2005

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Kirsten Benkendorff
Flinders University

Andrew R. Davis
University of Wollongong

Cary N. Rogers
University of Wollongong

John B. Bremner
University of Wollongong

Publication details

Postprint of: Benkendorff, K, Davis, AR, Rogers, CN & Bremner, JB 2005, 'Free fatty acids and sterols in the benthic spawn of aquatic molluscs, and their associated antimicrobial properties', *Journal of Experimental Marine Biology and Ecology*, vol. 316, no. 1, pp. 29-44. Publisher's version of this article is available at http://dx.doi.org/10.1016/j.jembe.2004.10.001

Free fatty acids and sterols in the benthic spawn of aquatic molluscs, and their associated antimicrobial properties.

Kirsten Benkendorff*, Andrew R. Davis[1], Cary N. Rogers[1] and John B. Bremner[2]

* Author to whom correspondence should be addressed

School of Biological Sciences, The Flinders University of South Australia, GPO Box 2100, Adelaide, S.A. 5001, Australia.

Phone: (61) 8 82013959; Fax: (61) 8 8201 3015

Email: kirsten.benkendorff@flinders.edu.au

1. School of Biological Sciences, 2. Department of Chemistry, University of Wollongong, NSW, 2522, Australia.

ABSTRACT – The free lipid content of extracts from the spawn of 17 molluscs were analysed by gas chromatography/mass spectrometry. These extracts encompass the encapsulated embryos and extra-embryonic structures from benthic gelatinous egg masses and leathery egg capsules covering five taxonomic groups. Palmitic and stearic acids were the dominant saturated fatty acids and oleic acid was the principal unsaturated acid found in the spawn. Cholesterol was the dominant sterol and the only sterol found in the spawn from every species. Extracts from gelatinous egg masses were found to contain proportionally more fatty acids compared to leathery egg capsules. No unsaturated fatty acids were found in any of the leathery egg capsules, including 5 neogastropods and one littorinimorph. Unsaturated fatty acids were present in all of the gelatinous egg masses, including two other

littorinimorphs. This is the first study to demonstrate that unsaturated fatty acids possess significant bacteriolytic activity against four aquatic pathogens. Encapsulated Anaspidea egg masses contain relatively high concentrations of these unsaturated fatty acids and a lipid mixture modeled on these extracts was strongly bacteriolytic at concentrations down to 0.0001mg/ml. By comparison, lipid mixtures modeled on extracts from the spawn of four other molluscan taxa with higher proportions of saturated fatty acid and cholesterol, were only partially active against some of the bacteria at 0.1mg/ml. Thus, unsaturated fatty acids could explain the antimicrobial activity reported from lipid extracts of some, but not most, molluscan spawn. MDS ordination and ANOSIM revealed significant taxonomic differences in the composition of free lipids from molluscan spawn, suggesting that lipid analyses may be useful in future systematic studies of the Mollusca.

Keywords

Antimicrobial activity, chemotaxonomy, leathery egg capsules, gelatinous egg mass, fatty acid, sterol.

Introduction

Lipids form a significant component of the fuel reserves in the muscles and organs of adult molluscs (Suryanarayanan and Alexander 1971; Jarzebski et al. 1986) and are thought to act as a storage product in the freely spawned eggs of bivalve molluscs (Vassallo 1973; Bayne et al. 1978; Kluytmans et al. 1985). However, the composition and significance of lipids in spawn, deposited as benthic egg masses and capsules by aquatic molluscs, remains unclear. Early histochemical studies provide little evidence for the presence of

lipids in the nutritive reserves of several benthic molluscan egg masses (Bayne 1968; Raven 1972). More recently, several studies have been undertaken on lipids in gelatinous Aplysiidae egg masses, revealing the presence of glycosphingolipids and a novel phosphonoglycosphingolipid (Yamaguchi et al. 1992a b, Yamada et al, 1995), as well as a mixture of sterols (Miyamoto et al. 1988; Yamaguchi et al. 1992c).

