SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 28, No. 1, pp. 123–132, 2009
© 2009 SETAC
Printed in the USA
0730-7268/09 $12.00 + .00

# FATTY ACID COMPOSITION OF THE ESTUARINE AMPHIPOD, *MELITA PLUMULOSA* (ZEIDLER): LINK BETWEEN DIET AND FECUNDITY

ROSS V. HYNE,*† FRANCISCO SÁNCHEZ-BAYO,‡ ASHLEY D. BRYAN,§ EMMA L. JOHNSTON,§ and REINIER M. MANN‡

†Centre for Ecotoxicology, Department of Environment and Climate Change,
‡Centre for Ecotoxicology, Department of Environmental Sciences, University of Technology–Sydney, P.O. Box 29, Lidcombe, New South Wales 1825, Australia
§Evolution and Ecology Research Centre, School of Biological, Earth and Environmental Sciences, University of New South Wales, New South Wales 2052, Australia

(*Received* 2 *May* 2008; *Accepted* 10 *July* 2008)

**Abstract**—The influence of various diets on the survival, fecundity, and the polyunsaturated fatty acid (PUFA) composition of the benthic estuarine amphipod *Melita plumulosa* (Zeidler) in laboratory cultures were determined. Apart from a natural silty sediment, six commercial food supplements were examined: an omega-6 PUFA enriched *Spirulina*-based dry powder, Sera® micron; a shrimp-based pellet food; an omega-3 PUFA enriched algal paste, Rotiselco®-ALG; an omega-6 PUFA enriched algal dry powder, AlgaMac-ARA (arachidonic acid); flaxseed meal; and an omega-3 PUFA enriched dry powder, Frippak®. We have previously established that *M. plumulosa* cultures perform poorly and eventually decline if provided with silty sediment alone, but will thrive if supplemented with Sera micron. Conversely, if the amphipods are cultured on a nutrient-depleted sand substrate, Sera micron alone does not constitute an adequate feed. The major difference in the fatty acid composition of *M. plumulosa* cultured on silty sediment compared to amphipods cultured on a sand substrate and both fed Sera micron was an increase in the ratio of omega-3 to omega-6 PUFAs, indicating that the silty sediment provides additional food sources rich in omega-3 PUFAs. Furthermore, amphipods cultured in sand and fed any of the three algal-based foods or the Frippak powder as the sole food source had poor survival rates, although Sera micron maintained the best survival—this was attributed to it containing high amounts of β-carotene and terpenoids. *Melita plumulosa* fed a mixture of Sera micron in conjunction with the omega-3 PUFA enriched Rotiselco-ALG and cultured on a silty substrate were found to have good fecundity with low variability.

**Keywords**—Polyunsaturated fatty acids    Amphipod    Fecundity    Carotenoids    Omega-3 fatty acids

## INTRODUCTION

*Melita plumulosa* is an epibenthic amphipod that has been recently adopted in Australia as a test organism for the evaluation of estuarine sediments using acute and sublethal sediment toxicity tests [1,2]. This gammarid amphipod is ideal for sediment toxicity testing as it obtains its food through scavenging detritus materials rather than through filter feeding alone. The impacts of contaminated sediments on *M. plumulosa* are clear but there is variability in the fecundity of gravid females in the control sediments [1,3], which presents a limitation in the use of reproduction as an endpoint in toxicity testing. A contributing factor for the variable fecundity may be the dietary requirements of the amphipod which are ill-defined. We have previously observed that *M. plumulosa* derives essential nutrients from silty sediment that are not supplied by the added *Spirulina*-based food, Sera micron [4].

Lipid accumulation is the most widespread long-term energy strategy in aquatic crustaceans and their reproductive potential is largely dictated by lipid content [5–7]. Triacylglycerol is the main storage lipid in benthic amphipods and in females of two iteroparous gammarid amphipods, with total lipid accumulated and the percentage of triacylglycerol increasing as the ovary matured [6]. In addition, eggs contain proportionally more polyunsaturated fatty acids (PUFAs) than females or males [6,8]. Polyunsaturated fatty acids are fatty acids of 18 carbons or more in length with two or more double bonds. Depending on the position of the first double bond proximate to the methyl end of fatty acids, PUFAs can be classified into two major groups, omega-6 or omega-3 families [9]. Fatty acids are obtained through diet or synthesized from two dietary essential fatty acids, linoleic acid (LOA, C18:2n-6) and α-linolenic acid (α-LA, C18:3n-3), via a desaturase–elongase system [9].

A key step to optimizing the essential fatty acid requirements is determining the dietary requirements for LOA and α-LA and other PUFAs. Dietary sources of fatty acids for marine invertebrates are derived either from plants, phytoplankton, or animal detritus. Normally only a small proportion of dietary LOA and α-LA can be converted to long PUFAs and most are β-oxidized to provide energy [10,11]. Essential dietary PUFAs include the plant-derived LOA and α-LA and some long chain fatty acids, which are essential for early crustacean egg development [12]. Among these, the most important long chain PUFAs are the omega-3 eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3), or docosapentaenoic acid (DPA, C22:5n-3) and the omega-6 fatty acid arachidonic acid (ARA, C20:4n-6).

Fatty acids such as EPA and DHA cannot be produced by most crustacean species in sufficient quantities for metabolic functioning [13], so they must be obtained from the diet [8,14,15]. Polyunsaturated fatty acids are important structural components of cells that confer membrane fluidity and selec-

* To whom correspondence may be addressed (ross.hyne@environment.nsw.gov.au).
Published on the Web 8/19/2008.

tive permeability. Docosahexaenoic acid and ARA occur in high concentrations in neuronal tissues such as the brain and in testis [16,17]. Several studies have shown that a key factor governing the nutritional food quality for *Daphnia magna* and other cladocerans is the essential fatty acid content and composition of their food [18,19]. Eicosapentaenoic acid is selectively stored in eggs of *D. magna* and reproduction is therefore the major drain of EPA from females. A diet that does not provide adequate amounts of fatty acids can potentially affect the life cycle of a crustacean as these acids play an essential role in cell membrane physiology and hormone metabolism [16,19]. Arachidonic acid is a precursor in the production of eicosanoids, which are signaling molecules that exert complex control over many bodily systems, mainly in inflammation or immunity, and are countered by dietary omega-3 PUFAs [16].

The objective of the present study was to determine the dietary PUFA requirements of *M. plumulosa* in laboratory cultures and subsequently to reduce the variability in the fecundity of *M. plumulosa* by manipulating the fatty acid composition of its diet.

## MATERIALS AND METHODS

### *Seawater and substrate*

Seawater was collected from Port Hacking, New South Wales, Australia, and stored in a fiberglass tank outside the laboratory until required. Seawater was filtered (0.45 μm), adjusted to the test salinity using Sydney tap water that had been passed through a mixed bed filter and activated carbon filter, and then allowed to stand for six weeks for dechlorination. It was then stored in a plastic tank in the laboratory to allow acclimation to the test temperature.

Control silty sediment was collected from Bonnet Bay, in the lower Woronora River that drains into Port Hacking, Sydney, Australia. This site was chosen because the land use in the catchment is primarily national park, with isolated urban areas, so the measured sediment-bound and dissolved porewater concentrations of metals and organic contaminants were low [20]. The Bonnet Bay sediment was suitable for a substrate as it was a hydrous (64–70% water) and sub-oxic (<0.5 μmol/g acid-volatile sulfide) silty sediment (96–99% particles <63 μm), with a pore-water salinity and pH of 29‰ and 7.3, respectively [3,20]. The subsurface layer (2–4 cm) of sediment was collected using hand shovels and was press-sieved (1.1 mm) on site. Sediment was stored in plastic bags at 4°C for a maximum of two months.

