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Aldehyde-encapsulating liposomes impair marine grazer survivorship

Isabella Buttino[1,*], Giuseppe De Rosa[2], Ylenia Carotenuto[1], Marialuisa Mazzella[2], Adrianna Ianora[1], Francesco Esposito[1], Valentina Vitiello[1], Fabiana Quaglia[2], Maria Immacolata La Rotonda[2] and Antonio Miralto[1]

[1]Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy and [2]Dipartimento di Chimica Farmaceutica e Tossicologica, Università degli Studi di Napoli Federico II, Via D. Montesano 49, 80131 Napoli, Italy

*Author for correspondence (e-mail: buttino@szn.it)

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SUMMARY

In the last decade, there has been an increased awareness that secondary metabolites produced by marine diatoms negatively impact the reproductive success of their principal predators, the copepods. Several oxylipins, products of the enzymatic oxidation of fatty acids, are produced when these unicellular algae are damaged, as occurs during grazing. In the past, the dinoflagellate *Prorocentrum minimum*, which does not produce the oxylipin 2-*trans*,4-*trans*-decadienal (DD), has been used as a live carrier to calculate daily ingestion rates of this molecule by copepod crustaceans. However, since the interaction between oxylipins and live carriers is unknown, the question as to how much and for how long ingestion of these molecules affects copepod reproduction remains a critical point to understanding the functional role of such compounds at sea. In the investigation presented here we used giant liposomes (~7 μm) as a delivery system for the oxylipin DD, prepared in the same size range as copepod food and containing known amounts of DD. The aim of this work was to relate the ingestion of DD to the reproductive failure of the copepods *Temora stylifera* and *Calanus helgolandicus*. Liposomes were very stable over time and after 10 days of feeding, liposomes encapsulating DD reduced egg hatching success and female survival with a concomitant appearance of apoptosis in both copepod embryos and female tissues. Concentrations of DD inducing blockage were one order of magnitude lower that those used in classical feeding experiments demonstrating that liposomes are a useful tool to quantitatively analyze the impact of toxins on copepods.

Key words: copepod, diatom, decadienal, reproduction, egg viability, apoptosis.

INTRODUCTION

In the last decade, there has been an increased awareness that secondary metabolites produced by diatoms, a major class of unicellular algae contributing to over 45% of the oceanic primary production, negatively impact the reproductive success of their principal zooplankton predators such as copepods (Ianora et al., 2003; Pohnert, 2005) with possible consequences on the transfer of energy through the marine food chain to top carnivores (Miralto et al., 1999; Ianora et al., 2004). This insidious mechanism, which does not deter the herbivore from feeding but impairs its recruitment, will restrain the cohort size of the next generation. The compounds responsible for reduced hatching success and abnormal larval development in these small crustaceans are several oxylipins, products of the enzymatic oxidation of fatty acids, which include polyunsaturated aldehydes (PUAs) as well as fatty acid hydroxides, epoxy alcohols, hydroperoxides and reactive oxygen species (ROS) (Fontana et al., 2007). These compounds are not constitutively present in the cells but are only produced when the cell is damaged as would occur during grazing (Pohnert, 2000). Owing to the teratogenic nature of diatom oxylipins, the mechanism of chemical defense in diatoms functions through induction of apoptosis which can occur in maturing oocytes (Poulet et al., 2007) or during embryo development (Romano et al., 2003) and in newly hatched nauplii (Ianora et al., 2004; Poulet et al., 2003). The toxicity of oxylipins, and more specifically of PUAs, has been demonstrated in classical feeding experiments using diatoms as copepod food (Ianora et al., 2003; Poulet et al., 2007) or in *in vitro* tests by incubating embryos and adults in known concentrations of pure molecules dissolved into seawater (Ianora et al., 1999; Romano et al., 2003; Adolph et al., 2004; Caldwell et al., 2005; Taylor et al., 2007).

Recently, Ianora et al. used the dinoflagellate *Prorocentrum minimum*, which does not produce oxylipins, as a live carrier of 2-*trans*,4-*trans*-decadienal (henceforth called decadienal or DD), which has been widely used as a model aldehyde to study the deleterious effects of these compounds on marine invertebrates (Ianora et al., 2004). The daily ingestion rate of DD by females of the copepod *Calanus helgolandicus* was indirectly calculated from the filtration rate of *P. minimum*, considering the amount of DD adsorbed onto the algal cells. However, since the interaction between PUAs and live carriers is unknown, the question as to how much and for how long ingestion of oxylipins affects copepod reproduction remains a critical point to understanding the functional role of such compounds in the marine system.

Until now, the lack of an efficient method to deliver realistic amounts of these compounds into the copepod body has represented a major limitation to a deeper investigation of the toxicological impact of oxylipins in copepods. Recently, several authors have discussed the need to develop inert carriers delivering known concentrations of oxylipins into copepods, and able to mimic their release as occurs during diatom feeding under natural conditions (Caldwell et al., 2004; Paffenhofer et al., 2005), in order to better understand the impact of these compounds on

copepod population control at sea. Although the use of liposomes as a delivery system for drugs and chemicals in zooplankton and in aquaculture is not new (Hontoria et al., 1994; Ozkizilcik and Chu, 1994; Koven et al., 1999; Touraki et al., 1995), this technique has only recently been proposed in marine copepods (Buttino et al., 2006). Buttino and co-workers demonstrated that giant liposomes of about 7 μm diameter, which corresponds to the same dimensional range as phytoplankton cells ingested by copepods, were actively ingested by the copepod *Temora stylifera*. Liposome uptake and palatability was confirmed using liposomes containing a fluorescent marker (fluorescein isothiocyanate–dextran), and ^{3}H-labelled liposomes were used to calculate the rate of liposome ingestion. These authors also showed that when administered with the dinoflagellate algae *P. minimum*, liposomes were actively ingested over a 48 h period, and copepods grazed twice as much as with a diet of liposomes alone. Moreover, a diet of liposomes had no supplementary effects on copepod egg production and egg viability, making them a good candidate as a delivery system for chemicals in copepods.