In 1923, Grün suggested that a “systematic study of the fats of lower animals and plants might lead to the discovery of relationships which should prove to be of biological interest” (cited in Bergmann 1949). Bergmann (1949) has also recommended the inclusion of sterols in comparative biochemical investigations because they are present in adequate diversity in all animals. Two classes of molluscs, the Gastropoda and Cephalopoda, are known to deposit embryos enclosed in benthic egg masses (Smith et al. 1989). Within the gastropods, two major forms of egg masses can be recognized: gelatinous egg masses and leathery egg capsules. Leathery egg capsules are primarily deposited by neogastropods (e.g. D’Asaro 1991; 1993), although some brooding cymatids in the Infraorder Littorinimorpha also deposit leathery egg capsules (e.g. Laxton 1969). Most other Littorinimorpha and most aquatic heterobranchs (Opisthobranchia and Pulmonata) deposit gelatinous egg masses (e.g. Smith et al. 1989, Przeslawski 2004). In this paper we use the term “spawn” to encompass either type of benthic egg mass (capsule and gelatinous), as well as their contents including eggs, embryos and intracapsular fluid.

Both types of benthic egg mass are thought to provide protection to the encapsulated embryos against predation and environmental stress (Pechenik 1979; 1983; Rawlings 1994; Przeslawski et al. 2004). Recently, extracts from the benthic spawn of a broad range of aquatic molluscs have also been shown to possess antimicrobial properties (Benkendorff et al. 2000a; 2001a). Activity against a broad spectrum of marine and human pathogenic bacteria is primarily concentrated in the lipophilic layer of solvent extracts of the spawn. Preliminary GC/MS analyses indicate that the predominant secondary metabolites in these extracts are free fatty acids and sterols (Benkendorff 1999).

The ability of fatty acids to interfere with bacterial growth and survival has been known for several decades (Kabara et al.1977; Kabara 1978; 1987; Ababouch et al. 1992), although their effectiveness against marine pathogens has never been tested. Structure-function relationship studies on free fatty acids against human pathogenic bacteria indicate that antimicrobial activity can depend on both the chain length and the degree of unsaturation (Kabara et al. 1977). It has also been demonstrated that compounds such as cholesterol can antagonize the antimicrobial properties of fatty acids (Galbraith et al. 1971). Consequently, both the composition and concentrations of free lipids in extracts from molluscan spawn could influence their antimicrobial properties.

This paper presents the first comparative study of the fatty acid and sterol composition in the benthic spawn of 17 molluscs from five taxonomic groups. The lipid extracts incorporate both encapsulated embryos and extra

embryonic structures from leathery egg capsules and gelatinous egg masses. The antimicrobial activities of dominant fatty acids are assessed against four marine pathogenic bacteria for the first time. Free lipid mixtures modeled on those found in the extracts from five molluscan taxa are then tested for antimicrobial activity to confirm whether or not these components could explain the previously reported antimicrobial activity in these spawn extracts (see Benkendorff *et al*., 2001a). By combining biological assays with a taxonomic treatment of the data, we contribute to the understanding of molluscan biochemical evolution and the role free fatty acids could potentially play in defense against marine pathogens.

Methods

Collection and preparation of biological material

The gelatinous egg masses and leathery egg capsules from 17 species of molluscs were collected along the Illawarra Coast, NSW, Australia (Table 1). Voucher specimens and/or photographs are held in a reference collection at the Department of Biological Sciences, University of Wollongong. All species were found in intertidal or shallow subtidal marine or estuarine habitats, with the exception of one freshwater species (Table 1). Immediately after collection, the egg masses and capsules were macerated and extracted along with their encapsulated embryos. This holistic approach is the most appropriate for taxonomic comparisons, but has some limitations for the investigation of antimicrobial defense because it is not possible to determine where the potential defense compounds reside. However, it is very difficult to separate embryos from the gelatinous matrix or intracapsular fluid of molluscan egg masses and therefore this approach of homogenizing the

whole spawn is routinely used in the published literature concerning bioactive compounds from molluscan spawn (e.g. Yamazaki et al., 1984; 1985; Roesner and Scheuer, 1986; Kisgui et al., 1987; Yamaguchi et al., 1992a,b,c; Yamada et al, 1995; Benkendorff et al., 2000b).

