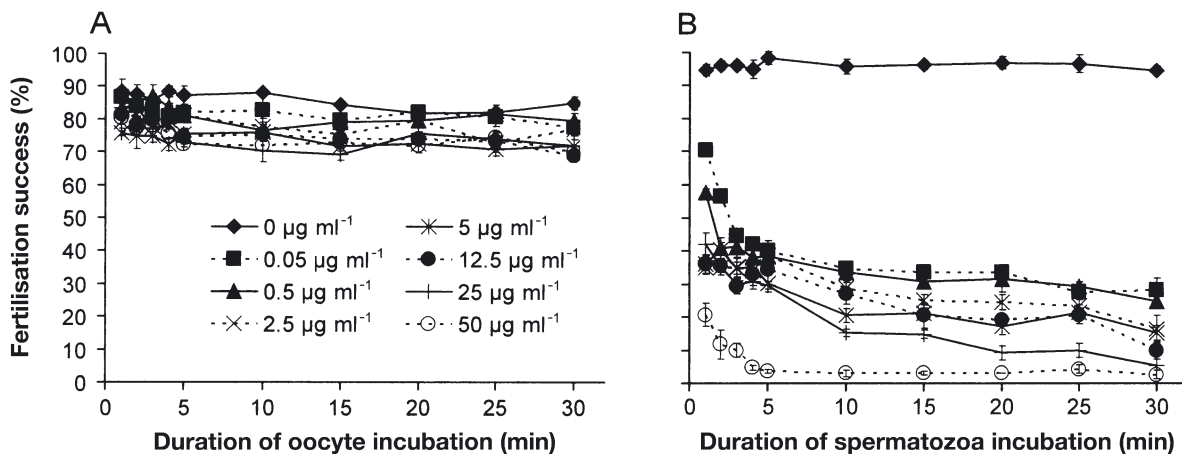


Fig. 2. *Asterias rubens*. Effect of incubating gametes in increasing concentrations of DD over time on subsequent fertilisation success. (A) Egg incubation; (B) sperm incubation. Mean (\pm SE) of 3 replicates for oocyte incubations and 4 replicates for sperm incubations

Table 1. *Asterias rubens*, *Psammechinus miliaris*, *Arenicola marina* and *Nereis virens*. Effect of oocyte incubation with 2E,4E-decadienal (DD) followed by fertilisation with control sperm on fertilisation success. Analysis of variance on arcsine-transformed fertilisation levels, with time and DD concentration as main effects

Source	df	SS	MS	F ratio	p-value
<i>A. rubens</i>					
Time	9	0.04087	0.004541	9.05	<0.001
DD conc.	7	0.122959	0.017566	35	<0.001
Interaction	63	0.030558	0.000485	0.97	0.552
Error	160	0.0803	0.000502		
<i>P. miliaris</i>					
Time	9	0.104048	0.011561	63.87	<0.001
DD conc.	7	0.019656	0.002808	15.51	<0.001
Interaction	63	0.012573	0.0002	1.1	0.298
Error	240	0.043444	0.000181		
<i>A. marina</i>					
Time	9	0.60359	0.06707	23.42	<0.001
DD conc.	7	0.13284	0.01898	6.63	<0.001
Interaction	63	0.03516	0.00056	0.19	1.0
Error	480	1.37445	0.00286		
<i>N. virens</i>					
Time	9	0.494952	0.054995	82	<0.001
DD conc.	7	0.718503	0.102643	153.06	<0.001
Interaction	63	0.120743	0.001917	2.86	<0.001
Error	160	0.107300	0.000671		

Table 2. *Asterias rubens*, *Psammechinus miliaris*, *Arenicola marina* and *Nereis virens*. Effect of sperm incubation with DD followed by fertilisation with control oocytes on fertilisation success. Analysis of variance on arcsine-transformed fertilisation levels, with time and DD concentration as main effects