Clean Sydney sand (180 μm < particle size < 1 mm) was purchased from Greenlife (Hornsley Park, Sydney, Australia) and washed with 25‰ seawater prior to use as an alternate substrate for the amphipod cultures.

### *Test organisms*

*Melita plumulosa* were cultured in the laboratory following procedures described by Hyne et al. [4]. Gravid females were distinguished from males, under a dissecting microscope, by the much smaller gnathopod 2 and the presence of eggs in the brood pouch. The amphipod cultures were fed fine-powdered fish fry food, Sera micron (0.5 mg per amphipod), and Sera Fishtamins (20 μl/L of overlying water) twice per week and the overlying 25‰ seawater was changed weekly.

### *Feeding experiments*

The feeding experiments were conducted in 1-L glass beakers. Test beakers contained 50 g of sediment or sand and were filled to 800 ml with seawater (25‰) one day before the tests began. Each feeding experiment consisted of three or four treatments replicated three times. For the duration of the feeding tests, beakers were kept in a temperature-controlled room at 24°C ± 1.0°C with a 12:12 h light:dark cycle and a light intensity of 200 to 600 lux. Cling wrap was placed tightly over the top of each beaker to limit evaporation, prior to being continuously lightly aerated.

On the day of test commencement, 80% of the water was drained from each beaker and replaced with fresh 25‰ seawater to remove any oxidized metals and compounds that often appear after the equilibration period of the sediment [20]. Physico-chemical parameters were measured in the overlying water before the addition of amphipods and at the end of the test, as described in detail previously [4]. To each replicate, consisting of paired beakers, 10 adult male amphipods were transferred with a plastic pipette to one beaker and 10 adult females added to the other beaker of that replicate pair. All treatments consisted of three replicate paired beakers containing 10 sexually mature amphipods of each sex housed separately for 14 d, to give a total of 60 amphipods for each treatment. Separation of sexes was necessary to ensure any eggs or embryos carried by females would be released at the end of that period of time [3], so that all females could be ready for mating in the subsequent phase in order to assess the fecundity resulting from a particular diet.

The treatments were then fed twice per week with each food type at a dose of 0.5 mg of food or food mixture per animal. Each food type or food mixture was prepared in a 25‰ seawater slurry that was continuously mixed on a magnetic stirrer, and 5 mg in 100 μl were transferred by pipette into each test beaker. The amphipods in the control treatment were fed exclusively Sera micron; all other treatments were fed a 1:1 (by weight) mixture of Sera micron and one of the alternative foods (see below).

After 14 d, the 10 male amphipods were removed from each replicate pair using a plastic pipette and were combined with the 10 nongravid females from the same food treatment. Following a further 5 d, surviving adults of both sexes were recorded and females from each replicate were transferred to 250 ml beakers that were labeled appropriately with the food type and replicate number. A plastic pipette was used to transfer each female amphipod to a watch glass containing minimal 25‰ seawater. Embryos were then gently teased from the marsupium of the female with fine tweezers before being counted and recorded, as described previously [3].

### *Food type*

Sera micron was obtained from Sera (Heinsberg, Germany); Rotiselco-ALG (INVE Technologies, Dendermonde, Belgium), Grobest T1 shrimp feed (Grobest, Beenleigh, Queensland, Australia), and Frippak No. 1 CAR encapsulated feed (INVE Technologies) were supplied by Primo Aquaculture (Coffs Harbour, NSW, Australia); AlgaMac-ARA (Aquafauna Bio-Marine, Hawthorne, CA, USA) was obtained from Aquatic Diagnostic Services International (Banora Point, NSW, Australia); and flaxseed meal was purchased from Stony Creek Oil Products (Talbot, VIC, Australia).

In an experiment investigating the effectiveness of various food types in supporting the fecundity of *M. plumulosa* cultured in silty sediment, the following four food treatments were initially tested: Sera micron only, Sera micron and shrimp pellet, Sera micron and omega-3 PUFA enriched Rotiselco-

ALG, or Sera micron and omega-6 PUFA enriched AlgaMac-ARA. In a second similar experiment, *M. plumulosa* cultured in silty sediment were fed Sera micron only, Sera micron and omega-3 PUFA enriched Rotiselco-ALG, Sera micron and omega-3 PUFA enriched Frippak, or Sera micron and omega-3 PUFA enriched flaxseed.

In a third food type experiment *M. plumulosa* cultured in sand were fed the following four food treatments to test for their ability to maintain the survival of the amphipods: Sera micron only, AlgaMac-ARA only, Rotiselco-ALG only, and Frippak only.

### *Extraction and esterification of fatty acids in food*

Fatty acids from each food type were extracted by the addition of 4 ml of a dichloromethane:methanol (2:1 v/v) mixture to 30 mg of each food type in a 5-ml Reacti-vial (Alltech, Grace Davidson, Sydney, Australia), based on the procedure described by von Elert and Stampfl [21]. After securing the lid, the vial and its contents were vigorously shaken by hand for 2 min. The Reacti-vials were then placed in a plastic centrifuge tube (50 ml, Falcon), and centrifuged at 2,000 *g* for 5 min. Using a pasteur pipette, the supernatant was then transferred to a 20-ml scintillation vial and evaporated to near dryness under a gentle stream of nitrogen.

The procedure used for esterification of the fatty acid extracts was based on the methylation method described by Mason et al. [22]. To the dried extract in each scintillation vial, 3 ml of 3 N methanolic HCl (Supelco, Sigma-Aldrich, Sydney, Australia) was added and the vial covered with a lid. The vial was incubated in a water bath at 50 to 60°C for 15 min. After the incubation, the vial was removed from the water bath and allowed to cool down on the bench at room temperature (23°C).

The methylated fatty esters were extracted by the addition of 3 ml of *n*-hexane to the scintillation vial together with 20 μl of internal standard solution (370 mg/L) of methyl nonadecanoate (C19:0). With the lid on, each vial was shaken gently by hand to mix the solvent with the esterified mixture. Using a pasteur pipette, the upper hexane layer was transferred to a new scintillation vial. The extraction procedure was repeated a second time, and the extracts combined in the new vial. The extracts were then evaporated to near dryness under a gentle stream of nitrogen, redissolved in 1 ml of *n*-hexane, and transferred to a 2-ml gas chromatograph (GC) vial (Alltech, Grace Davison, Sydney, Australia) for analysis.

The remaining aqueous phase was neutralized with 3 ml of 1 N NaOH and filtered (0.2 μm) to remove small amounts of soap that formed in the process. The unsaponifiable filtered extract was analyzed using a liquid chromatograph (LC) coupled to a triple quadrupole (QQQ) mass spectrometer (MS) as described below.

### *Extraction and methylation of fatty acids in amphipods*

Surviving animals collected from the various food treatments were placed on absorbent paper prior to weighing, so as to eliminate any excess water. The amphipods were grouped by sex, and females were sorted into gravid or nongravid groups. Approximately 30 mg amphipod wet weight (~12 males or 20 females) was needed for each analysis. After weighing, the amphipods were placed in a 5-ml glass pestle homogenizer (Glas-Col, Terre Haute, IN, USA). Following the addition of 1 ml of a dichloromethane:methanol (2:1 v/v) mixture, the animals were homogenized using the glass pestle until the homogenate did not contain any large particles. After allowing the homogenate to settle, the supernatant was transferred to a Reacti-vial. An additional 3 ml of the dichloromethane:methanol (2:1 v/v) mixture was then added to the homogenizer tube to extract again the remaining particles, and after settling, the supernatant was transferred to the Reacti-vial to make 4 ml of combined extract. The Reacti-vial was then placed in a plastic centrifuge tube (50 ml, Falcon), and centrifuged at 2,000 *g* for 5 min. Using a pasteur pipette, the supernatant was then transferred to a 20-ml scintillation vial and evaporated to near dryness under a gentle stream of nitrogen. The esterification and extraction of methylated fatty esters were then undertaken as described above for the food.

