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Giant liposomes as delivery system for ecophysiological studies in copepods

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Summary

Giant liposomes are proposed as a potential delivery system in marine copepods, the dominant constituent of the zooplankton. Liposomes were prepared in the same size range as the food ingested by copepods (mean diameter of about 7 μm). The encapsulation of a hydrophilic and high molecular mass fluorescent compound, fluorescein isothiocyanate-dextran (FitcDx), within the liposomes provided a means of verifying copepod ingestion when viewed with the confocal laser-scanning microscope. Females of the calanoid copepod *Temora stylifera* were fed with FitcDx-encapsulated liposomes alone or mixed with the dinoflagellate alga *Prorocentrum minimum*. Control copepods were incubated with the *P. minimum* diet alone. Egg production rates, percentage egg-hatching success and number of faecal pellets produced were evaluated after 24 h and 48 h of feeding. Epifluorescence of copepod gut and faecal pellets indicated that the liposomes were actively ingested by *T. stylifera* in both experimental food conditions, with or without the dinoflagellate diet. Ingestion rates calculated using ^{3}H-labelled liposomes indicated that females ingested more liposomes when *P. minimum* was added to the solution (16% *vs* 7.6% of uptake). When liposomes were supplied together with the algal diet, egg production rate, egg-hatching success and faecal pellet production were as high as those observed for the control diet. By contrary, egg production and hatching success were very low with a diet of liposomes alone and faecal pellet production was similar to that recorded in starved females. This results suggest that liposomes alone did not add any nutritive value to the diet, making them a good candidate as inert carriers to study the nutrient requirements or biological activity of different compounds. In particular, such liposomes are proposed as carriers for diatom-derived polyunsaturated aldehydes, which are known to impair copepod embryo viability. Other potential applications of liposomes as a delivery system of drugs and nutrients in copepod mass cultivation, or as carriers of pollutants to study copepod physiology in ecotoxicological experiments, are also discussed.

Key words: *Temora stylifera*, *Prorocentrum minimum*, giant liposome, diatom, unsaturated aldehyde, copepod egg viability, delivery system, confocal microscopy, feeding experiment.

Introduction

Copepods are the most abundant planktonic grazers in aquatic ecosystems, ranging from 30 to 80% of the total zooplankton biomass in marine areas (Longhurst, 1985). Since they represent the principal diet for many fish larvae, fluctuations in copepod biomass can dramatically influence fish species abundances at higher trophic levels (Beaugrand et al., 2003). Copepod egg production rates and egg-hatching success are key biological parameters to predict secondary production at sea (Poulet et al., 1995a), both of which are strongly influenced by quality and/or quantity of the available food (Ianora et al., 2003).

Several studies have focused on the biochemical composition of copepod diets in terms of fatty acids and amino acids (Jónasdóttir and Kiørboe, 1996; Kleppel et al., 1998; Laabir et al., 1999). Commonly, the methods used are to incubate copepods in different algal cultures with known biochemical composition and then compare reduction of egg production and hatching success to the lack of some essential nutrients. However, these methods are circuitous and more direct methods, e.g. microparticulate carriers (Caldwell et al., 2004) are required to assess the nutritional requirements of copepods.

Food quality can also be related to the presence of phytoplankton toxicants that negatively impact copepod reproductive fitness. For example, several studies have shown that some diatoms strongly reduce copepod egg viability or induce malformations in newly hatched larvae (Ianora et al., 2004 and references therein). The compounds responsible for these

effects are linear poly-unsaturated aldehydes (PUAs) produced by some diatom species within seconds after crushing of cells, as occurs during grazing by copepods (Miralto et al., 1999; Pohnert, 2000). Inhibition of egg-hatching success and teratogenic effects induced by these compounds were first demonstrated through classical feeding experiments, in which ripe copepod females were fed uni-algal diatom cultures (Ianora and Poulet, 1993; Poulet et al., 1995b). Successively, the negative effects of PUAs on embryo viability in different invertebrates were studied by directly incubating embryos in known concentrations of pure molecules (Caldwell et al., 2002; Caldwell et al., 2003; Romano et al., 2003; Tosti et al., 2003) or using the alga *Prorocentrum minimum* as live carrier cells (Ianora et al., 2004). However *in vitro* incubations are not appropriate to study *in vivo* effects mediated by female diets for several reasons: (i) in natural conditions, females are exposed to these compounds after grazing and crushing of the diatom cells; (ii) owing to the high volatility of these compounds, their concentration in the water may change with time; (iii) toxicants that are freely dissolved in the water may not only be ingested by females, but also adsorbed through the exoskeleton; (iv) live carrier cells could metabolically transform active molecules in non-active ones. Hence, new methods using carriers for nutrients, antibiotics or other substances to enhance copepod production for aquaculture, or to study the effects of algal toxicants, could be very advantageous.