Source	df	SS	MS	F ratio	p-value
<i>A. rubens</i>					
Time	9	2.14723	0.23858	84.5	<0.001
DD conc.	7	16.23309	2.31901	821.33	0.001
Interaction	63	1.16142	0.01844	6.53	<0.001
Error	240	0.67764	0.00282		
<i>P. miliaris</i>					
Time	9	3.44261	0.38251	165.16	<0.001
DD conc.	7	18.6674	2.66677	1151.43	<0.001
Interaction	63	1.13852	0.01807	7.8	<0.001
Error	240	0.55585	0.00232		
<i>A. marina</i>					
Time	9	5.03305	0.55923	390.63	<0.001
DD conc.	7	17.99141	2.5702	1795.31	<0.001
Interaction	63	1.17906	0.01872	13.07	<0.001
Error	240	0.34359	0.00143		
<i>N. virens</i>					
Time	9	3.20472	0.35608	130.08	<0.001
DD conc.	7	12.60436	1.80062	657.79	<0.001
Interaction	63	0.8784	0.01394	5.09	<0.001
Error	240	0.65697	0.00274		

vealed that there was a significant inhibitory effect due to both incubation time and concentration (Table 1), however there was no significant interaction between the factors. A similar pattern was observed for *Psammechinus miliaris* and *Arenicola marina* characterised by a general decline in fertilisation success with time, but the slope did not differ significantly from controls (data not shown). This pattern was not adhered to by *Nereis virens* oocytes (Table 1). Oocytes incubated for 30 min with $0.05 \mu\text{g ml}^{-1}$ had a mean fertilisation success of $71 \pm 1.8\%$, whereas controls had a mean fertilisation success of $92.4 \pm 0.6\%$. Increased concentration had little effect thereafter, with a mean fertilisation success of $61.5 \pm 1.6\%$.

The decrease in fertilisation success due solely to oocyte incubation was not sufficient to explain the results in Fig. 1. The effect of DD on sperm-related fertilisation success was therefore investigated.

Pre-incubation of sperm with DD

Incubation of sperm in DD had a profound effect on fertilisation success in *Asterias rubens* (Fig. 2B). Control sperm maintained a fertilisation rate of 94 to 100% throughout the experiment. There was a significant effect of both DD concentration and incubation period (Table 2). Interaction between concentration and time was also significant. Fertilisation success for sperm incubated with $0.05 \mu\text{g ml}^{-1}$ DD had declined to $70 \pm 0.82\%$ after 1 min, but the inhibition rate subsequently declined with time. After 30 min $28 \pm 3.5\%$ of oocytes were fertilised. Similar-shaped profiles were recorded for concentrations from 0.5 to $25 \mu\text{g ml}^{-1}$, with fertilisation success declining in a dose-dependent manner. Fertilisation success using sperm treated with $50 \mu\text{g ml}^{-1}$ declined rapidly, with only $20.8 \pm 3.3\%$ of oocytes fertilised after 1 min. Fertilisation success subsequently stabilised at ca. 2 to 5% by 4 min, and this success rate was maintained for the remainder of the experiment. A similar pattern was recorded for *Psammechinus miliaris* sperm (Fig. 3A, Table 2); however, a pronounced decline in fertilisation success for $0.05 \mu\text{g ml}^{-1}$ was not seen until