In the present work, we use giant liposomes as a delivery system for the PUA 2-*trans*,4-*trans* decadienal (DD), to study the effect of this molecule on egg production, egg hatching success, faecal pellet production and adult survival in the copepods *Temora stylifera* and *Calanus helgolandicus.* Giant liposomes containing known quantities of DD were prepared and fully characterized. Blank or DD-encapsulating liposomes and the control diet *P. minimum* were supplied for 10 days to both copepod species, which represent target species used in numerous aldehyde–copepod reproduction studies. In addition, the induction of apoptosis in embryos and females were also analyzed using specific fluorescent markers. The aim of this work was to relate the ingestion of DD, *via* a liposome-based delivery system, to the reproductive failure of copepods in order to better understand the ecological relevance of diatom-derived oxylipins for copepod recruitment at sea.

MATERIALS AND METHODS

Materials

Cholesterol, sea salts and 2-*trans*,4-*trans* decadienal (DD) were purchased from Sigma-Aldrich (Milan, Italy). Phosphatidylcholine from soybean lecithin (SPC) was kindly provided by Lipoid GmbH (Cam, Switzerland). Analytical grade chloroform, methanol, ethanol, 96% sulfuric acid and 2,4-dinitrophenylhydrazine were supplied by Carlo Erba Reagenti (Milan, Italy).

Liposome preparation

Liposomes were prepared by a modified hand-shaking method (Bangham et al., 1965). Briefly, a lipid mixture containing 110 mg SPC and 40 mg cholesterol in 5 ml of a chloroform–methanol solvent mixture (2:1 v/v) was introduced into a 250 ml round-bottomed flask. The solvent was removed in a rotary evaporator (Laborota 4010 Digital, Heidolph, Schwabach, Germany) until formation of a lipid film on the wall of the flask. To prepare blank liposomes, the lipid film was hydrated with 5 ml of 0.22 μm-filtered seawater (FSW). The resulting suspension was gently mixed in the presence of 0.5 g glass beads until the lipid layer was removed from the glass wall. The flask was then attached to the evaporator again, rotated at room temperature for about 30 min, and left at room temperature for 2 h. DD-encapsulating liposomes (LipoDD) were prepared by adding 500 μl of an ethanol solution containing 0.4 mg ml^{-1} DD to the organic solution containing lipids. After preparation, LipoDD was washed twice by centrifugation at 4470 ***g*** for 30 min at 4°C to remove un-encapsulated DD. All liposome formulations were stored at 4°C under nitrogen.

Liposome size analysis and lipid dosage

The mean diameter and size distribution of liposomes were analyzed by laser light scattering (Coulter LS, 100Q, Beckman Coulter, Miami, USA) on a dispersion of liposomes in FSW. Particle size was expressed as mean volume diameter ± standard deviation (s.d.) calculated on three different batches (N=3). The amount of lipids present in the liposome suspension after preparation was determined using the Stewart assay (Stewart, 1980). This test allows the quantification of SPC concentration in the suspension; total lipid content was calculated by assuming the same SPC:cholesterol ratio before and after preparation. Briefly, 0.1 ml of liposome suspension (approximately at a concentration of 0.1 mg ml^{-1}) was added to 1.9 ml of an aqueous 0.1 mol l^{-1} ammonium ferrothiocyanate solution in a test tube. The resulting suspension was mixed with 2 ml of chloroform for 15 s using a vortex, and then centrifuged for 5 min at 894 ***g*** The upper layer was recovered and analyzed at 485 nm by an UV-vis spectrophotometer (Shimadzu, Milano, Italy; model 1204), by comparison with a standard curve.

Dosage of 2-*trans*,4-*trans* decadienal

To quantify DD in water and liposomes, the aldehyde was previously transformed into the corresponding hydrazone by a reaction with 2,4-dinitrophenihydrazine (DNPH). DNPH solution was prepared as follows: 50 mg DNPH were dissolved in 0.5 ml of sulfuric acid and 4.5 ml of ethanol. DD was quantified by mixing 100 μl of the solution containing DD with 100 μl of the DNPH solution; the resulting solution was made up to 10 ml with ethanol. DD analysis was carried out by high-performance liquid chromatography (HPLC) using a Luna C18 (250×4.6 mm, 5 μm) column (Phenomenex, Klwid, Torrance, CA, USA), an HPLC LC-10AD pump (Shimadzu), a 7725i injection valve (Rheodyne, Rohnert Park, CA, USA) and a SPV-10A UV-Vis detector (Shimadzu) set at 360 nm. The system was controlled by a SCL-10A VP System Controller (Shimadzu) connected to a computer. Chromatograms were acquired and analysed by a Class VP Client/Server 7.2.1 program (Shimadzu). The analysis was performed with a mobile phase acetonitrile:water 15:85 (v/v) in isocratic conditions at a flow rate of 1 ml min^{-1}.

To determine the amount of DD loaded into liposomes, 100 μl of LipoDD was transferred to a 1.5 ml Eppendorf vial and centrifuged at 9615 ***g*** for 15 min (Mikro 20, Hettich, Town, Tuttlingen, Germany). The pellet and supernatant were separated and analyzed to determine DD content in liposomes and in the suspending medium, respectively. Briefly, 50 μl of supernatant was mixed with 50 μl of DNPH solution and, after vigorous mixing by vortexing, diluted to 5 ml with ethanol; the resulting solution was analyzed by HPLC. The pellet obtained by centrifugation was washed and re-suspended in FSW to a volume of 100 μl; the suspension was mixed with 100 μl of DNPH and, after complete liposome dissolution, diluted to 1 ml with ethanol. The samples were then centrifuged at 9615 ***g*** for 15 min at 4°C, the supernatant was withdrawn, diluted 1:10 with ethanol and analyzed by HPLC. Results were expressed as DD actually loaded (μg DD mg^{-1} total lipids) and encapsulation efficiency calculated as the ratio between DD actually loaded and DD theoretically loaded ×100. For each liposome formulation, DD actually loaded was determined soon after the preparation (t=0) and at 1, 3, 7, 9, 12 and 15 days, in order to follow the amount of DD administered during copepod feeding experiments.