Here we propose giant liposomes as a delivery system of different bioactive molecules, including essential nutrients or potential toxicants such as diatom PUAs, to study their effects on the physiology of copepods. Liposomes are small particles composed of a lipidic bilayer surrounding one or more aqueous cavities, largely used in the pharmaceutical and cosmetic fields. When natural lipids are employed, liposomes are biodegradable and not toxic; furthermore, the presence of hydrophobic and hydrophilic portions makes liposomes a very versatile carrier, allowing for the encapsulation of both lipophilic and hydrophilic compounds (Crommelin, 1994). In aquaculture, small liposomes have already been used as nutrient supplements or as drug carriers for first-feeding marine fish larvae (Koven et al., 1995) and the brine shrimp *Artemia* sp. (Hontoria et al., 1994; Touraki et al., 1995; Ozkizilcik and Chu, 1994). However, they have never been used as a delivery system for copepods. In this study, liposomes were prepared with a mean size in the same range as the food ingested by copepods. A fluorescent dye was encapsulated to follow copepod ingestion and palatability with the aid of confocal laser scanning microscopy (CLSM), and liposome ingestion rates were calculated using ^{3}H-labelled liposomes. Copepod egg production rate, percentage egg viability and faecal pellet production were also assessed in short-term feeding experiments, to evaluate the effect of the liposome diet alone, or combined with a dinoflagellate species, on copepod reproduction.

Material and methods

Materials

Cholesterol, sea salts, fluorescein isothiocyanate–dextran (FitcDx; average 70 kDa) and Triton X-100 were purchased from Sigma-Aldrich (Milan, Italy). Phosphatidylcholine from soybean lecithin (SPC) was kindly provided by Lipoid GMBH (Cam, Switzerland). Analytical grade chloroform and methanol were supplied by Carlo Erba Reagenti (Milan, Italy). Glutaraldehyde and osmium tetroxide were provided by TAAB Laboratories Equipment Ltd (Berkshire, UK). [7(n)-^{3}H]cholesterol was supplied by Amersham Biosciences (Milan, Italy).

Liposome preparation

Liposomes were prepared by a modified hand-shaking method (Bangham et al., 1965). Briefly, a lipid mixture containing 110 mg of SPC and 40 mg of cholesterol in 5 ml of a chloroform/methanol solvent mixture (2:1 v/v) was introduced into a 250 ml round-bottomed flask. In the case of ^{3}H-labelled liposomes (LIPOR), 1 mCi (37 MBq) of [7(n)-^{3}H]cholesterol, corresponding to 0.12% (w/w) of the total cholesterol, was added to the lipid mix. 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 blanks and ^{3}H-labelled liposomes or fluorescently labelled liposomes (LIPOF; FitcDx-encapsulating liposomes), the lipid film was hydrated with 5 ml of 0.22 μm filtered seawater (FSW) or FSW containing 0.05% (w/v) fluorescein isothiocyanate-dextran (FitcDx), respectively. The resulting suspension was gently mixed in the presence of glass beads (0.5 g) 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. After preparation, FitcDx loaded liposomes were washed two times as follows: the suspension was centrifuged at 7200 ***g*** for 20 min, the supernatant was removed, replaced with FSW, and resuspended by vortex. All liposome formulations were stored at 4°C under nitrogen until use in copepod feeding experiments.

Liposome characterization

The mean diameter and size distribution of liposomes were determined 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.) of values collected from three different batches.

Confocal laser scanning microscopy (CLSM; LSM-410, Zeiss, Jena, Germany) using a 63× water immersion objective was used to obtain images of LIPOF, after dilution in FSW and mounting with Confocal Matrix (Micro Tech Lab, Graz, Austria).

For transmission electron microscopy (TEM), liposomes were centrifuged at 7200 ***g*** for 20 min, the supernatant was removed and liposomes were fixed in 1% (v/v) glutaraldehyde solution in FSW for 1 h. After post-fixation in 1% osmium tetroxide (w/v) for 1 h and dehydration in a graded alcohol series, liposomes were embedded in EPON® 812 and sectioned with a Reichert ultramicrotome (Hamburg, Germany). Sections

were stained with uranyl acetate and lead citrate and examined with a Philips 400 transmission electron microscope (Eindhoven, The Netherlands).

To measure the amount of FitcDx entrapped within the liposomes, 0.9 ml of liposome suspension was mixed with 0.1 ml of Triton X-100 and fluorescence was quantified using a luminescence spectrometer (Perkin-Elmer Ltd, Buckinghamshire, UK). Encapsulation efficiency was calculated as percentage ratio between the amount of FitcDx entrapped and that initially added within liposomes. The results are presented as mean ± s.d. of three different experiments.

Copepod feeding experiments

Zooplankton were collected in the Gulf of Naples (Italy) from July 2003 to May 2004 with a 200 μm mesh net and transported, in an insulated box, to the laboratory where ripe male and female *Temora stylifera* (Dana) copepods were immediately sorted. *T. stylifera* couples were incubated individually in 50 ml crystallizing dishes containing 30 ml of 0.22 μm FSW (starved group; time 0). After 24 h, 25 couples were transferred to crystallizing dishes containing 30 ml FSW and the dinoflagellate *Prorocentrum minimum* Pavillard (Sciller) (PRO), in the exponential growth phase, at a final concentration of approx. 4×10^3 cells ml^{-1}, corresponding to a carbon daily intake of 720 μg C l^{-1} (Carotenuto et al., 2002). Another group of 25 couples was incubated in 30 ml FSW containing approx. 4×10^5 LIPOF ml^{-1}, and a third group of 25 couples was incubated in 30 ml FSW containing both PRO and LIPOF (LIPOF+PRO) at the same concentrations reported above. Liposome concentrations were assessed with the Coulter Multisizer II (Beckman Coulter). Each group of copepods was incubated in a temperature-controlled incubator at 20°C and on a 12 h:12 h light:dark cycle. After 24 h, couples were transferred to new crystallizing dishes containing either PRO, LIPOF or LIPOF+PRO, at the same concentrations, and eggs and faecal pellets were counted with an inverted Zeiss microscope. Percentage egg viability was calculated 48 h after spawning, as described by Ianora et al. (1995), by counting empty membranes and hatched nauplii. Egg production rates, egg viability and faecal pellet production were determined 24 and 48 h after feeding on PRO, LIPOF and LIPOF+PRO.