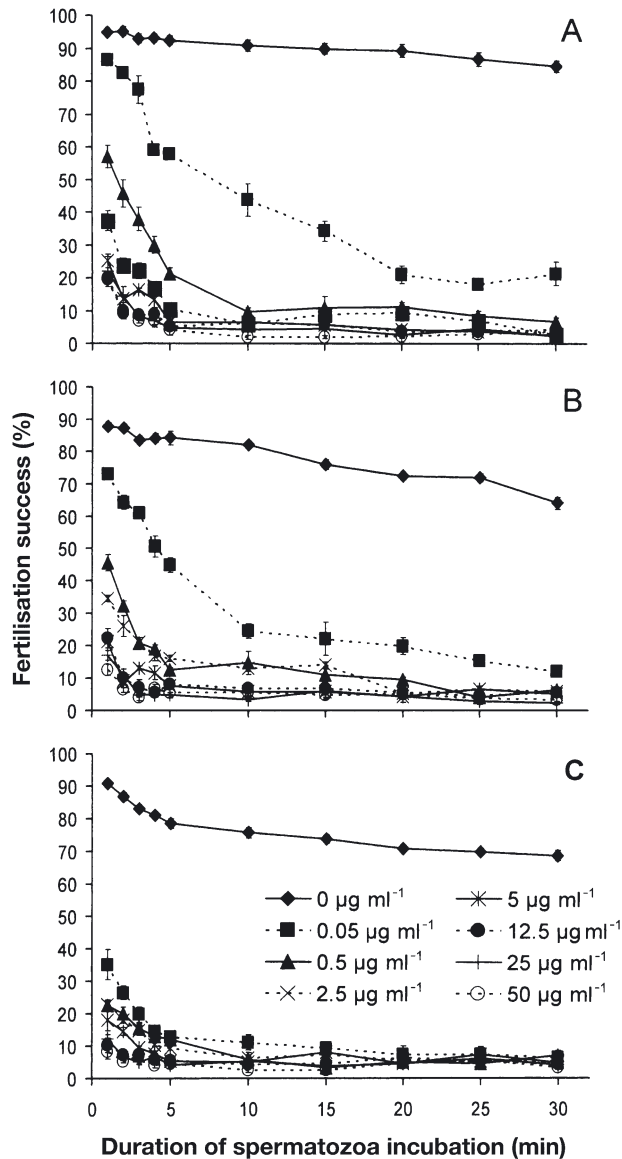


Fig. 3. *Psammechinus miliaris*. Effect of incubating sperm in increasing concentrations of DD over time on subsequent fertilisation success and effect of reduced sperm concentration. (A) 10^6 sperm ml^{-1} , (B) 10^5 sperm ml^{-1} , (C) 10^4 sperm ml^{-1} . Mean (\pm SE) of 4 replicate experiments for each concentration

4 min had elapsed. In both species, the rate of decline was most rapid within the first 5 min of incubation. A similar dose- and time-dependent pattern was displayed by the polychaetes (Table 2), also with the most rapid decline occurring in the first 5 min. A common feature among the 4 species was the occurrence of a residual level of fertilisation success, typically 2 to 7% at the maximum concentration tested. In oocytes that had fertilised, a fertilisation cone was frequently observed. Fertilisation cone-formation proceeded as normal at all DD concentrations.

Fig. 3 also shows the effect of increasing DD concentration coupled with a reduction in sperm concentration for *Psammechinus miliaris*. The proportion of fertilised oocytes declined significantly as sperm concentration was reduced (Table 3). The inhibitory effects of DD on fertilisation success were exacerbated in treatments with lower sperm concentrations. The most pronounced effect was observed when sperm concentration was reduced to 10^4 sperm ml^{-1} . For example, fertilisation success with sperm incubated with $0.05 \mu\text{g ml}^{-1}$ after 5 min was $57.8 \pm 1.2\%$ with 10^6 sperm ml^{-1} , $44.8 \pm 2.2\%$ for 10^5 sperm ml^{-1} and $12.75 \pm 1.2\%$ for 10^4 sperm ml^{-1} . The general linear model reveals that fertilisation inhibition is a function of DD concentration, period of sperm incubation and sperm concentration.

Sperm motility inhibition

The effects of increasing concentrations of DD on *Psammechinus miliaris* sperm migratory velocity is shown in Fig. 4. Motility was evident at all concentrations for the first 7 min, whereafter sperm exposed to $50 \mu\text{g ml}^{-1}$ were immobilised. Motility declined in a clear dose- and time-dependent manner (Table 4). Similarly shaped profiles were obtained for the other species (data not shown). The motility rates for *Asterias rubens*, *Arenicola marina* and *Nereis virens* are presented in Table 5. The data shows the ability of DD to profoundly inhibit forward movement of sperm populations even at a concentration of $0.05 \mu\text{g ml}^{-1}$. Microscopic examination of inhibited sperm, even those