The lipophilic extracts used in this study were the same samples as had been used previously to uncover antimicrobial properties in molluscan egg masses and capsules (Benkendorff et al. 2001a) and in the present study we aimed to investigate whether fatty acids could be responsible for the observed activity. These organic extracts were prepared in chloroform/methanol (1:1, v:v) and concentrated under vacuum pressure, as previously described for recovering marine fatty acids and sterols (e.g. Stoilov et al. 1984; Yamaguchi et al. 1992b; Carballeira et al. 1997; Carballeira et al. 2001). Polar components were removed from the extracts via the water/methanol fraction. Solvent controls were prepared by evaporating chloroform/methanol (1:1, v:v, 200ml).

Gas Chromatography-Mass Spectrometry

The volatile organic components in the egg masses and capsules were examined using a GC-17A (Shimadzu) gas chromatograph coupled to a QP-5000 (Shimadzu) mass spectrometer, using a DB-5MS column (30m x 0.32mm with 0.25 micron film, J&W Scientific). The samples were run in splitless mode and, in general, 50 μl of sample dissolved in dichloromethane (DCM) was injected, at an estimated concentration of 10mg/ml. The injector temperature was set at 260^{o}C. The oven temperature was held at 40^{o}C for 2 min then increased to 290^{o}C at a rate of 4^{o}C per min. The final oven

temperature was then held at 290^{o}C for 10 min. The carrier gas was helium and the flow rate 1.4 ml/min. The electron beam energy in the mass spectrometer was 70eV and the source temperature was 200^{o}C. Three replicate runs were performed on the same extract from the egg masses of *Dicathais orbita* and *Aplysia juliana* and no significant variation was detected between runs. Blank runs were performed sporadically to check for contaminants on the column or in the injection loop, by injecting 50 μl of DCM as a solvent control.

The retention time and intensity of each peak detected in the gas chromatograph was recorded for each sample. The composition of volatile organic compounds in the egg mass extracts was compared to the solvent control and blank runs to remove potential contaminants. The relative intensity of each peak was then calculated as a percent of the summed total. Fatty acids and sterols were identified by their characteristic mass spectral fragmentation patterns and by comparison with known compounds contained in the mass spectral library. Fatty acids and their respective methyl esters were combined due to the use of methanol in the extraction procedure and the esterification of some samples. Sterols other than cholesterol were generally present in concentrations that were too low to permit positive identification and so these were pooled as “all other sterols”.

Statistical analyses

All statistical analyses concentrated on the extracts taken from intertidal (marine and estuarine) Gastropoda. The differences in fatty acid and sterol

content between leathery egg capsules and gelatinous egg masses were analysed using independent *t*-tests on the SPSS statistical package. Prior to analysis, the data were examined visually for normal distribution and Cochran's C test was used to check for homogeneity of variances (Winer 1971).

Multivariate statistical techniques were used to examine phylogenetic similarities in the free lipid composition of gastropod egg masses and capsules using the PRIMER software package (Plymouth Marine Laboratories, UK). Bray-Curtis measures of similarity were used to examine the data matrices (concentration of each free lipid as a proportion of total extract) which were fourth-root transformed to reduce weighting given to abundant fatty acids (Clarke and Warwick, 1994). Two analyses were undertaken to assess taxonomic patterns in the free lipid composition. In the first analysis samples were grouped according to Superorder (Caenogastropoda & Heterobranchia), whereas in the second analysis samples were grouped according to Order or Infraorder (Anaspidea, Basommatophora, Littorinimorpha and Neogastropoda). Non-metric multi-dimensional scaling (nMDS) ordinations were constructed to graphically illustrate relationships between groups. The significance of any apparent differences among taxa was determined using one-way analysis of similarity (ANOSIM) tests (Clarke and Warwick 1994).