### *GC analysis of methylated fatty acids*

The analysis of the methylated fatty acids was undertaken using a Varian 3400 GC (Varian Associates, Walnut Creek, CA, USA) fitted with a Varian 8200 autosampler and a flame ionization detector on a fused silica HP-5MS column 30 m × 0.25 id × 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA), based on the procedures described by von Elert [23] and Tokuşoglu and Ünal [24]. The instrumental conditions for the analysis of the methylated fatty acids were as follows: the injector temperature was set at 250°C and held for 35 min and the detector temperature was set at 250°C. The chromatograph was programmed for an initial temperature of 150°C for 1 min, then raised to 170°C at 5°C/min and followed by a temperature ramp to 220°C at 2°C/min; the final temperature was held for 5 min to give a total running time of 35 min. The autosampler used a 10-μl injection using standard automated mode and the peak threshold was set at 10,000. Methylated fatty acid ester standards were purchased from Sigma Aldrich (Sydney, Australia). The detection limit was 1 μg/100 mg dry weight. A conversion factor of wet:dry weight of 4.65 and 4.34 was used for the female and male amphipods, respectively.

### *LC-MS analysis of unsaponifiable fractions*

It was observed that extracts from amphipods cultured in sand and fed Sera micron had a distinctive yellowish color, particularly in the aqueous phase, whereas those from amphipods fed other food types were colorless. The analysis of the unsaponifiable fraction of the aqueous phase was undertaken using an Agilent 1200 series LC coupled to a QQQ mass spectrometer (Wilmington, DE, USA) and Atmospheric Pressure Chemical Ionization to determine the nature of the compounds responsible for the color. A rapid resolution silica column (Zorbax Eclipse XDB-C18, 2.1 × 50 mm and 1.8-micron particle size; Agilent) at 40°C was used. The mobile phase was a mixture of methanol and methyl *tert*-butyl ether (70:30 v/v) and the flow rate 0.1 ml/min. The injection volume was 1 μl. Ionization conditions were as described by Lacker et al. [25] for analysis of carotenoids.

### *Statistical analysis*

The percent survival of amphipods and gravidity (% gravid females per total number of surviving females) were calculated from the raw data. Differences in the amphipod survival between the different food treatments were assessed by analysis of variance (ANOVA). Box and whisker plots [26] were plotted for the brood size of surviving gravid females in the various food treatments using Sigma Plot® (Ver 7, www.insightful.com). The relationships between the fatty acid composition of the amphipods and diet as well as the amphipods' sex or gra-

vidity were determined by the multivariate ordination method of principal components analysis, using S-plus® 2000, version 3 (Insightful, Seattle, WA, USA). Performance indicators of the treatments, i.e., fecundity and survival, were correlated to the loadings on the main components so as to assess any possible association with fatty acid composition. Differences in fecundity among food treatments were assessed by the nonparametric Mann–Whitney *U* test, whereas the variance of the brood size of the various food treatments was assessed with pair-wise use of either Levene's or Brown–Forsythe's tests [27].

## RESULTS

### *Fatty acid composition of the various food types*

The PUFA composition of the various food types selected for use as food for *M. plumulosa* was initially determined. Sera micron was dominated by omega-6 PUFAs, particularly the essential omega-6 metabolic precursor LOA, which makes up 19%, and gamma linoleic acid (γ-LA, C18:3n-6), which makes up an additional 10% of the total fatty acid content (Table 1). The saturated palmitic acid (PA, 16:0), was the major fatty acid forming 28% of the total fatty acid content. The omega-3 to omega-6 PUFAs ratio was $<1$ in Sera micron. Similarly, the omega-6 enriched algae, AlgaMac-ARA, was dominated by ARA, which made up to 30% of its fatty acid composition.

In contrast, the Rotiselco-ALG algal paste was enriched with omega-3 PUFAs, particularly the important dietary long-chain PUFAs, but no omega-6 LOA was detected (Table 1). Its main omega-3 PUFAs included EPA, DHA, DPA, and the metabolic intermediate *cis*-11,14,17-eicosatrienoic acid (ETrA, C20:3n-3), which gave an omega-3 to omega-6 PUFAs ratio of approximately 20. The Frippak dry powder was also enriched with omega-3 PUFAs, containing a high percentage of α-LA and some long-chain omega-3 PUFAs, with an omega-3 to omega-6 ratio of 15.

The shrimp-based pellet food was intermediate in its PUFA composition, with an omega-3 to omega-6 PUFAs ratio of 1.2 and containing twice as much omega-3 PUFAs as Sera micron (Table 1). The fatty acid composition of the flaxseed flakes were dominated by the essential omega-3 metabolic precursor α-LA, forming 85% of the total fatty acid composition of this type of food.

### *Fatty acids of amphipods fed Sera micron in sediment or sand*

The PUFA content of male and nongravid and gravid female *M. plumulosa* was determined after being fed Sera micron when cultured in silty sediment (Table 2). Twelve fatty acids were prominent, with only a few others being observed in the chromatographs, usually at concentrations below 5 μg/100 mg dry weight. A major feature of the PUFA composition of *M. plumulosa* was that both sexes contained a relatively high content of the long-chain omega-3 PUFAs EPA, ETrA, and DHA compared to the fatty acid composition of the Sera micron food source (Table 1). This is reflected in an omega-3 to omega-6 PUFAs ratio for the amphipods ranging from 1.6 to 1.9 (Table 2), compared a ratio of 0.4 for Sera micron (Table 1). The major differences in the PUFA composition between male and nongravid and gravid female *M. plumulosa* was the presence of the omega-3 DPA in nongravid females only and the absence of the monounsaturated fatty acid, palmitoleic acid (POA, C16:1n-7) in males.

To evaluate the contribution of the silty sediment as an additional food source, the amphipods were cultured in sand for 14 d with added Sera micron as the only food. The PUFA content of male and nongravid and gravid female *M. plumulosa* was then determined (Table 3). The omega-3 to omega-6 PUFAs ratio of the male and nongravid and gravid female amphipods cultured in sand decreased to a range from 1.1 to 1.3 in sand. The change in the ratio was in part due to an increased uptake of the two main omega-6 PUFAs, LOA and γ-LA acids, a characteristic of Sera micron, since this was the only food source. However, the amounts of the long-chain omega-3 PUFAs EPA, DHA, DPA, and ETrA in male and nongravid female amphipods cultured in sand decreased (Table 3) compared to their concentrations in amphipods cultured in the silty sediment (Table 2). With the exception of gravid females, animals cultured in sand had generally lower amounts of fatty acids than those cultured in silty sediment, even if the supplementary food was the same in both cases.

### *Fatty acid composition of amphipods fed various foods*

The fatty acid composition of amphipods fed the six commercial food supplements, either as the sole food fed to the amphipods cultured in sand or as binary mixtures with Sera micron fed to amphipods cultured in silty sediment was examined using the multivariate ordination method of principal components analysis (Fig. 1). Data values are the mean concentration of the 12 major fatty acids measured in the male and female adult amphipods collected from the cultures fed the various food types. The first component of the principal components analysis plot in Figure 1 (52% of variance) was positive for amphipods that accumulated larger amounts of omega-6 fatty acids and negative for amphipods that accumulated higher amounts of omega-3 fatty acids from their diet. In fact, the loadings for the food treatments on component 1 were strongly correlated with their ratio of omega-3 to omega-6 ($r^2 = 0.98$). The second component (25% of variance) in the principal components analysis showed a good correlation ($r^2 = 0.64$) with the ratio EPA:ARA, whereas neither component seemed to correlate with fecundity or survival of amphipods in the food treatments. The plot showed that *M. plumulosa* fed binary mixtures of omega-6 enriched Sera micron with either the three omega-3 enriched foods, whether shrimp powder, Rotiselco-ALG algal paste, or Frippak dry powder (diets 2, 3, and 5, respectively), accumulated a range of both omega-3 and omega-6 fatty acids. This indicated that these three diets were the more balanced diets for the amphipods for accumulating the essential PUFAs. In contrast, the fatty acid composition of amphipods fed an omega-6 enriched diet consisting of a binary mixture of Sera micron and AlgaMac-ARA (diet 1) was dominated by their accumulation of high amounts of ARA, but low amounts of the essential long-chain omega-3 EPA. Similarly, amphipods fed a binary food mixture of Sera micron and the flaxseed flakes (diet 4) accumulated a high amount of the omega-3 α-LA, which is characteristic of the flaxseed (Table 1).