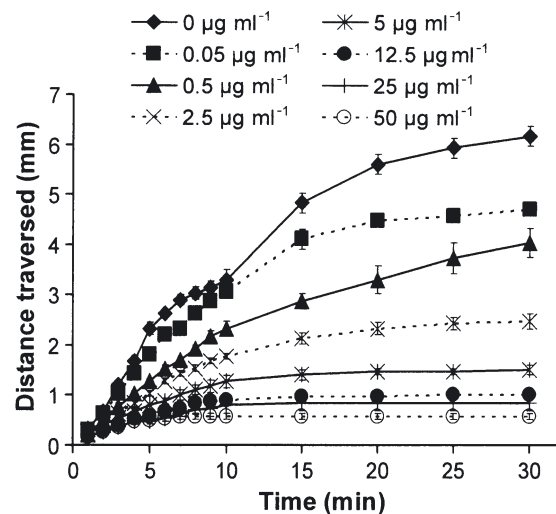


Fig. 4. *Psammechinus miliaris*. Effect of increasing concentration of DD over time on sperm-migration velocities. Mean (\pm SE) of 3 replicate experiments

Table 3. *Psammechinus miliaris*. Effect of sperm concentration, DD concentration and incubation period followed by fertilisation with control oocytes on fertilisation success. General linear model analysis of variance on arcsine-transformed fertilisation levels, with sperm concentration, time and DD concentration as main effects

Source	df	SS	MS	F ratio	p-value
Sperm conc.	1	14.5977	14.5977	774.28	<0.001
DD conc.	1	0.5401	0.5401	28.65	<0.001
DD conc. × Time	1	0.1306	0.1306	6.93	0.009
DD conc. × Sperm conc.	1	0.9503	0.9503	50.41	<0.001
Time	1	0.0118	0.0118	0.62	0.43
Time × Sperm conc.	1	0.1654	0.1654	8.77	0.003
DD conc. × Time × Sperm conc.	1	0.1263	0.1263	6.7	0.01
Error	952	17.9485	0.0189		

exposed to the maximum concentration of 50 µg ml⁻¹ for 30 min, demonstrated clearly that the sperm remained viable, as the sperm heads were still twitching despite there being no flagellar movement.

From Fig. 5 it can be seen that the proportion of sperm remaining motile declines in a time- and dose-dependent fashion. All sperm exposed to 50 µg ml⁻¹ were immobilised after 10 min, and all sperm exposed to 25 µg ml⁻¹ were immobilised after 15 min. By 30 min, sperm exposed to 12.5 µg ml⁻¹ were also stationary. Approximately 30% of sperm retained some degree of motility after 30 min when exposed to the lowest concentration of 0.05 µg ml⁻¹. Additionally, the 'rate' of sperm flagellar activity also declined in a time- and dose-dependent manner.

DISCUSSION

Using the same test species, it has been previously demonstrated that incubation with DD inhibits *in vitro* fertilisation success (Caldwell et al. 2002). Following the same protocol we have shown that the saturated aldehydes decanal and undecanal and the fatty acid EPA have no inhibitory effect on fertilisation success (data not shown). SCA toxicity is unrelated solely to the aldehyde functional group up to 20 µg ml⁻¹, but requires also the α,β,γ,δ-unsaturated reactive Michael acceptor (Pohnert et al. 2002, Adolph et al. 2003). It has recently been shown that the production of toxic SCAs is species- and strain-specific. Saturated aldehydes do not affect embryonic development or hatching success in echino-

derms or polychaetes at the concentrations used in our experiments (G. Caldwell unpubl.); however, oxoacids do inhibit cleavage in sea urchin embryos (Pohnert et al. 2002). EC₅₀ values were slightly lower for *Arenicola marina* and *Nereis virens* than those determined by Caldwell et al. (2002). Inhibitory concentrations were the same for *Asterias rubens*, but a considerably lower value was noted for *Psammechinus miliaris* (7.94 compared with 40.41 µg ml⁻¹). It is likely that the EC₅₀ concentrations for fertilisation are considerably higher than in reality. The protocol adopted here assessed fertilisation by scoring membrane ele-

vation. The cortical reaction which leads to membrane elevation is a secondary fertilisation process. It has recently been demonstrated that DD and the related decatrienal block early fertilisation processes in ascidians at a concentration of 2.2 µg ml⁻¹ (Tosti et al. 2003).