Model lipid mixtures

Five saturated fatty acids (pentadecanoic, palmitic, heptadecanoic, stearic and dodecanoic, Sigma), five unsaturated fatty acids (oleic, arachidonic and

cis-5,8,11,14,17-eicosapentaenoic (Aldrich); *cis*-6,9,12,15-octadecatetraenoic acid, and *cis*-7,10,13,16-docosatetraenoic (Sigma)), as well as cholesterol (>98% purity, Sigma) were prepared as mixtures to model the relative proportion of saturated fatty acids, unsaturated fatty acids and sterols found in five types of molluscan spawn; 1) the Aplysiidae family; 2) the Basommatophora; 3) the Littorinimorpha with gelatinous egg masses; 4) the Neogastropoda; and 5) a Cephalopoda *Sepioteuthis australis* (Table 2). The mean proportion of each of these fatty acids, as well as the combined sterols found in the spawn extracts, were calculated from a wide range of samples that had previously been tested for antimicrobial activity (Benkendorff 1999; Benkendorff *et al*. 2001). These included some extracts from freeze dried spawn, as well as some derivatised (methylated) samples of *Aplysia* extracts, that were otherwise excluded from the current study to reduce additional sources of variability in the data. The mean proportions of each fatty acid and sterol found in the fresh extracts used here (Table 1) are also presented in Table 2 for comparison. To obtain the five model mixtures, the relative proportion of each lipid was adjusted to obtain 100%, with care taken to distribute the acids according to both the degree of unsaturation and the length of the carbon chain (refer to Table 2). GC-MS analysis of each mixture confirmed that the fatty acids and cholesterol were recovered in the expected proportions (Benkendorff unpublished data). Each mixture was then tested for antimicrobial activity.

Antimicrobial assays

The five lipid mixtures, as well as the 10 pure fatty acids, were dissolved in acetone and tested for antimicrobial activity against the Gram-positive

Lactococcus garvieae and the three Gram-negative marine pathogens *Vibrio harveyi*, *Vibrio anguillarium* and *Vibrio alginocolyticus*. Exponentially growing cultures were prepared according to Benkendorff et al. (2000a b). Antimicrobial activity was established using the fluorescein diacetate (FDA) assay according to Benkendorff et al. (2000a b), followed by the broth dilution assay for bacteriolytic activity (Haltalin et al. 1973). The pure acids were tested at three concentrations ranging from 0.1-0.001mg/ml and the lipid mixtures were tested at four concentrations from 0.1-0.0001mg/ml. Each sample was tested in triplicate for the FDA assay and duplicate samples were used to create a serial dilution for the bacteriolytic assay. Each dilution was plated onto agar in duplicate and percent viability was determined by comparing the test cultures to a dilution series of the control cultures that were incubated with acetone only.

Results

The benthic molluscan spawn was found to contain a range of saturated fatty acids, primarily with even chain lengths between C14 and C22 (Table 3a). Palmitic (16:0) and stearic (18:0) acids were the dominant fatty acids and the only acids found in the full range of encapsulated egg masses and capsules examined. Myristic acid (14:0) occurred in most of the gelatinous egg masses, but not in the leathery egg capsules. Pentadecanoic acid (15:0) and heptadecanoic acid (17:0) were also found in the egg masses of the Heterobranchia, Littorinimorpha and Cephalopoda, but not in neogastropod egg capsules.

Unsaturated fatty acids were prevalent in the encapsulated gelatinous egg masses of many of the molluscs (Table 3b). The most widespread and abundant was oleic acid (18:1), although C16 and C20 monounsaturated acids were also common (Table 3b). Polyunsaturated acids including C18, C20 and C22 dieneoic, trienoic, tetraenoic and pentaenoic acids, were recorded in the gelatinous egg masses of some Heterobranchia and Littorinimorpha (Table 3b).