Differences were found in the fatty acid composition of male, nongravid female, and gravid female *M. plumulosa* cultured in silty sediment and fed Sera micron (Fig. 2). The first component (93% of variance) was related to total amounts of fatty acids. Male amphipods had reduced concentrations of the 12 major fatty acids compared to females. The second component (4% of variance) seems to distinguish between specific PUFAs present in females during their reproductive cycle.

Table 1. Fatty acid methyl ester composition of various food types used for feeding *Melita plumulosa*[a]

| Code[b] | Fatty acid | Sera micron mean ± CI[c] (%) μg/100 mg dry wt (*n* = 4) | Shrimp-pellet mean ± CI (%) μg/100 mg dry wt (*n* = 4) | Rotiselco-ALG mean ± CI (%) μg/100 mg wet wt (*n* = 2) | AlgaMac-ARA mean ± CI (%) μg/100 mg dry wt (*n* = 4) | Flaxseed mean ± CI (%) μg/100 mg dry wt (*n* = 2) | Frippak mean ± CI (%) μg/100 mg dry wt (*n* = 2) |
|---|---|---|---|---|---|---|---|
| POA | 16:1n-7 | 700 ± 293 (18) | 481 ± 174 (14) | 1,823 ± 137 (17) | 111 ± 7 (1) | 14 ± 27 (0) | 1,459 ± 269 (17) |
| PA | 16:0 | 1,058 ± 389 (28) | 617 ± 181 (18) | 3,652 ± 64 (34) | 2,790 ± 558 (21) | 304 ± 26 (8) | 1,465 ± 274 (18) |
| γ-LA | 18:3n-6 | 376 ± 114 (10) | 72 ± 15 (2) | 98 ± 8 (1) | 714 ± 435 (5) | <1 (0) | 93 ± 18 (1) |
| LOA | 18:2n-6 | 743 ± 313 (19) | 660 ± 244 (19) | <1 (0) | <1 (0) | <1 (0) | 96 ± 17 (1) |
| OA | 18:1n-9 | 397 ± 91 (10) | 485 ± 90 (14) | 1,782 ± 74 (17) | 1,651 ± 284 (13) | <1 (0) | 690 ± 127 (8) |
| α-LA | 18:3n-3 | 157 ± 32 (4) | 128 ± 38 (4) | 1,412 ± 27 (13) | 1,476 ± 325 (11) | 3,177 ± 239 (85) | 1,566 ± 285 (19) |
| SA | 18:0 | 108 ± 26 (3) | 189 ± 41 (6) | 500 ± 25 (5) | 1,630 ± 330 (12) | 167 ± 19 (4) | 370 ± 69 (4) |
| ARA | 20:4n-6 | 5 ± 6 (0) | 12 ± 14 (0) | 44 ± 6 (0) | 3,966 ± 805 (30) | 0 ± 0 (0) | 86 ± 18 (1) |
| EPA | 20:5n-3 | 93 ± 41 (2) | 149 ± 53 (4) | 270 ± 47 (3) | 329 ± 66 (2) | 0 ± 0 (0) | 288 ± 52 (4) |
| ETrA | 20:3n-3 | 143 ± 117 (4) | 318 ± 127 (9) | 563 ± 42 (5) | 370 ± 74 (3) | 30 ± 13 (1) | 1,532 ± 175 (18) |
| DHA | 22:6n-3 | 56 ± 19 (1) | 249 ± 71 (7) | 432 ± 61 (4) | 51 ± 39 (0) | 11 ± 10 (0) | 561 ± 103 (7) |
| DPA | 22:5n-3 | 12 ± 13 (0) | 47 ± 37 (1) | 92 ± 1 (1) | 62 ± 45 (0) | 29 ± 14 (1) | 147 ± 27 (2) |
| Totals | | 3,846 (100) | 3,408 (100) | 10,666 (100) | 13,148 (100) | 3,731 (100) | 8,354 (100) |
| Omega-3 | | 460 | 892 | 2,768 | 2,286 | 3,246 | 4,095 |
| Omega-6 | | 1,123 | 744 | 142 | 4,679 | <1 | 275 |
| Omega-3:omega-6 | | 0.4 | 1.2 | 19.5 | 0.5 | >3,250 | 14.9 |

[a] The food products were obtained from the following manufacturers: Sera micron (Sera, Heinsberg, Germany), shrimp feed (Grobest, Beenleigh, Queensland, Australia), Rotiselco-ALG (INVE Technologies, Dendermonde, Belgium), AlgaMac-ARA (Aquafauna Bio-Marine, Hawthorne, CA, USA), flaxseed meal (Stony Creek Oil Products, Talbot, VIC, Australia) and Frippak No. 1 CAR encapsulated feed (INVE Technologies).

[b] POA = palmitoleic acid; PA = palmitic acid; γ-LA = gamma linoleic acid; LOA = linoleic acid; OA = oleic acid; α-LA = alpha linolenic acid; SA = stearic acid; ARA = arachidonic acid; EPA = eicosapentaenoic acid; ETrA = *cis*-11,14,17-eicosatrienoic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid.

[c] Values shown are the mean ± 95% confidence interval (CI), and percentage of total in parentheses.

Table 2. Fatty acid methyl ester composition of *Melita plumulosa* cultured in silty sediment and fed on added Sera micron

| Code[a] | Fatty acid | Males mean ± CI[b] (%) μg/100 mg dry wt | Nongravid females mean ± CI (%) μg/100 mg dry wt | Gravid females mean ± CI (%) μg/100 mg dry wt |
|---|---|---|---|---|
| POA | 16:1n-7 | 0 (0) | 118 ± 48 (4) | 134 ± 24 (5) |
| PA | 16:0 | 219 ± 10 (18) | 552 ± 33 (17) | 518 ± 98 (20) |
| γ-LA | 18:3n-6 | 15 ± 17 (1) | 53 ± 25 (2) | 43 ± 30 (2) |
| LOA | 18:2n-6 | 113 ± 39 (9) | 353 ± 29 (11) | 284 ± 55 (11) |
| OA | 18:1n-9 | 272 ± 38 (22) | 660 ± 101 (21) | 592 ± 103 (23) |
| α-LA | 18:3n-3 | 102 ± 11 (8) | 205 ± 43 (6) | 173 ± 38 (7) |
| SA | 18:0 | 139 ± 12 (11) | 235 ± 26 (7) | 235 ± 62 (9) |
| ARA | 20:4n-6 | 83 ± 24 (7) | 163 ± 18 (5) | 110 ± 19 (4) |
| EPA | 20:5n-3 | 147 ± 38 (12) | 299 ± 31 (9) | 198 ± 32 (8) |
| ETrA | 20:3n-3 | 89 ± 61 (7) | 293 ± 33 (9) | 228 ± 38 (9) |
| DHA | 22:6n-3 | 65 ± 17 (5) | 166 ± 77 (5) | 89 ± 14 (3) |
| DPA | 22:5n-3 | 0 (0) | 77 ± 91 (2) | 0 (0) |
| Totals | | 1,245 (100) | 3,175 (100) | 2,602 (100) |
| Omega-3 | | 404 | 1,040 | 687 |
| Omega-6 | | 212 | 569 | 437 |
| Omega-3:omega-6 | | 1.9 | 1.8 | 1.6 |

[a] Refer to Table 1 for definitions of the codes.
[b] Values shown are the mean ± 95% confidence interval ($n = 4$), and percentage of total in parentheses.