In a separate experiment we also quantified copepod ingestion rates of liposomes, with and without PRO, using liposomes containing [7(n)-^{3}H]cholesterol (LIPOR). After 24 h starvation, three female *T. stylifera* were incubated in 50 ml bottles containing 30 ml of FSW and 30 μl of LIPOR (2×10^5 liposome ml^{-1}) or LIPOR + PRO (6000 cells ml^{-1}). After 30 min females were collected on a filter, rinsed in SW, placed individually in scintillation vials with 450 μl of Solvable® (Perkin-Elmer, Groningen, The Netherlands), and incubated overnight at 60°C. To differentiate radioactivity of non-ingested liposomes adhering to the copepod body from those ingested, the same experiments were performed with females killed with 4% formalin, and incubated for 30 min in LIPOR or LIPOR + PRO suspensions. Experiments were performed in triplicates. Activity (in d.p.m.) was measured in 5 ml of scintillation cocktail (Ultima Gold, Packard-Bioscience, Milan Italy) using a Beckman LS 6500 liquid scintillation counter.

Filtration rate (ml copepod^{-1} h^{-1}) was calculated as: $(R_f–R_d)0.45$ ml/$(R_s–R_{sw})30$ min, where R_f is the radioactivity of the female, R_d is the radioactivity of dead females; R_s is the radioactivity of the liposome suspension, and R_{sw} is the radioactivity of the seawater, all in d.p.m.

Confocal laser scanning microscopy

Temora stylifera fed PRO, LIPOF and LIPOF+PRO for 24 and 48 h, were observed with CLSM to detect gut fluorescence. Live fed and starved females were mounted on a slide, immobilized with a coverglass, and observed by CLSM using an inverted microscope (Zeiss LSM-410) equipped with a 10× water immersion objective. 488 and 543 nm wavelength (λ) lasers were used to excite fluorescein and chlorophyll, respectively. Each female was optically *z*-sectioned and the final image was reconstructed three-dimensionally using the Zeiss software. Light-transmitted images were acquired, at a single faecal plane, using a 633 nm λ laser. Focal pellets, produced by females and males fed PRO, LIPOF and LIPOF+PRO for 24 and 48 h, were also observed using CLSM, with the same setting reported above, to detect chlorophyll and FitcDx fluorescence.

Results

Liposome preparation was optimized in terms of lipid mix composition and hydration conditions in order to obtain giant vesicles in a dimension range suitable to be retained by copepods. The selected liposome formulation had a mean diameter of 6.8±0.2 μm and a narrow size distribution (size ranging from >1 to <20 μm), which remained dimensionally stable when stored at 4°C for 15 days (Fig. 1). Liposome morphology was investigated by CLSM and TEM; CLSM images of LIPOF suspension in fluorescent mode, transmitted light, and the superimposition of the first two, are reported in Fig. 2A–C, respectively. Liposomes appeared as multilamellar structures delimiting a large aqueous cavity, which contained

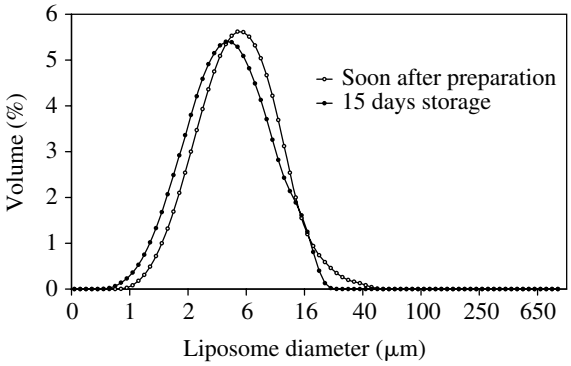

Fig. 1. Size distribution of liposomes soon after preparation and after 2 weeks of storage at 4°C.

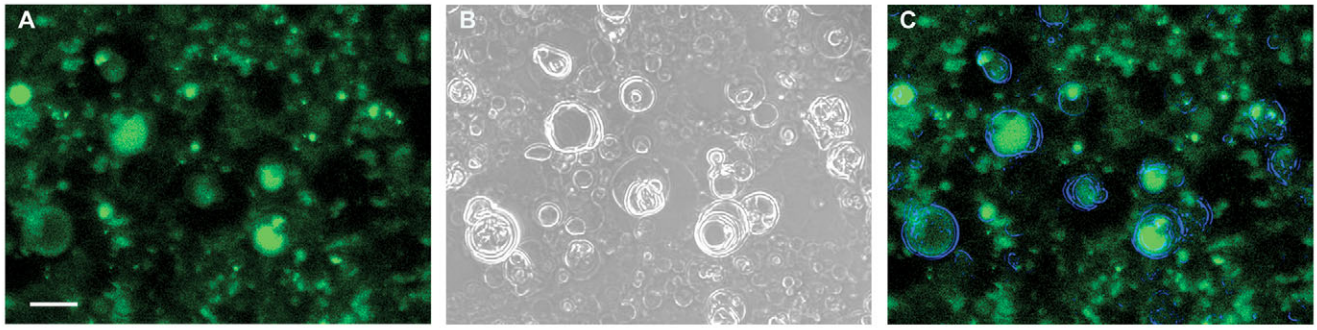

Fig. 2. Confocal laser scanning microscopy of FitcDx-encapsulating liposomes (LIPOF) in fluorescence (A) and in transmitted (B) mode; (C) merged image of A and B. Lipidic membranes are digitally coloured in blue. Bar, 13.0 μm.

the fluorescent dye. TEM revealed liposome morphology in greater detail (Fig. 3). Images showed vesicular structures consisting of large cavities surrounded by a thick wall, confirming liposome morphology observed with CLSM. Spectrophotometric analysis of LIPOF allowed for the quantification of the FitcDx entrapped in the aqueous cavities; we found an encapsulation efficiency of about 17±1.5% of the FitcDx initially used in the liposome preparation.