Extracts from the spawn of all the molluscs examined in this study contained a substantial amount of sterol lipid (Table 3c). Cholesterol (M^+ 386) was the only sterol recorded in the egg mass/capsules of every species and was the dominant sterol in all species (Table 3c). Some of the other sterols tentatively identified include; cholestanol (M^+ 388), cholesta-3,5-diene (M^+ 368), cholesta-4,6-dien-3-ol (M^+ 384), methylcholestenol (M^+ 400), methylcholestadienol (M^+ 398) and stigmast-4-en-3-one (M^+ 412). However, most sterols remained inconclusively identified due to the very small amounts that were present.

Leathery capsules vs. Gelatinous egg masses

The lipid composition of extracts from molluscan spawn differed substantially according to the structural type (Figure 1). Fatty acids were minor components in the encapsulated leathery egg capsules of gastropods, whereas a high concentration of fatty acids occurred in encapsulated gelatinous egg masses (Figure 1a): *t*-tests revealed a significant difference in both the number of fatty acids (equal variances not assumed; $t = 6.48$, DF = 16.63, $p < 0.001$) and the relative amount of fatty acids, as a proportion of all

volatile compounds found in the lipid extract (t = 5.63, DF = 21, p < 0.001) from these different types of egg mass. The most notable difference between leathery capsules and gelatinous masses was the complete absence of unsaturated fatty acids in the leathery capsules (Figure 1a). The proportion of saturated fatty acids is also significantly higher in extracts from gelatinous egg masses compared to leathery egg capsules (Figure 1a; t = 4.09, DF = 21, p =0.001).

On average the proportion of sterols was higher in encapsulated leathery egg capsules than gelatinous egg masses, although there was substantial variation within the extracts from leathery egg capsules (Figure 1b). A nonparametric t-test revealed no significance difference in the amount of sterols in capsules or egg masses (t = 1.78, DF = 12.36, p = 0.10). Cholesterol, the primary sterol present in both types of egg mass, appears to be the major contributing factor to the differences in sterol concentration (Figure 1b).

Major taxonomic differences in lipid composition

The fatty acid composition in extracts from molluscan spawn varied between broad taxonomic groupings (Figure 2). MDS ordination analysis revealed significantly different groupings for samples taken from the two gastropod superorders, Heterobranchia and Caenogastropoda (Global R = 0.168; p = 0.046). In general, the encapsulated heterobranch egg masses contain a wider range of both saturated and unsaturated fatty acids and the lowest proportion of sterols in their extracts (Table 3). Nevertheless, there was overlap between these two groupings, with greater variation occurring in the

Caenogastropoda (Figure 2a), which are represented by both leathery capsules and gelatinous masses.

At a finer taxonomic level, significant differences were observed between the samples taken from different orders/infraorders (Figure 2b; Global $R = 0.0482$; $p = 0.001$). In particular, the neogastropods were found to cluster separately from all other taxa (from Anaspidea $R = 0.918$, $p = 0.022$; Basommatophora $R = 0.575$, $p = 0.003$; Littorinimorpha $R = 0.575$, $p = 0.003$). The neogastropods contain relatively small amounts of exclusively even chain length, saturated fatty acids, and high concentrations of sterol (Table 2, 3). All other taxonomic groups contain at least some species with unsaturated fatty acids (Table 3). No significant differences were observed between pairs for the other groupings at this taxonomic level (Anaspidea - Basommatophora $R = 0.167$, $p = 0.25$; Anaspidea – Littorinimorpha, $R = 0.509$, $p = 0.095$; Basommatophora – Littorinimorpha, $R = 0.048$, $p = 0.284$).