Nongravid females were the only amphipods that contained omega-3 DPA, while gravid females that had their embryos removed could be distinguished from gravid females carrying eggs because they contained reduced concentrations of omega-3 ETrA.

### *Survival of amphipods fed various food types in sand*

The ability of the various food types to support the survival of *M. plumulosa* cultured in sand was determined. After 14 d, 75% of *M. plumulosa* survived when fed Sera micron as the only food source, compared to 70% of *M. plumulosa* that survived when fed exclusively AlgaMac-ARA and 60% of *M. plumulosa* that survived when fed exclusively on either omega-3 enriched Rotiselco-ALG algal paste or Frippak powder. These survival rates were not significantly different among the food treatments ($p > 0.05$, ANOVA).

### *Carotenoid content of the various food types*

The total ion chromatogram of the unsaponifiable extract fraction from amphipods cultured in sand and fed Sera micron showed three distinctive peaks with retention times at 1.44, 1.86, and 2.43 min. The corresponding main protonated fragments $[M+H]^+$ in their spectra were *m/z* 256.3, *m/z* 317.2, and *m/z* 537.4, respectively. The latter peak with *m/z* 537.4 matched the spectrum of carotene, but the retention time was shorter than that of an all-*trans* β-carotene standard used (4.15 min), suggesting it is probably a *cis* β-carotene isomer [25]. Based on the mass range of the other ionized fragments, it is suggested they may correspond to $C_{17}$ terpenoids (*m/z* 256.3) and perhaps diterpenoids (*m/z* 317.2), which are precursors of important compounds such as retinol and retinal. The colorless extracts of amphipods fed other foods (i.e., AlgaMac-ARA),

Table 3. Fatty acid methyl ester composition of *Melita plumulosa* cultured in sand and fed on added Sera micron

| Code[a] | Fatty acid | Males mean ± CI[b] (%) μg/100 mg dry wt | Nongravid females mean ± CI (%) μg/100 mg dry wt | Gravid females mean ± CI (%) μg/100 mg dry wt |
|---|---|---|---|---|
| POA | 16:1n-7 | 49 ± 96 (4) | 197 ± 83 (8) | 217 ± 97 (7) |
| PA | 16:0 | 210 ± 137 (18) | 468 ± 69 (19) | 602 ± 329 (19) |
| γ-LA | 18:3n-6 | 45 ± 31 (4) | 84 ± 29 (3) | 100 ± 45 (3) |
| LOA | 18:2n-6 | 147 ± 62 (12) | 336 ± 189 (13) | 474 ± 300 (15) |
| OA | 18:1n-9 | 224 ± 63 (19) | 519 ± 143 (21) | 668 ± 419 (21) |
| α-LA | 18:3n-3 | 81 ± 31 (7) | 164 ± 33 (7) | 186 ± 98 (6) |
| SA | 18:0 | 107 ± 26 (9) | 179 ± 17 (7) | 212 ± 125 (7) |
| ARA | 20:4n-6 | 69 ± 24 (6) | 103 ± 25 (4) | 127 ± 73 (4) |
| EPA | 20:5n-3 | 120 ± 30 (10) | 226 ± 124 (9) | 298 ± 198 (9) |
| ETrA | 20:3n-3 | 76 ± 41 (6) | 147 ± 11 (6) | 178 ± 85 (6) |
| DHA | 22:6n-3 | 57 ± 19 (5) | 92 ± 22 (4) | 117 ± 73 (4) |
| DPA | 22:5n-3 | 0 (0) | 0 (0) | 0 (0) |
| Totals | | 1,185 (100) | 2,514 (100) | 3,177 (100) |
| Omega-3 | | 335 | 629 | 779 |
| Omega-6 | | 261 | 523 | 701 |
| Omega-3:omega-6 | | 1.3 | 1.2 | 1.1 |

[a] Refer to Table 1 for definitions of the codes.
[b] Values shown are the mean ± 95% confidence interval ($n = 3$), and percentage of total in parentheses.

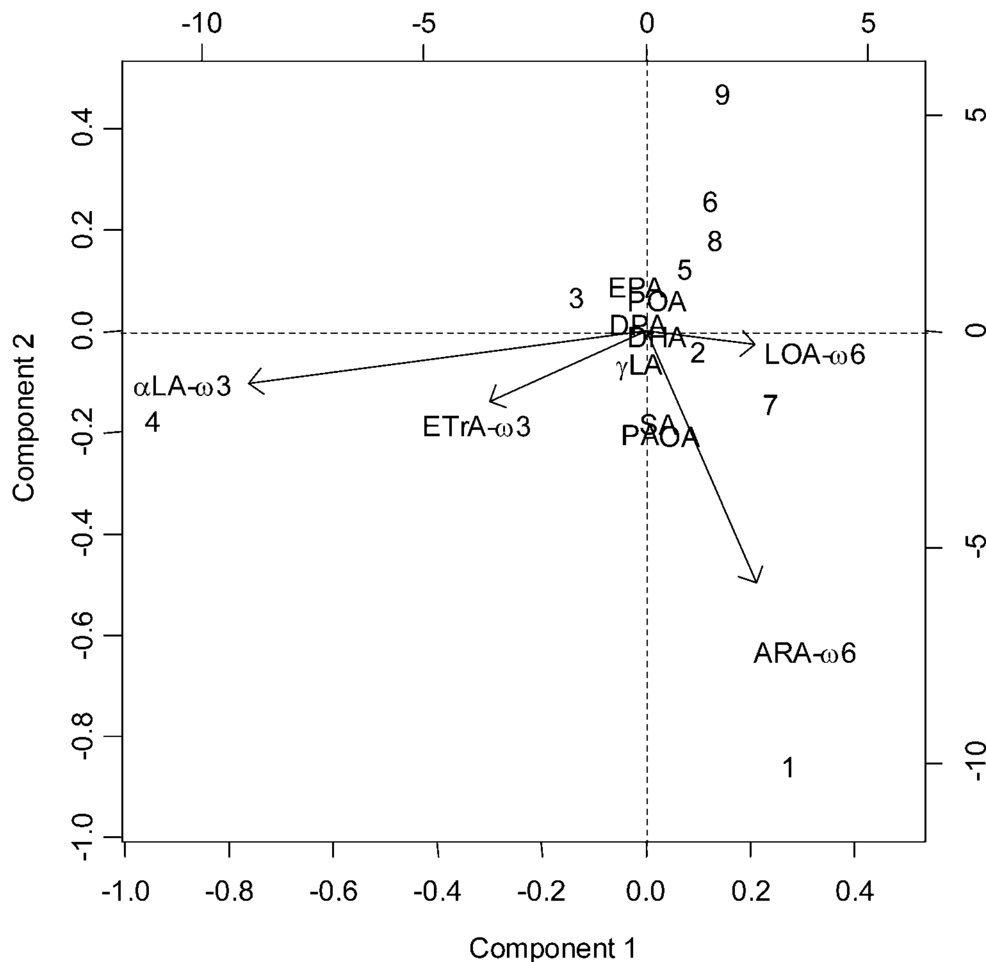


Fig. 1. Principal components analysis of the accumulated fatty acid compositional data of *Melita plumulosa* after being fed various foods for 19 d. The concentrations of the 12 major fatty acids were the mean values of male and female amphipods in the cultures. The abbreviations for the fatty acids are shown in Table 1. The first axis explains 52% of the variation in the fatty aid composition, while the second axis explains 25%. The diet centroids (1–9) are as follows: 1 = Sera micron (Sera, Heinsberg, Germany) and AlgaMac-ARA (Aquafauna Bio-Marine, Hawthorne, CA, USA) in silty sediment; 2 = Sera micron and shrimp pellet (Grobest, Beenleigh, Queensland, Australia) in silty sediment; 3 = Sera micron and Rotiselco-ALG (INVE Technologies, Dendermonde, Belgium) in silty sediment; 4 = Sera micron and flaxseed (Creek Oil Products, Talbot, VIC, Australia) in silty sediment; 5 = Sera micron and Frippak (INVE Technologies) in silty sediment; 6 = Sera micron only in silty sediment; 7 = AlgaMac-ARA only in sand; 8 = Rotiselco-ALG in sand; 9 = Sera micron only in sand.

did not show any peaks when analyzed by LC-MS under the same conditions.