Copepod experiments

Zooplankton were collected in the Gulf of Naples from March to November 2006 using a 200 μm mesh plankton net, and immediately transported to the laboratory in an insulated box. Mature males and females of the copepod *Temora stylifera* Dana were isolated under a Leica stereomicroscope and one male and 1 female were incubated in 100 ml crystallizing dishes containing 60 ml of 50 μm filtered seawater. After 24 h of acclimatization, couples were transferred to new crystallizing dishes containing 60 ml of 0.22 μm filtered seawater and the dinoflagellate *Prorocentrum minimum* Pavillard (Sciller) at a final concentration of 8000 cells ml^{-1}. This microalga was used as the copepod food source in our liposome experiments since it does not produce aldehydes or other oxylipins (Fontana et al., 2007).

A group of twenty *T. stylifera* couples were incubated with *P. minimum* alone, at the above mentioned concentrations, and used as the control group (hereafter referred to as Pro). In order to verify any toxic effect of liposome formulation *per se*, we first tested blank liposomes at different lipid concentrations on the feeding activity and survival of *T. stylifera* females (Table 1). Three groups of *T. stylifera* couples (N=10) were incubated as for the Pro group and a specific volume of liposome suspension was added to reach final lipid concentrations of 7.5 μg ml^{-1} (Lipo A), 4 μg ml^{-1} (Lipo B) and 2.0 μg ml^{-1} (Lipo C; Table 1). In order to test the biological activity of DD on *T. stylifera* reproduction, couples (N=10) were incubated with DD-encapsulating liposomes at the lipid concentration used for Lipo C (the lipid concentration that was found not to affect survival of *T. stylifera*) which resulted in a DD concentration of 2.9±0.23 ng ml^{-1} (hereafter referred to as LipoDD).

In another set of experiments, wild females of the copepod *Calanus helgolandicus* Claus were sorted from the zooplankton collected in the North Adriatic Sea during April 2006 and March 2007. About 30 *C. helgolandicus* females were incubated in 1 l bottles filled with 50 μm-filtered seawater and transported within 48 h to the Stazione Zoologica in Naples in an insulated box. *C. helgolandicus* females (N=10) were individually incubated in 100 ml crystallizing dishes containing 60 ml of 50 μm-filtered seawater. Males were not sorted since this species does not require remating for the continued production of viable eggs as in the case of *T. stylifera*. After 24 h of acclimatization, females were transferred to new 100 ml crystallizing dishes containing 60 ml of 0.22 μm-filtered seawater and *P. minimum* at a final concentration of 8000 cells ml^{-1} (control treatment, hereafter referred to as Pro). Another group of *C. helgolandicus* females (N=10) was incubated as for the Pro group with a liposome suspension at 6.3 μg ml^{-1} final lipid concentration (hereafter referred to as Lipo Cal; Table 1). A last group of *C. helgolandicus* females (N=10) was incubated with DD-encapsulating liposomes at the same lipid concentration as Lipo Cal (lipid concentration that was found not to affect survival of *C. helgolandicus*) which resulted in a DD concentration in the medium of 3.6±0.3 ng ml^{-1} (hereafter referred to as LipoDD Cal; Table 1).

All groups of copepods were incubated in a temperature-controlled chamber at 20°C and 12 h:12 h light:dark cycle, for 10 days. Each day, *T. stylifera* couples and *C. helgolandicus* females were transferred to new crystallizing dishes containing fresh medium. Eggs and faecal pellets were counted under a Zeiss inverted microscope; eggs were left to hatch for another 48 h and percentage egg viability was calculated as described elsewhere (Ianora et al., 1995). Survival of *T. stylifera* and *C. helgolandicus* females under different food conditions was also assessed.

Fluorescent staining

Embryos produced by *C. helgolandicus* females fed for 8 days with LipoDD were collected, rinsed in FSW and incubated for 30 min in 1 i.u. ml^{-1} chitinase as reported elsewhere (Buttino et al., 2004) to permeabilize the chitinous wall. After rinsing in FSW embryos were incubated for 30 min with Annexin V-FITC (Alexis Biochemicals, Bingham, Nottingham, UK) at a concentration of 250 μl ml^{-1} FSW, rinsed again in FSW and observed with the Zeiss inverted fluorescence microscope using 10× and 20× objectives. Green fluorescence reveals apoptosis. Annexin V-FITC is, in fact, a vital fluorescent probe able to bind the phosphatidylserine that is externalized on the plasma membrane surface during early phases of apoptotic cells (Aubry et al., 1999). Annexin V was therefore used for measuring apoptosis in the early stages of cellular cytotoxicity.

To verify whether DD-encapsulating liposomes also induced apoptosis in adults, three *T. stylifera* females fed for 6 and 10 days on Pro, Lipo D and LipoDD and three *C. helgolandicus* females fed for 9 days on Pro, Lipo Cal and LipoDD Cal were fixed in 4% paraformaldehyde in FSW for 24 h at room temperature. Before TUNEL analysis (terminal deoxy-nucleotidyl-transferase-mediated dUTP nick end labelling; Roche Diagnostics GmbH, Mannheim, Germany), copepods were frozen and thawed three times in liquid nitrogen to fracture the carapace. The cephalosome of *C. helgolandicus* females was cut to facilitate penetration of the dye. Samples were then treated as reported by Ianora et al. (Ianora et al., 2004) and observed using a Zeiss LSM META-510 confocal laser scanning microscope (CLSM) with a 488 nm wavelength argon laser and 10× or 25× water immersion objectives.