A reasonably large amount of variation in the fatty acid and sterol composition occurred within each taxonomic group (Table 2, 3, Figure 2). Despite forming a distinct cluster the Neogastropods appear to separate into two groups (Figure 2b). Some of this variability was due to replicate samples from *Dicathais orbita* and hence there seems to be as much intra- as inter-species variation in the data. This was also the case with the Littorinimorpha, where high amount of intraspecific variation was observed in the lipid composition of replicate extracts from *Conuber* c.f. *sordidus* and *Bembicium nanum*. Interestingly however, *Cabestana spengleri* (a Littorinimorpha with leathery

capsules) was found to cluster with the other Littorinimorpha (with gelatinous masses) rather than with the neogastropods (Figure 2b).

The lipid composition in the encapsulated egg masses from pulmonates revealed striking differences between three families that occur in different aquatic habitats (Table 1, 3). The freshwater species *Isidorella hainesi* was found to contain much lower concentrations of fatty acids, and much higher concentrations of sterol compared to the marine and estuarine species (Table 3). The two estuarine (*Salinator*) species appear to have a greater content of unsaturated fatty acids than the two marine (Siphonariidae) species (Table 3c). Most of the variation in lipid composition observed within the marine and estuarine families was due to intraspecific variation rather than any apparent differences between the species. One pulmonate sample (Basommatophora, Heterobranchia) appears to be an outlier in the ordination analysis (Figure 2). This was a sample taken from *Salinator fragilis* egg masses and was found to contain high concentrations of molecular sulfur (S_6 and S_8) (Benkendorff 1999). This affected the total concentration of fatty acids recorded in the egg masses, which varied from 28% in the first sample to 51% in the second sample (Table 3).

Notably, the cephalopod spawn samples did not differ greatly from the gastropods in terms of their fatty acid composition, despite being from a different class (Table 2, 3). Overall, *Sepioteuthis australis* encapsulated egg masses contained relatively high concentrations of saturated fatty acids, but small amounts of unsaturated fatty acids were also detected (Table 2, 3). This species can also be distinguished by high concentrations of sterol, which is

exclusively cholesterol, unlike the gastropod egg masses, which contain a mixture of sterols (Table 3c).

Antimicrobial activity of fatty acids and lipid mixtures

A range of fatty acids found in the spawn extracts were tested for bacteriolytic activity against four marine pathogens (Table 4). In general, the FDA assay was not successful for detecting antimicrobial activity in these fatty acids even where the broth dilution method showed that significant cell death had occurred. Since the FDA assay has a relatively short incubation time, it is likely the fatty acids are relatively slow acting and any residual metabolic activity in the surviving cells was responsible for producing the false negative results observed using this assay.

In the broth dilution assay, the Gram-negative *Lactococcus garvieae* was found to be the most resistant to all test acids and was only strongly affected by the unsaturated fatty acids (Table 4). The three Gram-positive *Vibrio* spp. were also more susceptible to the unsaturated than the saturated fatty acids. In addition, saturated acids with shorter chain lengths (e.g. C15) generally caused more cell death than those with longer chain lengths (e.g. C18, Table 4). Oleic acid (C18:1) was at least as active as the polyunsaturated acids. *Vibrio harveyi* was the least resistant to all fatty acids, with less than 10% viability remaining in cultures incubated with only 0.001mg/ml of some unsaturated acids (Table 4).

The five lipid mixtures modeled on the composition of molluscan spawn extracts all showed some activity against the test bacteria (Table 2). The

Aplysiidae-equivalent mixture caused high levels of cell death against all three marine bacteria (Table 5). This mixture showed greater activity against *L. garvieae* than any of the individual fatty acids (Table 4), with less than 1% viability remaining after incubation with 0.0001mg/ml of the mixture (Table 5). The remaining lipid mixtures showed relatively low levels of activity, with significant cell death only occurring at the maximum test concentration (0.1mg/ml) against one or two species of bacteria (Table 5). The neogastropod-equivalent mixture, containing only saturated acids and sterols, showed the least activity with the majority of the cell cultures remaining viable after incubation with the lipid mixture at 0.1mg/ml.