Fig. 4 shows fluorescent, reconstructed three-dimensional images of *T. stylifera* females (Fig. 4A,C,E,G) and the same images in transmitted light (Fig. 4B,D,F,H), observed using CLSM. Females that had been starved for 24 h showed only an external autofluorescence (yellow colour) due to the chitinous wall (Fig. 4A). The chitin lining the oesophagus also appeared autofluorescent while no fluorescence was detected inside the body. In transmitted light, dark and mature gonads and diverticula of the oviducts were clearly visible (Fig. 4B). The gut of females fed the dinoflagellate PRO for 24 and 48 h has strong red fluorescence, due to ingested phytoplankton cells (Control, Fig. 4C). In transmitted light (Fig. 4D), fluorescent regions appeared dark and the faecal pellet was clearly visible in the hindgut (Fig. 4D). Females fed the diet LIPOF for 24 and 48 h, showed a strong green fluorescence in the gut, indicating that liposomes had been ingested (Fig. 4E). Fig. 4G-H is an example of a female fed the mixed diet LIPOF+PRO for 24 h; the oesophagus and hindgut emitted both green and red fluorescence. In transmitted light, the same fluorescent regions appeared dark and the faecal pellet was clearly visible in the hindgut (Fig. 4H).

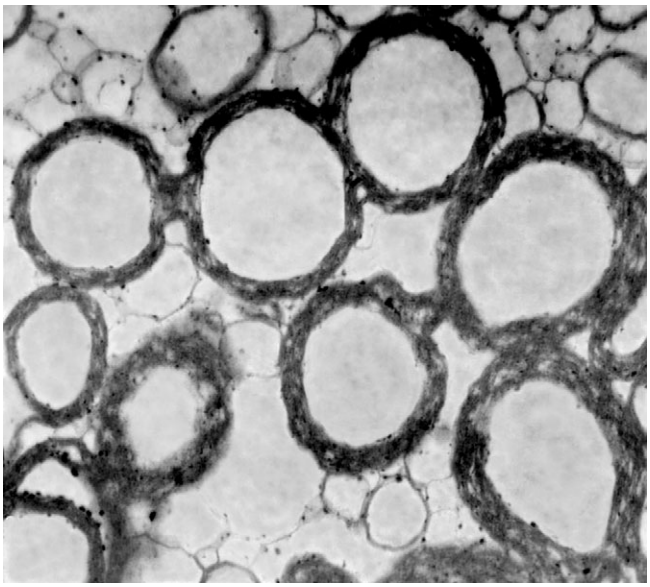

Fig. 3. Section of a liposome suspension observed with transmission electron microscopy (TEM; ×17 000).

Ingestion rate calculated using ^{3}H-labelled liposomes was higher when females were fed with a mixed diet of liposomes and *P. minimum* (28515.5±10832.2 liposomes female^{-1} h^{-1}; mean ± s.d.) compared to a diet of liposomes alone (14161.6±1930.5 liposomes female^{-1} h^{-1}). These values correspond to a 16% and 7.6% uptake of liposomes, respectively. Student's *t*-test analysis confirms that the difference is statistically significant (P<0.05; t=2.26; d.f.=4), suggesting that the presence of the algal food was necessary to enhance feeding by the copepods.

Faecal pellets, produced by *T. stylifera* fed PRO, LIPOF and LIPOF+PRO, were collected and observed with CLSM in fluorescence and transmitted mode (Fig. 5). Fig. 5A shows an example of faecal pellets produced by copepods fed the dinoflagellate PRO for 24 h. PRO cells were clearly visible inside the pellet, because of the red fluorescence of chlorophyll. In transmitted light, the same faecal pellet consisted mainly of empty dinoflagellate cell walls (Fig. 5B). Faecal pellets produced by copepods fed LIPOF for 24 h (Fig. 5C) showed only green fluorescence and in transmitted light they appeared dense and dark (Fig. 5D). Copepods fed LIPOF+PRO produced faecal pellets that fluoresced both green and red (Fig. 5E). In transmitted light, they appeared dark and their structure was similar to faecal pellets produced by copepods fed LIPOF (Fig. 5F).