Fertilisation success was reduced by pre-incubating oocytes with DD followed by repeated washing (Fig. 2A, Table 1). However, the lower fertilisation success was insufficient to fully account for the previous *in vitro* fertilisation failure. The fact that DD-treated oocytes retained a high fertilisation success suggests

Table 4. *Asterias rubens*, *Psammechinus miliaris*, *Arenicola marina* and *Nereis virens*. Effect of DD concentration and exposure period on forward progression of sperm fronts using a capillary motility assay. Analysis of variance, with time and DD concentration as main effects

Source	df	SS	MS	F ratio	p-value
<i>A. rubens</i>					
Time	13	145.5156	11.1935	3369.48	<0.001
DD conc.	7	293.7064	41.9581	13000	<0.001
Interaction	91	119.9151	1.3177	3369.48	<0.001
Error	224	0.7441	0.0033		
<i>P. miliaris</i>					
Time	13	192.652	14.8194	1394.77	<0.001
DD conc.	7	282.5637	40.3662	3799.18	<0.001
Interaction	91	121.9413	1.34	126.12	<0.001
Error	224	2.38	0.0106		
<i>A. marina</i>					
Time	13	98.1593	7.5507	5369.4	<0.001
DD conc.	7	367.1467	52.4495	37000	<0.001
Interaction	91	149.4927	1.6428	1168.2	<0.001
Error	224	0.315	0.0014		
<i>N. virens</i>					
Time	13	157.0175	12.0783	677.44	<0.001
DD conc.	7	252.5899	36.0843	2023.89	<0.001
Interaction	91	106.6514	1.172	65.73	<0.001
Error	224	3.9937	0.0178		

Table 5. *Asterias rubens*, *Psammechinus miliaris*, *Arenicola marina* and *Nereis virens*. Migration velocities ($\mu\text{m s}^{-1}$) of sperm incubated with increasing concentrations of DD. Migration velocities calculated from curve height after 15 min. Mean \pm SE, n = 3

DD conc. ($\mu\text{g ml}^{-1}$)	<i>A. rubens</i>	<i>P. miliaris</i>	<i>N. virens</i>	<i>A. marina</i>
0	5.16 \pm 0.1	5.37 \pm 0.23	4.53 \pm 0.28	5.59 \pm 0.08
0.05	4.18 \pm 0.06	4.56 \pm 0.22	3.72 \pm 0.09	4.27 \pm 0.05
0.5	2.67 \pm 0.04	3.19 \pm 0.17	3.04 \pm 0.03	1.81 \pm 0.03
2.5	2.15 \pm 0.06	2.37 \pm 0.13	2.73 \pm 0.06	1.34 \pm 0.07
5	1.21 \pm 0.04	1.56 \pm 0.11	1.49 \pm 0.03	0.58 \pm 0.02
12.5	0.78 \pm 0.15	1.07 \pm 0.06	0.82 \pm 0.41	0.44 \pm 0.01
25	0.69 \pm 0.05	0.93 \pm 0.17	0.81 \pm 0.07	0.32 \pm 0.02
50	0.48 \pm 0.02	0.63 \pm 0.06	0.51 \pm 0.02	0.29 \pm 0.02

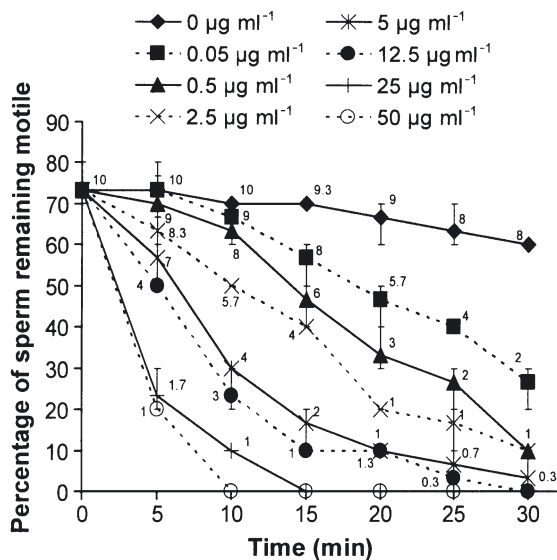


Fig. 5. *Psammechinus miliaris*. Effect of length of exposure to increasing concentration of DD upon per cent of sperm remaining motile. Number by each data point is rate of motility observed for the sample at that time. Error bars are range of values observed, n = 3