*Fecundity of amphipods fed various food types in sediment*

A two-factor ANOVA with trial and each food treatment and the interaction after logarithmic transformation found no significant difference in fecundity ($p > 0.05$, two-factor ANOVA), so the replicates of each food treatment were combined. *Melita plumulosa* fed a mixture of the omega-6 rich Sera micron in conjunction with the omega-3 enriched Rotiselco-ALG and cultured on a silty substrate were found to have good fecundity with significantly lower variability in the brood size ($p = 0.0045$, Brown–Forsythe's test) compared to that of amphipods fed Sera micron only (Fig. 3). Amphipods fed a 1:1 (w/w) mixture of Sera micron and the shrimp-based pellet, which contained some omega-3 PUFAs, also had less variability in their fecundity ($p < 0.05$, Levene's test) than that of amphipods fed Sera micron only. In contrast, the variability in brood size of amphipods fed a 1:1 (w/w) mixture of Sera micron and either flaxseed (data not shown), AlgaMac-ARA, or Frippak was as large as that of amphipods fed Sera micron only ($p > 0.05$, Levene's test). Since there was a difference in the variance between the food treatments of the combined data, it is not possible to use ANOVA to test for differences between the mean fecundity of *M. plumulosa* among the food treatments. However, the nonparametric Mann–Whitney test found there was no significant difference between the mean fecundity of *M. plumulosa* (8.08 eggs per female) among the food treatments ($p > 0.58$, Mann–Whitney).

## DISCUSSION

*Fatty acid metabolism of crustaceans*

Crustaceans obtain a majority of their omega-3 PUFAs by consumption of marine microalgae [28], which are considered to be the primary producers of long-chain omega-3 PUFAs. We have previously shown that the addition of various algae to *M. plumulosa* cultures in silty sediment without any other added food did not support good survival rates compared to *M. plumulosa* cultures fed Sera micron [4]. This suggests that the source of the omega-3 PUFAs accumulated by the amphipods from the silty sediment is more likely to be organic detritus or fungi. This proposal is consistent with the observation in the field that high population densities of *M. plu-*

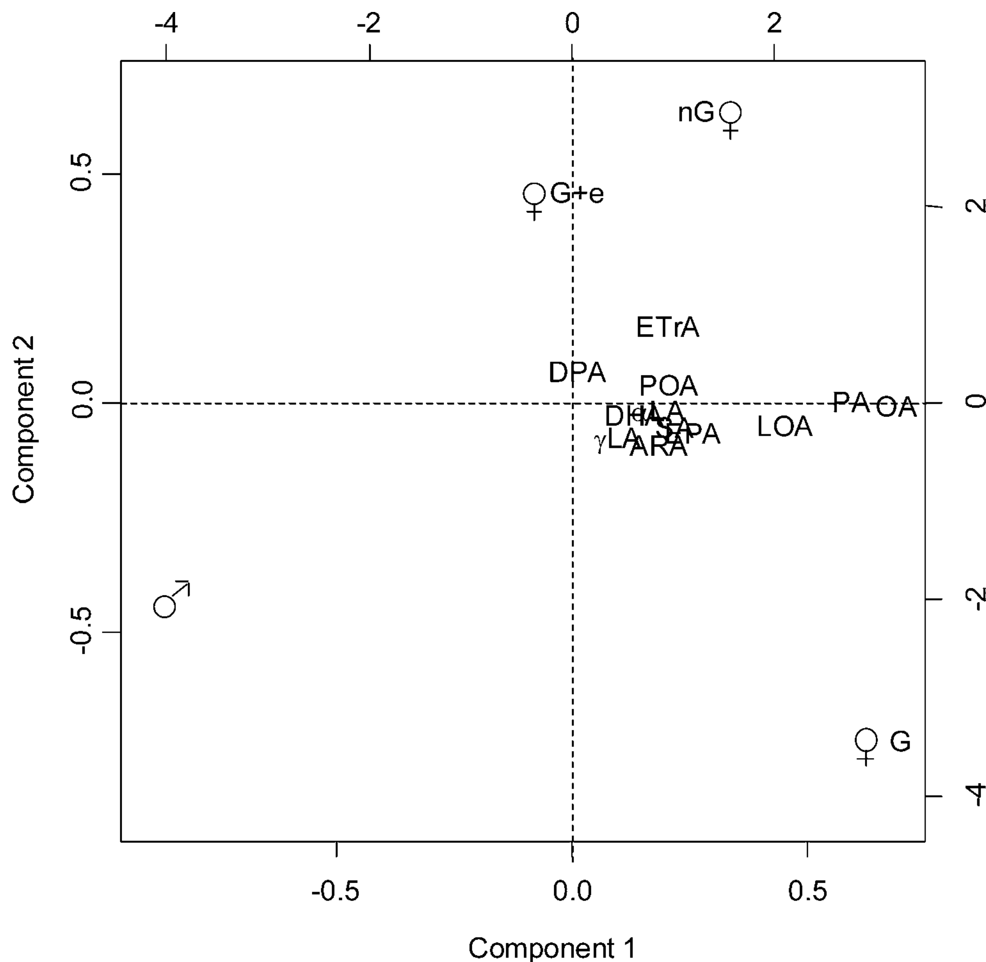


Fig. 2. Principal components analysis of the accumulated fatty acid compositional data of *Melita plumulosa* and sex or gravidity of the amphipods. The amphipods were cultured in silty sediment for 19 d and fed Sera micron (Sera, Heinsberg, Germany) only. The abbreviations for the fatty acids are shown in Table 1. The first axis explains 93% of the variation in the fatty aid composition, while the second axis explains 4%. The amphipod centroids are as follows: ♀G+e = gravid females; ♀G = gravid females after their embryos were removed; nG♀ = nongravid females; ♂ = males.

*mulosa* are found associated with fragments of oyster shells [4].

Long-chain PUFA biosynthesis involves the addition of two carbon moieties to the essential fatty acids LOA (omega-6) and α-LA (omega-3), which crustaceans must obtain from their diet. Each cycle of elongation of a fatty acid carbon chain requires the four enzymatic reactions of condensation, reduction, dehydration, and further reduction [9]. In general, elongation of fatty acids is catalyzed by a multi-enzyme ‘‘elongating enzyme complex’’ [9]. Within this complex, the condensing enzyme (elongase), is the most significant because it catalyzes the rate-limiting condensation step in the four-step reaction and determines the substrate specificity of the entire complex [29–31]. Studies on the biosynthesis pathways for the formation of PUFAs in crustaceans are limited, and it is unknown if amphipods possess the capacity for elongation and desaturation of the precursors LOA and α-LA. Recent studies have shown that prawns and cladocerans can elongate and desaturate these fatty acids to longer chain PUFAs, but at rates insufficient to satisfy their PUFA requirements [14,23,32]. The fatty acid biosynthesis ability of crustaceans also varies with species. The larval kuruma shrimp *Marsupenaeus japonicus*, for example, was found to have the ability to convert α-LA into the long-chain omega-3 PUFA EPA, and EPA into DHA, while *Penaeus chinensis* cannot [32]. The results of the present study suggest that some components of the so-called omega-3 $\Delta^8$ desaturase enzyme pathway that leads to the formation of EPA and DHA in lower eukaryotes [33] are active in *M. plumulosa*. Hence, elongation of the dietary LOA and α-LA would lead to the accumulation of eicosadienoic acid (C20: 3n-6) and ETrA, respectively, in the amphipods. The omega-3 ETrA was found to accumulate in *M. plumulosa*, with particularly high concentrations found in nongravid females (Table 2).