Egg and faecal pellet production were calculated for groups of females fed the diets PRO, LIPOF and LIPOF+PRO for 24 and 48 h. At the start of the experiments (time 0), the females produced 34±4.5 eggs per female (mean ± s.e.); the rate decreased after 24 h when females were fed PRO, LIPOF and

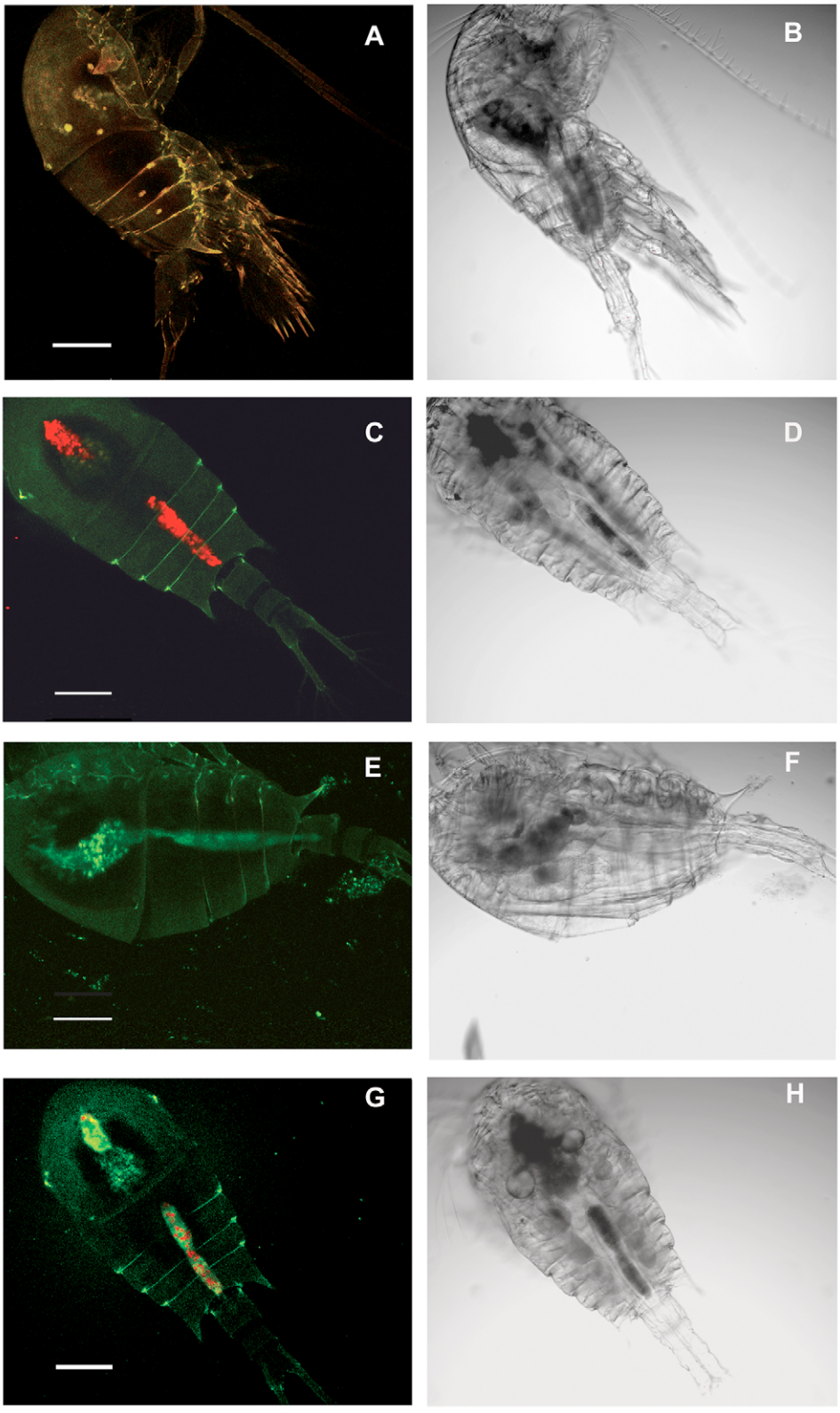

Fig. 4. *Temora stylifera* female copepods. (A,C,E,G) Reconstructed three-dimensional confocal laser scanning microscopy images obtained using both the 488 and 543 nm λ lasers to excite fluorescein and chlorophyll, respectively. (B,D,F,H) The same copepods as in A,C,E,G, seen in transmitted light. (A,B) A starved female (time=0). In A the chitinous wall is autofluorescent, the yellow colour is the result of superimposition of green and red fluorescence. (C,D) A female fed the dinoflagellate *Prorocentrum minimum* for 48 h. In C the red fluorescence inside the gut is due to chlorophyll emission. (E,F) A female fed FitcDx-encapsulating liposomes (LIPOF) for 48 h. In E the green fluorescence inside the gut is due to the emission of FitcDx. (G,H) Fluorescent 3-D image of a female fed a mixed diet of LIPOF and *P. minimum* for 24 h. In G the green fluorescence in the gut does not overlap with the red fluorescence of chlorophyll. Bar, 0.2 mm.

LIPOF+PRO (Fig. 6A). In particular, females fed PRO and LIPOF+PRO for 24 h had similar egg production rates with mean values of 13.8±2.4 and 12.1±3.2 eggs female^{-1}, respectively, whereas the lowest egg production rate (2.7±0.9 eggs female^{-1}) was found for females fed LIPOF for 24 h. Statistical analysis [one way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test] showed that the number of eggs produced by females fed LIPOF for 24 h was