Table 1. Liposome formulations used in feeding experiments with *Temora stylifera* and *Calanus helgolandicus* copepods

Copepod species	Liposome formulation		
	Abbreviation	[Lipid] (μg ml^{-1})	[DD] (ng ml^{-1})
Temora stylifera	Lipo A	7.5	
	Lipo B	4.0	
	Lipo C	2.0	
	LipoDD	2.0	2.9±0.23
Calanus helgolandicus	Lipo Cal	6.3	
	LipoDD Cal	6.3	3.6±0.3

[DD] (2-*trans*,4-*trans* decadienal concentration) given as mean ± s.d. (N=3).
All liposome diets were supplied with the control food *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1} (Pro).

RESULTS

Giant liposomes have a mean diameter similar to the preferred food of copepods (7.0 μm) and this size did not significantly change after storage at 4°C for 1 month (data not shown). DD was entrapped with an encapsulation efficiency of about 14%, corresponding to a DD loading of about 1.9 μg mg^{-1} lipids. DD content did not significantly change during storage at 4°C for 15 days (Fig. 1). To verify the ingestion of blank liposomes at a lipid concentration of 7.5 μg ml^{-1} (Lipo A) and 4.0 μg ml^{-1} (Lipo B), faecal pellets produced by copepods were monitored during the

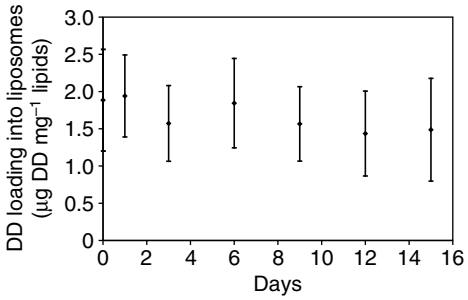

Fig. 1. 2-*trans*,4-*trans* decadienal loaded into liposomes, expressed as μg mg^{-1} (means ± s.d., *N*=3) of the total lipids during storage at 4°C for 15 days.

10 days of experiments (Fig. 2A). In the first 3 days of feeding, the mean faecal pellets produced by couples fed Lipo A and Lipo B were double the control group fed Pro (82.6; 91.9; 46.5, respectively). From day 4 to 10, faecal pellets produced by couples fed Lipo A and B decreased to <50 couple^{-1} day^{-1}. With the control food Pro, faecal pellet production was comparatively more stable than with Lipo A and B even if, on average, all groups had similar rates with a total mean production of 60.1, 58.1 and 54.1 faecal pellets couple^{-1} day^{-1} for Lipo A, B, and Pro, respectively. Experiments with Lipo A terminated on day 8 because no females survived beyond this day.

Fig. 2B shows the percentage survival of females incubated with Lipo A and B and the control Pro. Treatment with Lipo A strongly reduced *T. stylifera* female survival during the experiment: survival dropped to 45% after 7 days and all females died on day 9. Less than 30% of females survived with Lipo B at the end of the experiment whereas females fed Pro had the highest survival rate over 10 days (90.7±10.6). Since Lipo A and B affected female

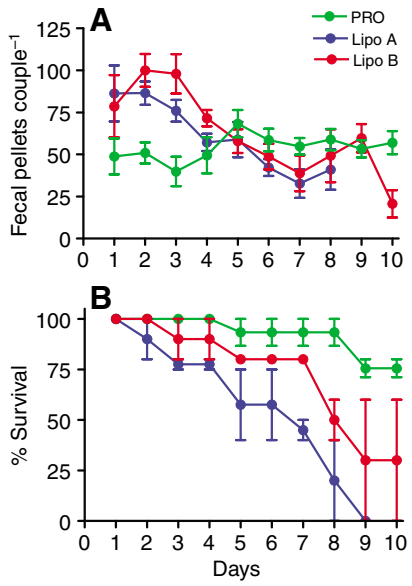

Fig. 2. *Temora stylifera* daily faecal pellet production (A) and percentage female survival (B) for couples fed liposomes at a lipid concentration of either 7.5 μg ml^{-1} lipids (Lipo A) or at 4.0 μg ml^{-1} lipids (Lipo B) supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1} and compared with a control diet of *P. minimum* alone (Pro) at the same cell concentration. Values are means ± s.e.m. (*N*=10).

survival, we used a lower liposome formulation with a concentration of 2 μg ml^{-1} lipids (Lipo C) as a carrier for decadienal.

Fig. 3 shows egg and faecal pellet production rates and egg hatching success of *T. stylifera* females fed blank liposomes (Lipo C), DD-encapsulating liposomes (LipoDD) and the control food Pro. All three groups had the highest egg production rates at the beginning of the experiment (day 1) with a mean of 48.4 eggs female^{-1} day^{-1} (Fig. 3A). Egg production decreased on day 2 to the end of the experiment in each treatment, more dramatically in the Lipo C group, with an egg production rate of <1 egg female^{-1} day^{-1} compared to 10 eggs female^{-1} day^{-1} produced by females fed Pro and LipoDD on day 10. On average, *T. stylifera* females fed Pro, LipoDD and Lipo C produced 17.1 eggs female^{-1} day^{-1}, 16.9 eggs female^{-1} day^{-1}, and 9.2 eggs female^{-1} day^{-1}, respectively, during the experiment. These values were not statistically different from each other (one-way ANOVA, $F_{2,27}$=1.08; P>0.05).

Hatching success for *T. stylifera* females fed Pro and Lipo C was very high for the duration of the experiment, ranging from 100% to 66.7%, and with a mean hatching success of 87.7% and 79.7%, respectively (Fig. 3B). By contrast, hatching success decreased in the group fed LipoDD to about 40% after 8 days of feeding. Statistical analysis showed that, on average, hatching success for females fed LipoDD (49.7%) was significantly lower than that for females fed Pro and Lipo C (one-way ANOVA, $F_{2,27}$=21.0; P<0.001; Tukey's *post-hoc* test P<0.001).

The average number of faecal pellets produced by *T. stylifera* couples fed LipoDD was 65.5 pellets couple^{-1} day^{-1} similar to those of females fed Pro (54.0 pellets couple^{-1} day^{-1}; Fig. 3C). Higher pellet production was initially recorded for couples fed Lipo C during the first 6 days of the experiment, with a mean of 97.2, but by day 7, the value resembled those of the Pro and LipoDD groups. On average, the Lipo C group produced significantly more pellets than the Pro and LipoDD groups (70.5 pellets couple^{-1} day^{-1}, one-way variance ANOVA, $F_{2,27}$=3.8; P<0.05; Tukey's *post-hoc* test P<0.05).