We have previously demonstrated that mature *M. plumulosa* in laboratory cultures of silty substrate breed continuously, with the fertilization and intramarsupial development of embryos until hatching completes within 7 d [3,4]. The presence of the omega-3 DPA only in nongravid adult females suggests that during the period leading to the formation of the next generation of ovulating oocytes in the ovaries, some PUFA metabolic pathways support de novo synthesis of DPA in the females. The DPA would need to be selectively obtained by the nongravid females directly from their diet or more likely synthesized from the accumulated ETrA and EPA [9,33]. This emphasizes the importance of long-chain omega-3 PUFAs in the diet of *M. plumulosa* for females to maintain high fecundity.

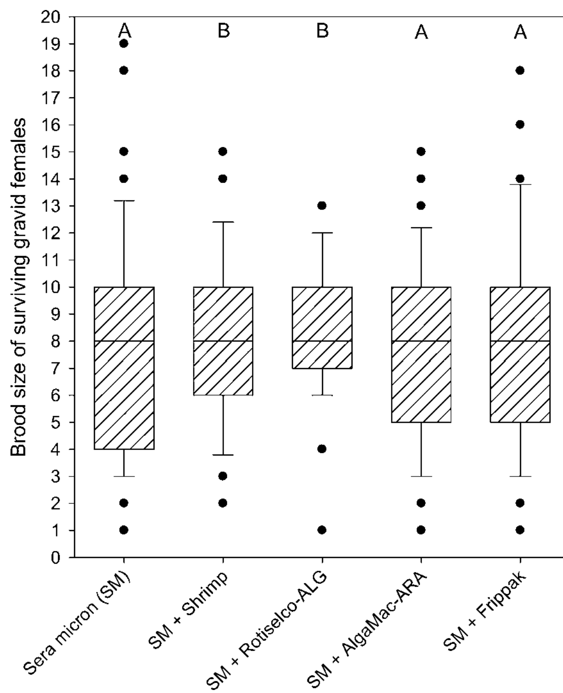


Fig. 3. Box plots showing the median and range of the total brood size of surviving gravid females *Melita plumulosa* fed various diets for 19 d. Box plots represent the limits of the middle half of the data and whiskers represent the extent of the data span (1.5× interquartile range) from the 25 and 75% quartiles. Points outside the whiskers are shown individually as closed symbols. Values shown are the median and range of the brood size from two or three combined experiments of surviving gravid females fed Sera micron (Sera, Heinsberg, Germany) ($n$ = 86), shrimp pellet (Grobest, Beenleigh, Queensland, Australia) ($n$ = 47), Rotiselco-ARG (INVE Technologies, Dendermonde, Belgium) ($n$ = 43), AlgaMac-ARA (Aquafauna Bio-Marine, Hawthorne, CA, USA) ($n$ = 47), and Frippak (INVE Technologies, Dendermonde, Belgium) ($n$ = 41). Mean fecundity among the treatments was not significantly different ($p > 0.05$, Mann–Whitney). Different letters indicate significant variance compared to amphipods fed Sera micron only ($p = 0.0045$, Brown–Forsythe's test).

*Crustacean dietary nutrient requirements*

In addition to protein, fatty acids, and carbohydrate, it is known that minerals, vitamins, and other nutrients such as sterols and carotenoids are essential in the crustacean diet for normal development [32,34]. Previous studies have reported *Spirulina* spp. to contain high concentrations of sterols and carotenoids [35]. Dietary carotenoids and their precursor terpenoids are the sole biological precursors of retinoids in crustaceans and dietary deficiency is associated with a loss of carotenoids in the ovaries of mature females [36]. Crustacean vitellin, the major egg yolk protein, is a lipoglycoprotein associated with carotenoids [36,37]. Crustacean eggs accumulate carotenoids in significant amounts, suggesting a possible function during vitellogenesis. The presence of receptors of retinoic acid in crustaceans also suggests a role for metabolism of carotenoids into retinoids that regulate crustacean physiology [36]. The *Spirulina*-based Sera micron is a good source for β-carotene and terpenoids compared to the other feeds investigated in the present study, perhaps explaining why Sera micron as the sole food supported the best survival of *M. plumulosa* cultured in a nutrient-depleted sand substrate.

*Fecundity of* M. plumulosa

*Melita plumulosa* fed a mixture of the omega-6 PUFA enriched Sera micron in conjunction with the omega-3 PUFA enriched Rotiselco-ALG and cultured on a silty substrate were found to have good fecundity with low variability compared to other food combinations. Similarly, the best growth of the prawn *Penaeus monodon* was obtained with a diet containing a balanced LOA and α-LA content [14]. These fatty acids interact for access to the $\Delta^6$ desaturase enzyme system [9] required for synthesis of the longer PUFAs such as ARA, EPA, and DHA [38]. Excess LOA or α-LA can suppress the de novo fatty acid synthesis [39]. Maintaining the balance between LOA and α-LA precursors may be important for maximizing the response to dietary ARA, EPA, and DHA, since an excess of either precursor can affect the fatty acid biosynthesis [39].

The present study shows that short-term changes in dietary PUFA content can influence the fecundity of *M. plumulosa*. Moulting and ovulation would certainly be a stressful period during the female amphipod's short reproductive cycle, during which the demand for eicosanoid production from ARA is likely to be elevated. *Melita plumulosa* cultured on silty sediment and fed a mixture of the Sera micron in conjunction with the Rotiselco-ALG food had higher concentrations of ARA as well as the long-chain omega-3 PUFAs α-LA and ETrA compared to amphipods fed Sera micron only. Amphipods fed Sera micron only were found to accumulate omega-3 PUFAs from the sediment, but the omega-3 PUFA content in sediment would vary within catchments depending on the sediment composition, location, and the season it was collected.

## CONCLUSION

The benthic amphipod *M. plumulosa*, maintained in laboratory cultures containing natural silty sediment and fed Sera micron, maintained good survival rates and obtained omega-3 PUFAs from the sediment, with females accumulating higher amounts than males. The omega-3 to omega-6 PUFAs ratio for male and nongravid and gravid female *M. plumulosa* ranged from 1.6 to 1.9. In contrast, when *M. plumulosa* were maintained in cultures containing nutrient-depleted sand substrate, Sera micron did not support a good survival rate and the omega-3 to omega-6 PUFAs ratio for the male and nongravid and gravid female amphipods decreased, ranging from 1.1 to 1.3. Furthermore, amphipods cultured in sand and fed any of the algal-based foods as the sole food source had poor survival rates, although Sera micron maintained the best survival. This was attributed to *Spirulina*-based Sera micron containing high amounts of carotenoids and terpenoids. It was concluded that a diet containing a balanced mixture of omega-3 and omega-6 PUFAs is essential for enhancing fecundity in *M. plumulosa*, whereas Sera micron supplements appear to help survival by providing other essential nutrients in the dietary intake of the amphipod.

*Acknowledgement*—This research was funded in part by a grant from the New South Wales Government's Environmental Trust (Environmental Research Project 2005/RD/0020). We thank Geoff Gordon, Brad Green, and anonymous referees.