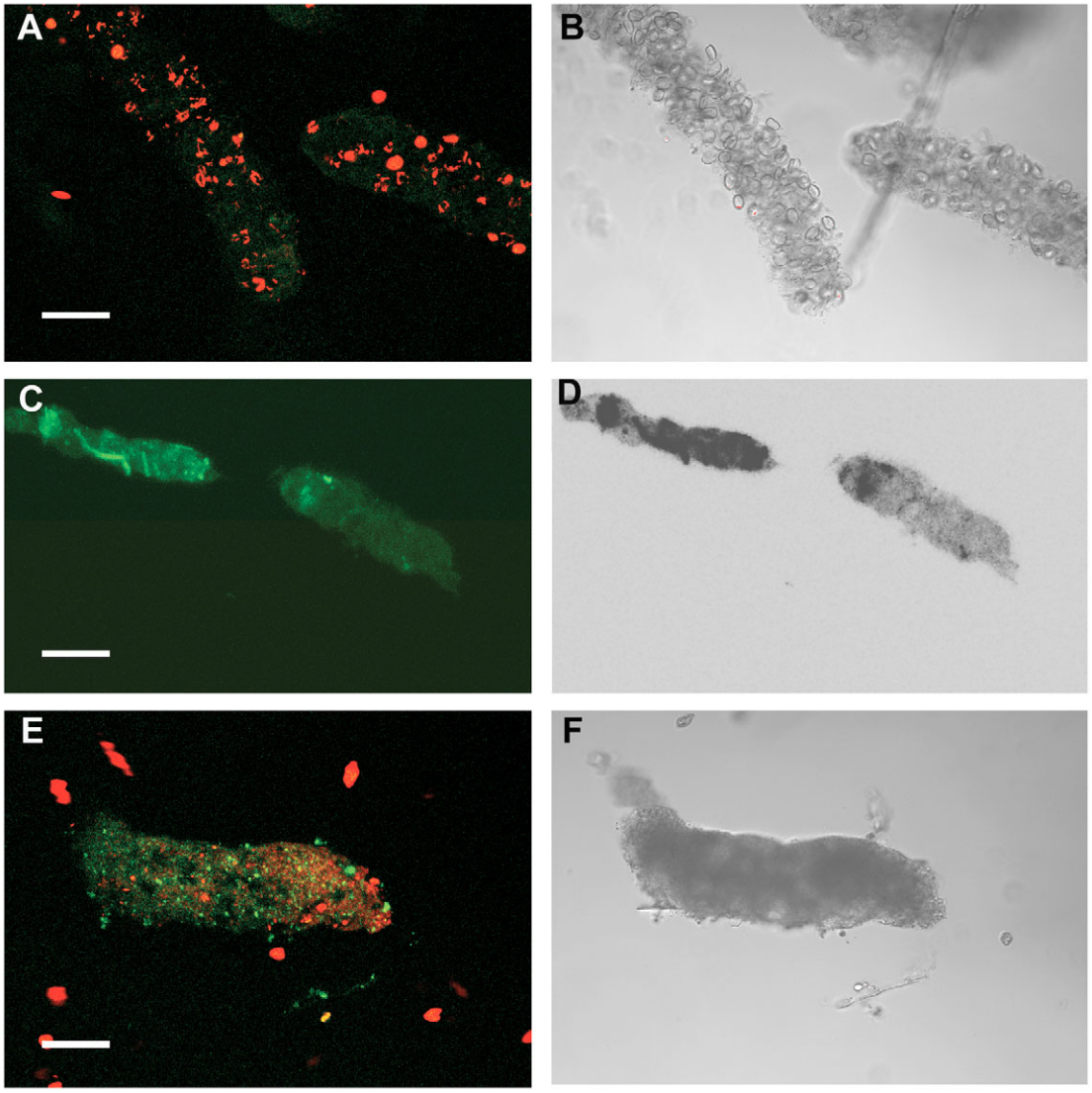

Fig. 5. Faecal pellets of *Temora stylifera* male and female copepods. Reconstructed three-dimensional confocal laser scanning microscopy images obtained using both 488 and 543 nm λ lasers to excite fluorescein and chlorophyll, respectively (A,C,E) and observed in transmitted light (B,D,F). (A,B) Faecal pellets produced by copepods fed the dinoflagellate *Prorocentrum minimum* for 24 h. (A) The red fluorescence is due to chlorophyll. Bar, 61.4 μm. (B) The faecal pellets can be seen to contain mainly *Prorocentrum minimum* cell walls. (C,D) Faecal pellets produced by copepods fed FitcDx-encapsulating liposomes (LIPOF) for 24 h. The green fluorescence in C is due to FitcDx. Bar, 80.0 μm. (E) Faecal pellets produced by copepods fed a mixed diet of LIPOF and *P. minimum* for 48 h. In E the green spots are due to FitcDx and the red spots are due to chlorophyll. Bar, 76.5 μm.

significantly lower than that produced by females fed PRO and LIPOF+PRO for 24 h (Table 1). After 48 h of feeding, egg production rates, calculated for females fed PRO and LIPOF+PRO, remained constant at 14.7±3.3 and 10.1±3.0 eggs female^{-1}, respectively, whereas those fed LIPOF for 48 h produced only 0.7±0.4 eggs female^{-1}. This value was statistically lower than that recorded for females fed the PRO diet (Table 1).

Initial egg viability was 86–88% in all females (Fig. 6B). This value remained constant after feeding on the dinoflagellate diet PRO for 24 h (91.9±3.0) and 48 h (96.1±2.9). Percentage egg viability decreased slightly after 48 h, to 79.4±8.7%, in females fed LIPOF+PRO, but this value was not significantly different from the PRO diet (Table 1). However, percentage egg hatching success calculated for females fed LIPOF, dropped dramatically to 49.1±12.8% after 24 h, and to 37.5±23.9% after 48 h. These values were significantly lower than those recorded for both PRO and LIPOF+PRO diets after 24 and 48 h (Table 1).