Fig. 3D shows the percentage survival of *T. stylifera* females after 10 days of feeding with Pro, Lipo C and LipoDD. The percentage survival was very high for both Pro and Lipo C diets during the whole experiment, with a mean of 92.4% and 95%, respectively. By contrast, the survivorship of females fed LipoDD declined steadily throughout the experiment, with only 47% live females by day 10. The average percentage survival was 76.0% in the LipoDD group, which was statistically lower than the Lipo C and Pro groups (one-way ANOVA, $F_{2,27}$=6.1; P<0.01; Tukey's *post-hoc* test P<0.05).

Egg production rate, hatching success and faecal pellet production of *C. helgolandicus* females fed Lipo Cal, LipoDD Cal and Pro are reported in Fig. 4. The daily pattern with a LipoDD diet was very stable during the experiment and similar to that of Lipo Cal and Pro groups, with an average of 8.9 eggs female^{-1} day^{-1}, 8.9 eggs female^{-1} day^{-1} and 11.7 eggs female^{-1} day^{-1}, respectively (Fig. 4A). Hatching success for the three treatments remained high until day 7 after which hatching success for females fed LipoDD Cal was reduced to <50% and fell to 0% by the end of the experiment (Fig. 4B). By contrast, Pro and Lipo Cal diets did not reduce hatching success, which was on average 76.7% and 71.6%, respectively. These values were statistically higher than those recorded for females fed LipoDD Cal (49.8%; one-way ANOVA, $F_{2,27}$=9.2; P<0.001; Tukey's *post-hoc* test P<0.01).

C. helgolandicus females had similar daily patterns for faecal pellet production when feeding on Pro and Lipo Cal (Fig. 4C),

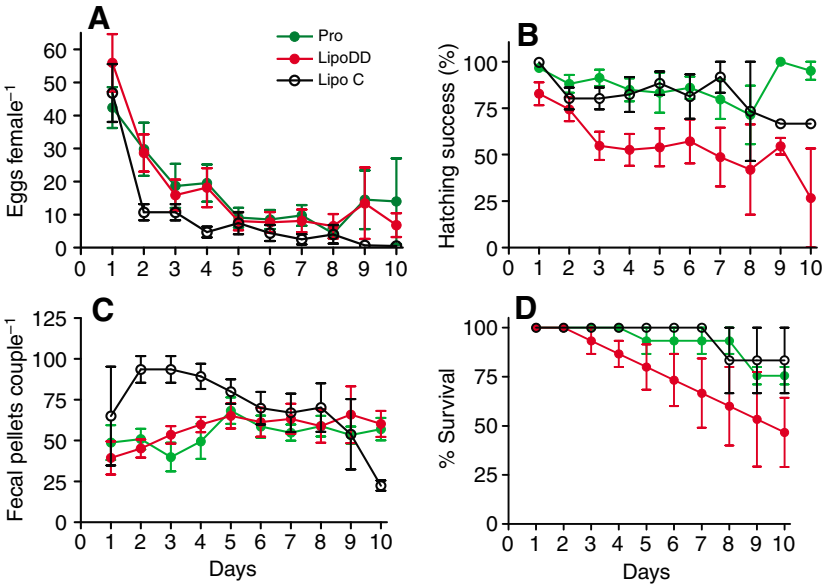

Fig. 3. *Temora stylifera* couples fed blank liposomes at a lipid concentration of 2 μg ml^{-1} (Lipo C) and liposomes encapsulating decadienal (DD) at 2.9 ng ml^{-1} (LipoDD) supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1}, and the control diet *P. minimum* alone (Pro) at the same cell concentration. (A) Daily egg production rates per female; (B) percentage egg hatching success; (C) faecal pellet production rates per couple; (D) percentage female survival. Values are means ± s.e.m. (*N*=10).

with an average of 53.8 pellets female^{-1} day^{-1} and 55.5 pellets female^{-1} day^{-1}, respectively. A higher number of pellets was produced by females fed LipoDD Cal, with an average of 80 pellets female^{-1} day^{-1}. This value was statistically higher than those recorded for the Pro and Lipo Cal groups (one-way ANOVA, $F_{2,27}$=13.7; P<0.001; Tukey's *post-hoc* test P<0.001).

Survival of *C. helgolandicus* Pro and Lipo Cal groups slightly decreased over the experiment, but remained above 70% on day 10 (Fig. 4D); only 45% of the females in the LipoDD Cal group were alive by the end of the experiment. However, the average percentage female survival was not statistically different from the other groups (one-way ANOVA, $F_{2,27}$=2.08; P>0.05).

To verify if DD-encapsulating liposomes induced apoptosis in embryos, *C. helgolandicus* embryos produced by females fed LipoDD Cal for 8 days were stained with annexin V-FITC. Most of these embryos fluoresced green indicating that an apoptotic process had started (Fig. 5A). By contrast, embryos in the control Lipo Cal group appeared dark (Fig. 5B), as for the control group fed Pro (not shown).

To verify if liposomes encapsulating DD induced apoptosis also in adults, *C. helgolandicus* females fed for 9 days on LipoDD Cal were stained with TUNEL. Fig. 6A shows the whole prosome strongly fluorescent, indicating the occurrence of apoptosis in these body tissues. At the highest magnification, the oocyte cavities in the ovary show small green fluorescent spots (Fig. 6B). By contrast, *C. helgolandicus* females fed Lipo Cal (Fig. 6C) and Pro (image not shown) were not fluorescent. An induction of apoptosis was also observed in *T. stylifera* females fed for 6 days on the LipoDD suspension and stained with TUNEL (Fig. 7). Confocal laser scanning sections revealed that one of the two gonads was positively stained during the apoptotic process (Fig. 7A,B). Control females fed Lipo C did not show any fluorescence except for an external autofluorescence of the chitinous wall (Fig. 7C). The same was true for females of the Pro group (image not shown). *T. stylifera* females fed LipoDD suspension for 10 days were stained green throughout the entire body, including muscles, suggesting that all organs underwent an apoptotic process in these females (Fig. 8A,B).