## REFERENCES

1. Gale SA, King CK, Hyne RV. 2006. Chronic sublethal sediment toxicity testing using the estuarine amphipod, *Melita plumulosa* (Zeidler): Evaluation using metal-spiked and field-contaminated sediments. *Environ Toxicol Chem* 25:1887–1898.
2. King CK, Gale SA, Hyne RV, Stauber JL, Simpson SL, Hickey CW. 2006. Sensitivities of Australian and New Zealand amphipods to copper and zinc in waters and spiked-sediments. *Chemosphere* 63:1466–1476.
3. Mann RM, Hyne RV. 2008. Embryological development of the

Australian amphipod, *Melita plumulosa* Zeidler, 1989 (Amphipoda, Gammaridea, Melitidae). *Crustaceana* 81:57–66.
4. Hyne RV, Gale SA, King CK. 2005. Laboratory culture and life cycle experiments with the benthic amphipod *Melita plumulosa* (Zeidler). *Environ Toxicol Chem* 24:2065–2073.
5. Middleditch BS, Missler SR, Hines HB, McVey JP, Brown A, Ward DG, Lawrence AL. 1980. Metabolic profiles of penaeid shrimp: Dietary lipids and ovarian maturation. *J Chromatogr A* 195:359–368.
6. Clarke A, Skadsheim A, Holmes LJ. 1985. Lipid biochemistry and reproductive biology in two species of Gammaridae (Crustacea:Amphipoda). *Mar Biol* 88:247–263.
7. Cahu C, Guillaume JC, Stéphan G, Chim L. 1994. Influence of phospholipid and highly unsaturated fatty acids on spawning rate and egg and tissue composition in *Penaeus vannamei* fed semipurified diets. *Aquaculture* 126:159–170.
8. Narciso L, Morais S. 2001. Fatty acid profile of *Palaemon serratus* (Palaemonidae) eggs and larvae during embryonic and larval development using different live diets. *J Crustac Biol* 21: 566–574.
9. Leonard AE, Pereira SL, Sprecher H, Huang Y-S. 2004. Elongation of long-chain fatty acids. *Prog Lipid Res* 43:36–54.
10. Kanazawa A, Teshima S-I, Ono K. 1979. Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. *Comp Biochem Physiol B* 63:295–298.
11. Cunnane SG, Anderson MJ. 1997. The majority of dietary linoleate in growing rats is β-oxidised or stored in visceral fat. *J Nutr* 127:146–152.
12. Rosa R, Morais S, Calado R, Narciso L, Nunes ML. 2003. Biochemical changes during embryonic development of Norway lobster, *Nephrops norvegicus*. *Aquaculture* 221:507–522.
13. Goulden CE, Place AR. 1990. Fatty acid synthesis and accumulation rates in Daphniids. *J Exp Zool* 256:168–178.
14. Glencross BD, Smith DM. 1999. The dietary linoleic and linolenic fatty acids requirements of the prawn *Penaeus monodon*. *Aquac Nutr* 5:53–63.
15. Phleger CF, Nelson MM, Mooney BD, Nichols PD, Ritar AJ, Smith GG, Hart PR, Jeffs AG. 2001. Lipids and nutrition of the southern rock lobster, *Jasus edwardsii*, from hatch to puerulus. *Mar Freshw Res* 52:1475–1486.
16. Arts MT, Achman RG, Holub BJ. 2001. ''Essential fatty acids'' in aquatic ecosystems: A crucial link between diet and human health and evolution. *Can J Fish Aquat Sci* 58:122–137.
17. Turner N, Else PL, Hulbert AJ. 2003. Docosahexaenoic acid (DHA) content of membranes determines molecular activity of the sodium pump: Implications for disease states and metabolism. *Naturwissenschaften* 90:521–523.
18. Sundbom M, Vrede T. 1997. Effects of fatty acid and phosphorus content of food on the growth, survival and reproduction of *Daphnia*. *Freshw Biol* 38:665–674.
19. Park S, Brett MT, Müller-Navarra DC, Goldman CR. 2002. Essential fatty acid content and the phosphorus to carbon ratio in cultured algae as indicators of food quality for *Daphnia*. *Freshw Biol* 47:1377–1390.
20. Simpson SL, Angel BM, Jolley DF. 2004. Metal equilibration in laboratory-contaminated (spiked) sediments used for the development of whole-sediment toxicity tests. *Chemosphere* 54:597–609.
21. von Elert E, Stampfl P. 2000. Food quality for *Eudiaptomus gracilis*: The importance of particularly highly unsaturated fatty acids. *Freshw Biol* 45:189–200.
22. Mason ME, Eager ME, Waller GR. 1964. A procedure for the simultaneous quantitative determination of glycerol and fatty acid contents of fats and oils. *Anal Chem* 36:587–590.
23. von Elert E. 2002. Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnol Oceanogr* 47:1764–1773.
24. Tokuşoglu Ö, Ünal MK. 2003. Biomass nutrient profiles of three microalgae; *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrisis galbana*. *J Food Sci* 68:1144–1148.
25. Lacker T, Strohschein S, Albert K. 1999. Separation and identification of various carotenoids by $C_{30}$ reversed-phase high performance liquid chromatography coupled to UV and atmospheric pressure chemical ionisation mass spectrometric detection. *J Chromatogr A* 854:37–44.
26. Hoaglin DC, Mosteller F, Tukey JW, eds. 1983. *Understanding Robust and Exploratory Data Analysis.* John Wiley, New York, NY, USA.
27. Conover WJ, Johnson ME, Johnson MM. 1981. A comparative study of tests for homogeneity of variances, with applications to the outer continental shelf bidding data. *Technometrics* 23:351–361.
28. De Pauw N, Personne G. 1988. Micro-algae for aquaculture. In Borowitzka MA, Borowitzka JL, eds, *Microalgal Biotechnology.* Cambridge University, Cambridge, MA, USA, pp 197–221.
29. Millar AA, Kunst L. 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *Plant J* 12:121–131.
30. Moon Y-A, Shah NA, Mohapatra S, Warrington JA, Horton JD. 2001. Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. *J Biol Chem* 276:45358–45366.
31. Fehling E, Mukherjee KD. 1991. Acyl-CoA elongase from a higher plant (*Lunaria annua*): Metabolic intermediates of a very-long-chain acyl-CoA products and substrate specificity. *Biochim Biophys Acta* 1082:239–246.
32. Takeuchi T, Murakami K. 2007. Crustacean nutrition and larval feed, with emphasis on Japanese spiny lobster, *Panulirus japonicus*. *Bulletin of the Fisheries Research Agency* 20:15–23.
33. Pereira SL, Leonard AE, Mukerji P. 2003. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandins Leukot Essent Fatty Acids* 68:97–106.
34. D'Abramo LR, Wright JS, Wright KH, Bordner CE, Conklin DE. 1985. Sterol requirement of cultured juvenile crayfish *Pacifastacus leniusculus*. *Aquaculture* 49:245–255.
35. Vonshak A, ed. 1997. *Spirulina platensis* (*Arthrospira*): Physiology, Cell-biology and Biotechnology. Taylor and Francis, London, UK.
36. Liñán-Cabello MA, Paniagua-Michel J, Hopkins PM. 2002. Bioactive roles of carotenoids and retinoids in crustaceans. *Aquac Nutr* 8:299–309.
37. Avarre J-C, Michelis R, Tietz A, Lubzens E. 2003. Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. *Biol Reprod* 69:355–364.
38. Garg ML, Thomson ABR, Clandinin MT. 1990. Interactions of saturated, n-6 and n-3 polyunsaturated fatty acids to modulate arachidonic acid metabolism. *J Lipid Res* 31:271–277.
39. Sargent J, Bell G, McEvoy L, Tocher D, Estevez A. 1999. Recent developments in the essential fatty acid nutrition of fish. *Aquaculture* 177:191–199.