Initial faecal pellet production rates were very low (mean of 11.5 faecal pellets couple^{-1}) but increased after 24 h with PRO (30.8±3.8) and doubled with LIPOF+PRO (57.8±3.4) diets. By contrast, the number of faecal pellets produced by couples fed LIPOF for 24 and 48 h, remained similar to that recorded at

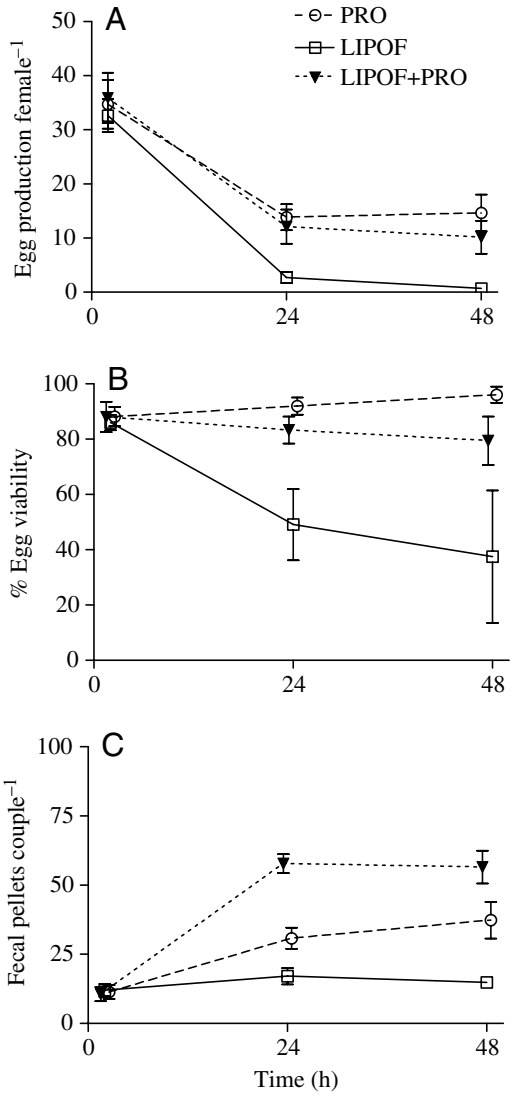

Fig. 6. *Temora stylifera*. Effect of different diets, *Prorocentrum minimum* (PRO), FitcDx-encapsulating liposomes (LIPOF) and both (PRO+LIPOF), on egg production rate (A), percentage egg viability (B) and faecal pellet production (C).

time zero (17.1±2.9 and 14.9±1.8, respectively). All three treatments showed statistically different faecal pellet production rates (Table 1), with maximum production recorded for couples fed the LIPOF+PRO diet.

Discussion

Giant liposomes, with dimensions (mean diameter of about 7 μm) suitable for retention by feeding appendages of copepods, remained stable for up to 15 days (Fig. 1). Our findings differ from those reported by Ravet et al. (Ravet et al. 2003), who found that liposomes >5 μm diameter were unstable and unsuitable for multiday periods. This difference is probably due to the different composition and preparation conditions of liposomes used in the two studies.

Liposomes observed with TEM and CLSM appeared as vesicles formed by a single large aqueous cavity surrounded by a thick membrane. A similar morphology has already been described for multilamellar vesicles composed entirely of neutral lipids, which tend to form very packed multilayer assemblies, with the adjacent bilayer stacked very closely upon the other, and with very little aqueous space between them (New, 1990). The sealing of bilayer sheets, observed in TEM images, may also have contributed to high liposome stability during storage.

Liposomes were loaded with FitcDx to follow the fate of liposomes in the copepod gut. We used a fluorescent dye covalently bonded to a high molecular mass hydrophilic compound to limit the escape of the fluorescent dye from the aqueous cavities. FitcDx was successfully entrapped within liposomes with an encapsulation efficiency higher than usually expected with this preparation technique for hydrophilic compounds (New, 1990). Females fed liposomes alone produced the lowest number of eggs, suggesting that liposomes were not sufficient to sustain copepod reproduction. As a consequence of this very low egg production (<1 egg female^{-1}), percentage egg hatching success was highly variable, ranging from 0–85%. In addition, the number of faecal pellets produced with the

Table 1. *Results of egg production, egg viability and faecal pellet production of Temora stylifera fed the diets Prorocentrum minimum, Liposomes or Liposomes + P. minimum, for 24 h and 48 h*

	Time fed (h)							
	24				48			
	PRO	LIPOF	LIPOF+PRO	ANOVA	PRO	LIPOF	LIPOF+PRO	ANOVA
Egg production (eggs female^{-1} day^{-1})	A	B	A	$F_{2,65}$=6.59 (P<0.001)	A	B	A,B	$F_{2,49}$=5.85 (P<0.01)
Egg viability (%)	A	B	A	$F_{2,43}$=11.45 (P<0.001)	A	B	A	$F_{2,20}$=8.23 (P<0.001)
Fecal pellet (pellets couple^{-1} day^{-1})	A	B	C	$F_{2,65}$=35.28 (P<0.001)	A	B	C	$F_{2,49}$=12.23 (P<0.001)

Diets were *Prorocentrum minimum* (PRO), Liposomes (LIPOF) and Liposomes + *P. minimum* (LIPOF+PRO).
Treatments with the same letter were not significantly different (one-way ANOVA).