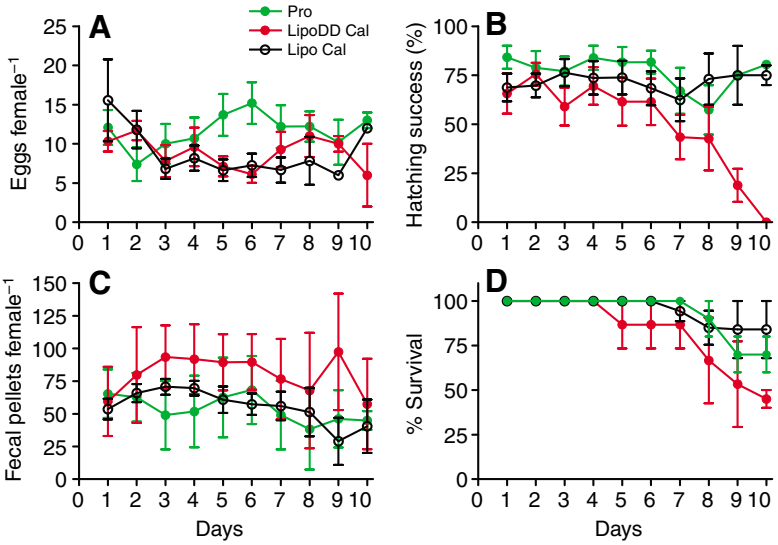

Fig. 4. *Calanus helgolandicus* females fed blank liposomes at a lipid concentration of 6.3 μg ml^{-1} (Lipo Cal) and liposomes encapsulating decadienal at 3.6 ng ml^{-1} (LipoDD Cal) supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1}, and the control diet of *P. minimum* alone (Pro) at the same cell concentration. (A) Daily egg production rates per female; (B) percentage egg hatching success; (C) faecal pellet production rates per female; (D) percentage female survival. Values are means ± s.e.m. (*N*=10).

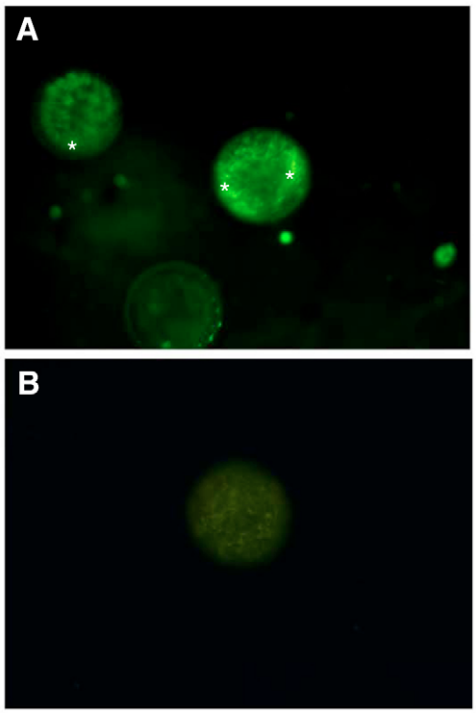

Fig. 5. *Calanus helgolandicus* embryos produced by females fed for 8 days with liposomes encapsulating decadienal at a concentration of 3.6 ng ml^{-1} (LipoDD Cal; A) and blank liposomes at a lipid concentration of 6.3 μg ml^{-1} lipid (Lipo Cal; B) and supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1}. Embryos were stained with the vital fluorescent probe Annexin V-FITC. Green fluorescence indicates that nuclei were apoptotic (asterisks). Magnification: ×200.

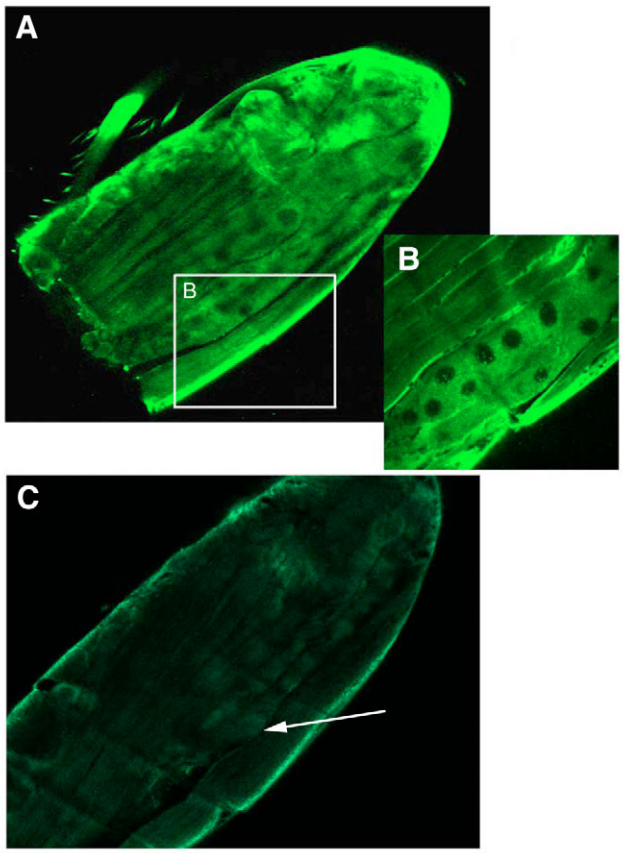

Fig. 6. *Calanus helgolandicus* females fed for 9 days with liposomes encapsulating decadienal at a concentration of 3.6 ng ml^{-1} (LipoDD Cal; A,B) and blank liposomes at a lipid concentration of 6.3 μg ml^{-1} lipid (Lipo Cal; C) and supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1}. TUNEL was used to detect apoptosis. Green fluorescence indicates that tissues were apoptotic. Arrow in C indicates the same region of the gonad as in B but no green fluorescence is present. Magnification: (A,C) ×100; (B) ×250.