Discussion

To date, few studies have investigated the biochemical composition of molluscan spawn. Here we have shown that methanol:chloroforrm extracts from the benthic spawn of 17 aquatic molluscs contain a wide range of long-chain fatty acids and sterols (see Table 3). By comparison, a previous histochemical study by Bayne (1968) found methanol:chloroform soluble lipids in just two of seven gastropod egg masses and capsules tested. Most of the species studied by Bayne (1968) were terrestrial and notably lipids were reported in the egg capsules of the only marine species analyzed (*Nucella lapillus*). However, lipids were not detected in the spawn of one freshwater Basommatophoran (Bayne, 1968), whereas in this study, extracts from the spawn of four marine and estuarine Basommatophora species showed reasonably high proportions of fatty acid (22-56%) and sterol (8-18%, Table 3). Fatty acids were also detected in extracts from the spawn of a freshwater Eupulmonata (Table 3), suggesting the GC/MS analysis of lipid extracts in this

study may be more sensitive that Bayne's (1968) histochemistry. Nevertheless, the potential for habitat related differences is worthy of further investigation since the fresh water species here examined contained relatively low concentrations of fatty acids (5.1%), but a high proportion of sterol (39%), when compared to all marine and estuarine species (Table 3). Past studies on adult molluscs support the idea that freshwater species have lower lipid content than marine species (Voogt 1968).

The composition of free fatty acids and sterols found in molluscan spawn varied according to both structural type (leathery capsules vs. gelatinous masses, Figure 1) and taxonomic origin of the species (Figure 2). Palmitic (16:0) and stearic (18:0) acids were the dominant fatty acids found in all the spawn extracts, which is consistent with previous studies on the lipids of adult molluscs (Tibaldi 1966; Beninger and Stephan 1985; Yamaguchi et al. 1992a b; Rakshit et al. 1997; Carballeira et al. 2001). *De novo* synthesis of these two saturated fatty acids has been reported to precede spawning in a bivalve mollusc (Kluytmans et al. 1985) and this may also be the case in other molluscan taxa. Saturated fatty acids with an uneven chain length (heptadecanoic and pentadecanoic acids) were detected in the extracts of several molluscan taxa (Table 3a) and likewise have been previously reported from molluscan tissue (Beninger and Stephan 1985; Rakshit et al. 1997). Notably, these uneven length acids were entirely absent from the Neogastropoda spawn, although they were detected in the encapsulated leathery egg masses of a Littorinimorpha (Table 3). This suggests that the biosynthetic pathways for producing these fatty acids and/or allocating them to neogastropod spawn may have been lost in this relatively recent Infraorder.

Further studies on adult neogastropods are required to determine whether or not they are capable of producing these compounds.

Unsaturated fatty acids were detected in extracts from encapsulated gelatinous gastropod egg masses, as well as the spawn of a cephalopod (see Figure 1, Table 3). Consistent with studies on adult molluscs (Beninger and Stephan 1985; Rakshit et al. 1997), oleic acid (C18:1) was the most abundant and widespread unsaturated fatty acid found in the spawn. A number of other predominately even chained unsaturated acids were found, including some polyunsaturated acids (Table 3b), which are characteristic of many marine lipid extracts (e.g. Yamada and Hayashi 1975). Palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2) and arachidonic (C20:4) acids are known to possess antimicrobial activity against human pathogens (Kabara 1978). Our study now confirms that these long-chain unsaturated fatty acids are also active against aquatic pathogenic bacteria at relatively low concentrations (see Table 4). Unsaturated fatty acids could thus account for some of the antimicrobial activity that has been previously recorded in these same lipophilic extracts from molluscan spawn (see Benkendorff et al. 2001).