that DD does not strongly associate with the oocyte surface membrane where it may affect sperm/egg binding. This observation is further supported by the discovery that rinsing within minutes of fertilisation prevents embryotoxic effects in ascidians (Tosti et al. 2003). Sperm incorporation and the formation of the fertilisation cone proceeded as normal in oocytes treated in this way, in contrast with fertilisation in the presence of DD above a concentration of $1 \mu\text{g ml}^{-1}$. This observation corroborates those made on ascidian embryos (Tosti et al. 2003), whereby DD interfered with actin microfilament reorganisation. Additionally, water-soluble extracts of *Thalassiosira rotula* are known to depolymerise microtubules in sea urchin

embryos (Buttino et al. 1999). It is therefore apparent that the cytoskeleton is a crucial cellular target for bioactive SCAs. The implications of this are profound when trying to understand the effects of SCAs on fertilisation biology. A co-ordinated sequence of motility events mediated by both microtubules and microfilaments are required for successful fertilisation to occur. Microtubules are the functional cytoskeletal component during sperm motility, pronuclear migrations and syngamy, whereas microfilaments mediate extrusion of the sperm acrosomal process, the formation of the fertilisation cone and the block to polyspermy (Tilney et al. 1973, Schatten & Schatten 1981).

When sperm were incubated with DD, the effects on fertilisation success with untreated oocytes was striking (Fig. 2B). A pronounced reduction in fertilisation success was observed between controls and the minimum concentration tested ($0.05 \mu\text{g ml}^{-1}$), with inhibitory effects in evidence after only 1 min. This concentration lies comfortably within known levels of SCA production by diatoms (in excess of $1.5 \mu\text{g ml}^{-1}$, Pohnert 2000). The combination of DD incubation and reduced sperm concentration caused a further reduction in fertilisation success (Fig. 3). Relatively high fertilisation success rates were achieved at dilutions of 10^6 and 10^5 sperm ml^{-1} combined with the lowest DD concentration, but once sperm concentration was reduced to 10^4 sperm ml^{-1} , fertilisation success became heavily impacted at all concentrations tested. The observed effect of sperm concentration on fertilisation efficiency is probably related to the ratio of sperm to eggs. Alternatively, detoxification systems such as glutathione are known to detoxify metals and oxyradicals in oyster gonads (Ringwood & Connors 2000). If such a system were functional in polychaete and echinoderm sperm, elevated sperm densities might result in a more rapid detoxification of DD, facilitating higher fertilisation rates.

To determine if this 'sperm effect' could be explained by motility inhibition, a simple and economical assay procedure for sperm motility was employed. The capillary motility assay (Fig. 4) clearly demonstrates the ability of DD to inhibit the linear forward progression of *Psammechinus miliaris* sperm. The concept of motility inhibition is further supported by the second microscopical assay (Fig. 5), which documents the inhibitory effects of DD on flagellatory vigour and shows a time- and dose-dependent reduction in the percentage of sperm remaining motile. Pronounced inhibitory effects were recorded at the minimum concentration tested ($0.05 \mu\text{g ml}^{-1}$). This clearly shows that DD causes an easily detectable inhibitory effect on invertebrate sperm motility.

Examples of algal products affecting fertilisation biology are seldom encountered in the literature.

Wicklund (1954) described inhibitory effects due to crude extracts from furoid algae. The inhibitor appeared to block sperm/egg interactions but did not inhibit sperm motility; on the contrary, the period of motility was prolonged. Other macroalgae-derived metabolites, including aldehydes such as udoteal and halimedatrial, are known to inhibit sperm motility (Paul & Fenical 1986). Microalgae examples include a toxin produced by *Chrysochromulina polylepsis* that has been shown to inhibit fertilisation in mussels and ascidians (Granmo et al. 1988). The ciguatera poison, maitotoxin, inhibits sea urchin fertilisation by oocyte exocytosis but has no effect on sperm (Pesando et al. 1991). Recently, cellular extracts of *Phaeocystis pouchetti* have also been demonstrated to inhibit sperm motility (Hansen et al. 2003).