DISCUSSION

In a previous study, we demonstrated that giant liposomes were efficiently ingested by the copepod *Temora stylifera* (Buttino et al., 2006). These results suggested that liposomes could be a very useful tool to deliver bioactive molecules into the gastrointestinal tract of aquatic organisms. Here we prepared giant liposomes encapsulating DD to unequivocally clarify the role of polyunsaturated diatom-derived aldehydes in copepod reproduction. Experiments in which copepods had been incubated in the presence of DD had already been carried out (Miralto et al., 1999; Ianora et al., 2004). However, in these studies the relationship between the observed effect and the amount of DD ingested by copepods was probably overestimated due to the high volatility of this aldehyde in the incubation medium (Miralto et al., 1999). Moreover, DD may have been transformed prior to delivery when live carriers were used (Ianora et al., 2004). By contrast, liposomes are inert carriers and able to entrap molecules with different chemical-physical characteristics. In particular, hydrophilic molecules can be entrapped into the aqueous internal cavity, whereas lipophilic compounds can enter into the lipid bilayer. This is what occurs with DD, a very lipophilic molecule that can be dissolved in the organic solution containing the lipid mix, before liposome preparation. We found that DD loading into liposomes did not significantly change during the time frame considered, probably due to a hydrophobic interaction between DD and the lipids. Therefore, we performed long-term incubation experiments with DD-encapsulating liposomes and tested the effects of these on the reproduction of two copepod species. Blank liposomes were also tested to evaluate lipid toxicity on copepods, with faecal pellet production used as a proxy for copepod ingestion.

Interestingly, our results show that the production of faecal pellets increased when *T. stylifera* females were fed a mixed diet of liposomes and Pro with respect to the single diet Pro. Previous results (Buttino et al., 2006) demonstrated that copepod ingestion rate, calculated using radiolabelled cholesterol, was double when liposomes where supplied together with Pro. An increase in the number of faecal pellets was also recorded during the experiments using lipo DD. However, whereas the number of pellets produced by *T. stylifera* was significantly higher with a diet of blank liposomes than with LipoDD, for *C. helgolandicus* the opposite was true. Much more LipoDD was ingested by *C. helgolandicus* than liposomes without DD. These results suggest different behaviors for the two copepod species, with one presumably more attracted by the 'flavor' of decadienal than the other. Odorous compounds such as the aldehyde decatrienal were liberated into water after cell disruption of some benthic diatoms, acting as a repellent to pelagic freshwater crustaceans (Jüttner, 2005). Such repellence consisted of reduced swimming movements and grazing activity. In our experiments, a similar repellent reaction was not evident, at least in one of the two species.

An increase in liposomes ingested was not matched by increased egg production rates for either copepod species, confirming that liposomes had no supplementary effect on egg production as also

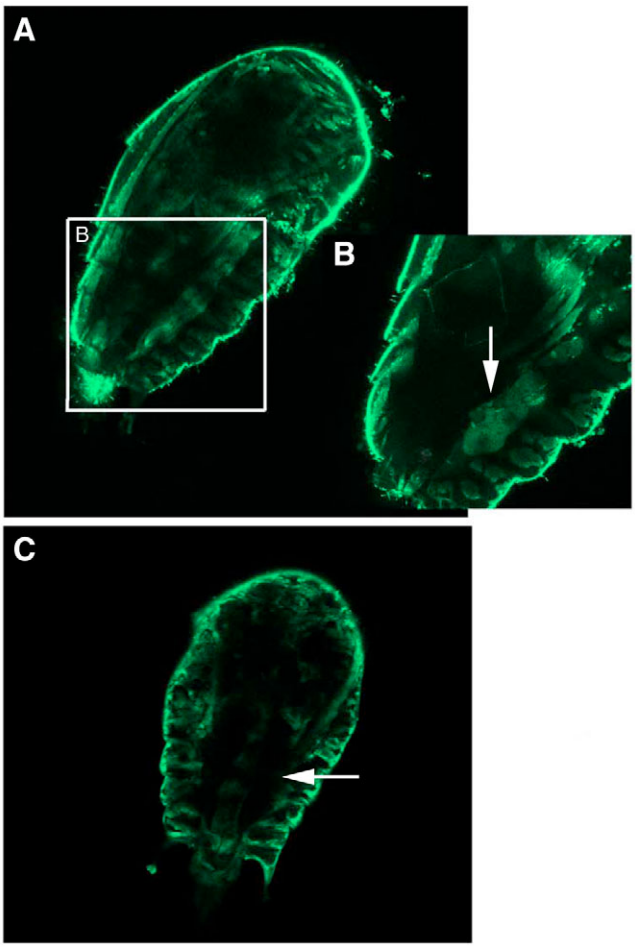

Fig. 7. *Temora stylifera* females fed for 6 days with liposomes encapsulating decadienal at a concentration of 2.9 ng ml^{-1} (LipoDD; A,B) and blank liposomes at a lipid concentration of 2.0 μg ml^{-1} lipid (Lipo C; C) and supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1}. TUNEL was used to detect apoptosis. The green fluorescence in the gonad (arrow in B) indicates that tissues were apoptotic. In C, the arrow indicates the same region of the gonad as in B but no green fluorescence is present. Magnification: (A,C)×100; B ×250).

reported by Buttino et al. (Buttino et al., 2006). Our results showed that when liposomes were supplied at lipid concentrations ranging from 7.5 to 4.0 μg lipids ml^{-1} (Lipo A and B) most *T. stylifera* females were dead a few days later and no adults survived more than 8 days with a Lipo B formulation. This effect was not attributed to lipid toxicity but rather to the obstruction of copepod mouthparts by highly concentrated liposome particles that were observed in some females (I.B., personal observations). This is why we used the lowest lipid concentration to incubate DD into liposomes for this species. By contrast, *C. helgolandicus* did not show a reduction in adult survival at the liposome formulation used. Different responses among the two species suggest that blank liposome incubations must be assessed for each copepod species before using them in toxicological experiments.