liposome diet alone was similar to the number produced by starved animals (time 0). With a mixed diet (dinoflagellate and liposomes together), however, both egg production rate and egg viability were similar to values recorded for females fed the control diet. This suggests that the liposome formulation *per se* had no supplementary effect on copepod egg production and did not impair egg-hatching success. Interestingly, the number of faecal pellets produced with the mixed diet LIPOF+PRO was double that recorded for the control diet PRO, indicating that significant numbers of liposomes were ingested when they were included in the algal diet. Previous studies have shown that copepods can discriminate between flavoured or unflavoured foods (De Mott, 1988; Kerfoot and Kirk, 1991). Our results on liposome ingestion rate, calculated using radiolabelled cholesterol, confirm that liposomes were actively ingested by copepods, in both experimental feeding conditions, with or without the dinoflagellate diet. However, with a diet of liposomes and *P. minimum*, copepods grazed twice as much compared to a diet of liposomes alone. Also in other experiments with inert particles, copepods grazed more if particles were coated with fresh algal exudates (Kerfoot and Kirk, 1991). In our protocol, the presence of algal cells increased ingestion rates with respect to an unflavoured liposome diet.

Recently, several authors have speculated on the use of liposomes as a standardized carrier system for diatom-derived PUAs in order to calculate the relationship between ingestion of these PUAs and toxicity in copepods (Caldwell et al., 2004; Paffenhöfer et al., 2005). Since ingestion of diatoms reduce copepod embryo viability and block embryonic divisions in several other marine organisms (Buttino et al., 1999; Miralto et al., 1999; Caldwell et al., 2002; Caldwell et al., 2003; Romano et al., 2003; Tosti et al., 2003) there is a need to better understand the molecular and cellular target(s) of these compounds. Here, we propose liposomes as a vehicle system to relate aldehyde ingestion to reproductive response, such as egg mortality or induction of teratogenesis, in copepods. Moreover, liposomes containing labelled compounds, such as radioisotopes, or fluorescent probes, could also be used as carriers to verify the fate of toxins in grazers. At present, diatom species have been shown to produce several different short-chain PUAs (Miralto et al., 1999; d'Ippolito et al., 2002a; d'Ippolito et al., 2002b; d'Ippolito et al., 2003); liposomes might make it possible to differentiate the effect of one chemical with respect to another, or to study the antagonistic/synergic effect of different diatom-derived aldehydes on the reproductive physiology of copepods. Testing the combined effects of different chemicals is, in fact, a requisite to understand phytoplankton-herbivore interactions at sea. A major debate in the study of diatom-copepod interactions has focused on whether reduced hatching is due to the lack of some essential nutrients in diatoms, or to the presence of antimitotic compounds (Ianora et al., 1999; Paffenhöfer et al., 2005). Since liposomes do not add any nutritional value to the diet, they could also be used as carriers of specific nutrients to evaluate if a deficiency of nutritive compounds reduces egg and embryo viability in copepods. The use of liposomes could clarify this controversy, differentiating toxic from nutritive effects.

On a more general basis, our giant liposomes could be used in studies on food quality effects and nutritional requirements of herbivorous copepods, a topic that has been poorly addressed in copepods compared to other zooplankters. For example, the nutritional needs for growth and reproduction in the freshwater crustacean, *Daphnia* sp., have been extensively investigated using different supplementation methods such as lipid emulsions (De Mott and Müller-Navarra, 1997), bovine serum albumin beads (von Elert and Wolffrom, 2001), inert algal carriers (von Elert, 2002; Becker and Boersma, 2003) and liposomes <5 μm diameter (Ravet et al., 2003). To our knowledge, only Hasset (Hasset, 2004) used homogenate or gelatine-acacia microcapsules, as carriers for cholesterol, to supplement copepod diets. In comparison with these carriers, liposomes seem to be more versatile because of the possibility of entrapping molecules of a different chemical nature.

Liposomes have already been employed for nutrient enrichment of brine shrimp (*Artemia salina*) larvae, which is one of the key organisms used as food for larval fish rearing in commercial mariculture (for a review, see Coutteau and Sorgeloos, 1997), and have recently been proposed as carriers of nutrients and therapeutic agents in mollusc aquaculture (Lai et al., 2004). Considering the increasing attention that copepods are receiving as alternative live preys to *Artemia* (Støttrup, 2000; McKinnon et al., 2003), we suggest the use of liposomes as specific nutrient and/or drug carriers for copepod mass cultivation in aquaculture.

Since marine copepods constitute an important pathway in the trophic transfer to top-level carnivores, they are recommended in baseline studies for xenobiotic toxicity (AMAP, 1995). Another application for liposomes could therefore be in ecotoxicological studies; until now, toxicity of chemicals on copepods have been tested by dissolving contaminants in the water, or by exposing copepods to contaminated food (Hook and Fisher, 2001; Willis and Ling, 2004). As an alternative, giant liposomes could be used to test toxicity of poorly water-soluble substances. This technique has already been applied to test pesticide toxicity on the freshwater cladoceran *Daphnia* (Fliedner, 1997). Our liposomes could be used to deliver different pollutants to copepods, allowing for more in-depth studies on bioaccumulation processes through the marine food chain.

List of abbreviations

CLSM	confocal laser scanning microscope
FitcDx	fluorescein isothiocyanate-dextran
FSW	filtered seawater
LIPOF	FitcDx-encapsulating liposomes
LIPOR	^{3}H-labelled liposomes
PRO	*Prorocentrum minimum*
PUAs	polyunsaturated aldehydes
SPC	phosphatidylcholine
TEM	transmission electron microscope

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