Motile ect-aquasperm, such as those in broadcast-spawning species, where fertilisation is external and sperm are not packaged for transfer, e.g. in spermatophores (Rouse 1999), are an essential prerequisite for fertilisation of broadcast-spawning marine invertebrates. The sperm of the species used in this study have a motile life of several hours (echinoderms, *Nereis virens*) to more than a day (*Arenicola marina*) (Williams & Bentley 2002). Therefore, the rapid onset of DD-mediated motility inhibition could provide a potent block to *in situ* fertilisation success. Although flagellar activity was arrested, the sperm remained viable, as evidenced by prolonged oscillation of the sperm head. Flagellar beating is not always essential for sperm incorporation upon contact between oocyte and sperm (Epel et al. 1977). This may account for the presence of a basal fertilisation rate (ca. 2 to 7%), even when sperm were rendered completely immotile. This suggests that mixing during sperm addition to the oocyte suspension facilitated a degree of passive fertilisation by immotile but otherwise functionally viable sperm. This is likely to be an artefact of the *in vitro* fertilisation technique.

The relationship between sperm/egg density and fertilisation efficiency, both *in vitro* and *in situ*, is of critical importance in fertilisation ecology (Williams 1999). Broadcast-spawning species discharge their gametes directly into the environment, whereupon numerous physical constraints can significantly impact fertilisation success (Mead & Denny 1995). Adverse tidal, weather or current conditions may result in considerable gamete dilution. Sperm dilution results in low fertilisation rates, which can vary from 15% in sheltered conditions (Pennington 1985, Levitan 1991, Levitan et al. 1992) to less than 1% on wave-exposed shores (Denny & Shibata 1989). The proportion of eggs fertilised is dependent upon the concentration of viable sperm. Following cell damage, the cytoplasm-associated enzymes responsible for SCA formation are

active for over 20 min (Pohnert 2002). Therefore, if spawning were to coincide with a diatom bloom under intense grazing pressure, it might be feasible that the concentration of free SCAs in seawater derived from 'sloppy feeding' might limit *in situ* fertilisation success. Fig. 3 clearly shows that DD-mediated fertilisation failure can further compound the fertilisation efficiency impacts of sperm limitation. As such, the potential for successful fertilisation of oocytes exposed to depressed sperm concentrations is greatly reduced by the presence of diatom SCAs.

This study utilised 2 polychaete and 2 echinoderm species as representative of broadcast-spawning species. The potential effects of bioactive aldehydes to sperm motility are equally applicable to any species which spawns during periods of high diatom productivity. A particularly interesting group to study would be filter-feeding bivalves, which are capable of consuming substantial quantities of diatoms. As SCAs are liberated following cell damage, there exists a strong possibility that aldehydes produced during feeding could affect sperm quality, particularly during storage in the gonads. A further area of concern relates to aquaculture. Diatoms are frequently used as a live food component. The effects of SCA-producing diatoms on the reproductive success of commercially important animals such as oysters are unknown, and clearly warrant investigation. We have previously suggested that diatom productivity may represent a strong selective pressure forcing seasonally reproducing broadcast-spawning species to spawn in suboptimal conditions (Caldwell et al. 2002). It is possible that the production of anti-reproduction chemicals by diatoms (SCAs and oxo-acids) will exert a strong influence on the evolution of life-history strategies in marine invertebrates. Future work designed to further elucidate the responses of invertebrate gametes and embryos, and grazer immunological responses to diatom toxins will shed much light on the role diatom blooms have in structuring the reproductive biology of marine animals.