Until now, the toxicity of PUAs in an aqueous medium has only been tested by exposing organisms *in vitro* to dissolved PUAs in water or by using PUA-producing diatoms as food. Our results indicate that the concentration affecting hatching success in both copepod species was one order of magnitude lower than those recorded in previous incubation experiments [e.g. 1 μg ml^{-1} in incubation experiments (Miralto et al., 1999; Ceballos and Ianora, 2003; Taylor et al., 2007)]. Concentrations blocking hatching success in our experiments were much lower in both *T. stylifera* (97 ng DD) and *C. helgolandicus* (121 ng DD) considering filtration rates of 0.14 ml h^{-1} per copepod (Buttino et al., 2006) and incubation concentrations of 2.9 ng ml^{-1} and 3.6 ng ml^{-1} DD, respectively, for 10 days. Adolph et al. (Adolph et al., 2004) calculated similar values for feeding experiments where 30 ng ml^{-1} potential oxylipins equivalent concentrations reduced hatching success to 30% after 5 days of feeding on a diatom diet.

Another interesting finding here is that when the percentage of egg viability for *C. helgolandicus* was below 25% and adult survival was less than 50% (Fig. 4B,D), gonad tissues of some females appeared positively stained by TUNEL (Fig. 6A), suggesting that they were undergoing apoptosis. The apoptotic region corresponded to oocyte 3-stage producers (OS3) (Poulet et al., 2007). Follicle chambers with apoptotic body-like spots were similar to structures found in histological sections by these authors (Fig. 6B). Poulet and co-workers reported a concomitant arrest of OS3 maturation, characterized by cell fragmentation and by the presence of apoptotic bodies, when *C. helgolandicus* females were fed some diatom diets that reduced both egg production and hatching. Our results also indicate a concomitance between a reduction in egg hatching success and induction of apoptosis even if egg production was similar to the control. Because TUNEL detects early apoptotic events, it is probable that eggs are released but they die later, and the observed effect indicates a reduction in hatching success rather than fecundity. Similarly, a concomitance between TUNEL positivity and a reduction in hatching success (≈50%) occurred for *T. stylifera* females fed LipoDD for 6 days (Fig. 3B, Fig. 7A,B).

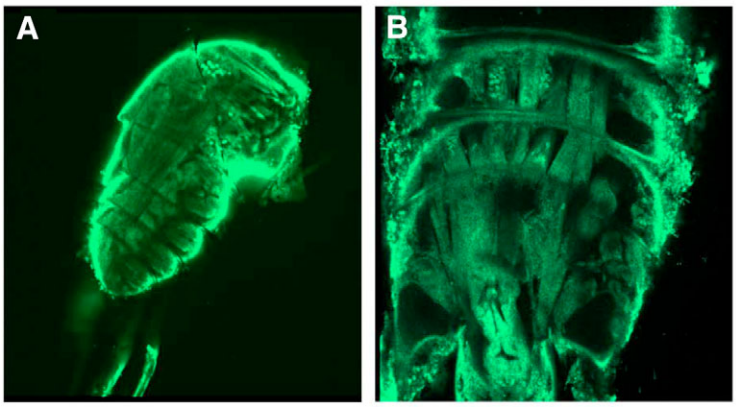

Fig. 8. *Temora stylifera* females fed for 10 days with liposomes encapsulating decadienal at a concentration of 2.9 ng ml^{-1} (LipoDD; A,B) and supplied with *Prorocentrum minimum* at a concentration of 8000 cells ml^{-1}. TUNEL was used to detect apoptosis. Green fluorescence indicates that tissues were apoptotic. Magnification: (A) ×100; (B) ×250.

Moreover, our results also show, for the first time, that DD affected adult survivorship. Ceballos and Ianora (Ceballos and Ianora, 2003) reported a similar reduction in adult survival when *T. stylifera* was fed the diatom *Skeletonema costatum* for more than 10 days. Presumably this decrease in adult survival was due to DD and not to poor food quality. Females of both species appeared strongly positive for apoptosis coincidentally with reduced survival (≤50%; Fig. 8A,B, Fig. 3D). Hence the mechanism of chemical defense in diatoms not only functions by reducing grazing effects of subsequent generations of copepods, as hitherto believed, but also targets the direct predator. These compounds are of lower acute toxicity to adult predators compared to other feeding deterrents such as dinoflagellate toxins even though they eventually induce death if ingested for a sufficient length of time, and lead to post-digestive reduction in fecundity or depressed viability of the offspring. Grazing pressure is thus reduced, allowing diatom blooms to persist when grazing pressure would otherwise have caused them to crash.

An important application of our work is the possibility of delivering a known quantity of toxin and being able to calculate the efficiency of adsorption and longevity of the toxins once encapsulated. Liposomes have been extensively used in the pharmaceutical and chemical industries for the past 30 years and in aquaculture to deliver food supplements in the diet [Buttino et al. (Buttino et al., 2006) and references therein]. However, until recently liposomes >6 μm were unstable over multiday experiments (Ravet et al., 2003) and were only used for short-term incubations. Here we were able to produce giant (≥7 μm diameter) DD-encapsulating liposomes that were very stable over time allowing us to carry out long-term incubations necessary to test the effects of PUAs on copepod reproductive fitness. Further studies using liposomes are in progress to test synergistic or antagonistic effects of different chemicals and varied nutritional content of copepods, providing a tool to qualitatively and quantitatively analyze the impact of toxins and nutrient supplements on copepod grazers.

LIST OF ABBREVIATIONS

DD	2-*trans*,4-*trans* decadienal
DNPH	2,4-dinitrophenihydrazine
FSW	filtered seawater
HPLC	high-performance liquid chromatography
LipoDD	DD-encapsulating liposome
PUA	polyunsaturated aldehyde
ROS	reactive oxygen species
SPC	soybean lecithin

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