We present strong evidence that DD affects sperm motility, so influencing fertilisation success from the paternal side (Figs. 4 & 5). However, maternal effects cannot be discounted. Poulet et al. (1994) have advanced the hypothesis that diatom toxins are incorporated into oocytes during oogenesis. It is conceivable that SCA-contaminated oocytes could greatly limit fertilisation success. Such an effect may not be manifested by blockage of fertilisation membrane elevation as assessed in this study, but may be expressed at an alternate stage when ooplasmic cytoskeletal elements are critical. There is as yet no definitive data for the effects of diatom SCAs on fertilisation success in copepods. Previous studies have obtained viable oocytes

from diatom-fed females, but there appears to be no information specific to fertilisation success. The dinoflagellates *Prorocentrum micans*, *Lingulodinium polyedra* and *Gymnodinium sanguinuum* have been identified as inducing poor sperm quality in the copepod *Temora stylifera* (Ianora et al. 1999, Laabir et al. 2001). Ianora et al. (1999) suggested that the dinoflagellates interfere with spermatogenic processes. These negative effects appear not to be related to major sperm ultrastructural aberrations, but fine-scale ultrastructural or molecular modifications cannot be discounted. Ianora & Poulet (1993) found that spermatophore production in *T. stylifera* was lower when males were fed *Thalassiosira rotula* compared with the dinoflagellate *Prorocentrum minimum*, and Ianora et al. (1995) made similar observations for *T. stylifera* fed *Skeletonema costatum*. Ianora et al. (1995) suggested that this was due to poor digestibility and assimilation of *S. costatum*, as egg production was also reduced. Whether these effects are toxin-induced or due to nutritional deficiency is as yet unclear.

Microtubule-inhibiting compounds have been described from the Cyanophyta, Chlorophyta and Pyrrophyta (Lincoln et al. 1990, Blokhin et al. 1995). Inhibitors of microtubule assembly, such as colchicine, are capable of inhibiting sperm motility (Haimov-Kochman & Ben-Chetrit 1998). However, there are other factors such as changes in intracellular pH that may impact sperm motility. It has recently been demonstrated that DD inhibits the fertilisation current in ascidians together with the voltage-gated calcium current activity (Tosti et al. 2003). Calcium ions play an essential role in the normal functioning of marine invertebrate sperm, greatly influencing the pattern and shape of flagellar bending (Su & Vacquier 2002). In sea urchin sperm, elevated Ca^{2+} increases flagellar beat asymmetry and reversibly blocks beating at 0.1 to 0.2 mM (Brokaw et al. 1974, Brokaw 1979, Gibbons & Gibbons 1980). In view of the current evidence regarding SCA-mediated cytoskeletal disruption and interference with calcium signalling, it would seem likely that a similar effect would significantly contribute towards motility inhibition in sperm. We suggest that sperm may represent a useful cellular model to further elucidate the cellular and molecular targets of novel diatom compounds.

SCAs and other volatile organic compounds are key pheromone molecules in terrestrial and aquatic organisms. Furthermore, Spehr et al. (2003) recently identified a suite of aromatic aldehydes as agonists to a human sperm olfactory receptor, with the SCA undecanal a potent antagonist to these molecules. Undecanal is liberated by diatoms following wounding (Pohnert et al. 2002), and interestingly occurs in *Nereis virens* oocytes, together with other SCAs that have

undergone gametogenesis during periods of high diatom production (G. S. C. unpubl.). These worms are characterised by low fertilisation efficiency and high embryonic mortality. Such bioactive aldehydes, including DD, are formed following lipid peroxidation of unsaturated eicosanoid precursors, both in diatom and metazoan systems including humans. Reactive aldehydes may represent a significant chemical agent regulating reproductive success both at the individual level and at an ecosystem scale.

The importance of volatile hydrocarbons as signalling molecules in biological systems is increasingly being recognised. Their utility in cell–cell communication includes application as defensive molecules and in the evolution of species-specific reproductive strategies. Chemical communication between sperm and egg is a key factor mediating sexual reproduction, and has been demonstrated in broadcast-spawning marine plants and animals, and in internally fertilising terrestrial organisms, including humans (Spehr et al. 2003). Chemically mediated control of gamete functionality and behaviour, whether promoting or impeding fertilisation efficiency, is likely to be a key component in the regulation of reproductive success and the evolution of life-history strategies in eukaryotes. The inhibitory effect of diatoms on grazer reproduction represents a significant paradigm shift in aquatic systems biology. Lipid peroxidation products offer a tantalising research subject to further examine fertility processes in higher organisms, and humans in particular.

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