Both the type and quantity of free fatty acids in the spawn could have implications for their potential role in defense against microbial invasion, providing these metabolites are present in a freely available form. Previous studies have found chemical defense associated with eggs, embryos and larvae of many marine invertebrates, but few studies to date provide any data on the localization of metabolites (reviewed by Lindquist, 2002). Likewise the embryos were not separated from the extra-embryonic supporting layers or

intracapsular fluid for lipid extraction in this study. Future studies should endeavor to address whether or not unsaturated fatty acids are bioavailable in the extra-embryonic material, thus providing a clear mechanism for defending the encapsulated embryos against aquatic pathogens. Nevertheless, it is apparent from our study that the whole composition of a lipid mixture will influence the level of bacteriolytic activity against a suite of aquatic bacteria (Table 4, 5). Bioactive unsaturated fatty acids were not present in sufficiently high concentrations in the whole spawn extracts of most molluscan taxa to provide any potential sterilization. Indeed of the five lipid mixtures modeled on extracts from different molluscan taxa (see Table 2) only one, the Aplysiidae-equivalent mixture, was active at sufficiently low concentrations to account for all the previously recorded antimicrobial activity (Table 5; Benkendorff et al. 2001a). This suggests that activity in the extracts from the other taxa must be mediated by alternative or additional components. Notably, a number of bioactive brominated alkaloids have been identified in the spawn of Muricidae (Benkendorff et al. 2000b; 2001b; 2004). The capsule wall could also provide a barrier against microbial invasion in leathery egg capsules of caenogastropods, as suggested by Lord (1986). Preliminary analysis of all the gelatinous spawn extracts studied here have revealed mixtures of secondary metabolites (Benkendorff 1999), which may act synergistically with fatty acids and/or complement their activity.

Sterols, such as cholesterol, are known to antagonize the antimicrobial activity of fatty acids (Galbraith et al. 1971). Consequently, high cholesterol levels (>25% w/w) may explain the relatively low activity observed in the lipid mixtures from all taxon groups except the Aplysiidae (Table 2 & 5).

Cholesterol was the most abundant sterol and the only sterol found in the spawn of every species examined (see Table 3c), which is consistent with previous studies on sterols in adult gastropods and cephalopods (Bergmann 1949; Idler and Wiseman 1972). With the exception of *Sepioteuthis australis*, all species contained a mixture of sterols in their encapsulated spawn (see Table 3c). Previous studies indicate that cholesterol is the only sterol occurring in cephalopods (Idler and Wiseman 1972). Numerous other sterols were detected in the gastropod spawn (see Table 3c), but in such small quantities that most remain unidentified.

The functional role of the free lipids in molluscan spawn remains uncertain at this stage, but is likely to vary between species according to both the composition of fatty acids and where they are located. In the caenogastropod *Nucella lapillus* lipid globules were found amongst spheres of nutritive material (Bayne, 1968) and thus these may represent additional energy reserves for the embryos. Bayne (1968) also reported lipids on the surface of the egg capsules from a terrestrial pulmonate and suggested these could play a role in reducing desiccation. Pechenik (1983) has proposed that non-diffusing organic molecules (such as free lipids) could play a role in protecting encapsulated embryos from salinity stress. Protection from both desiccation and salinity fluctuations could be important adaptive functions for lipids on the surface of spawn from intertidal gastropods, such as those examined here. Finally, unsaturated fatty acids may contribute towards the maintenance of cell membrane fluidity, in addition to their antimicrobial activity, in the gelatinous spawn of some molluscs. Long-chain unsaturated fatty acids are generally thought to be an adaptation to cold temperatures (Holland 1978).

However, all species were collected from the same temperate location in this study so climatic differences cannot directly explain the fact that these metabolites are present in encapsulated gelatinous egg masses but not leathery capsules. It remains possible that the caenogastropods first evolved leathery egg capsules in warmer climates with fewer requirements for unsaturated fatty acids. Alternatively, the wall of fibrous proteins found in leathery egg capsules (Flower et al., 1969; Hunt, 1971) may effectively buffer the encapsulated embryos from environmental fluctuations (e.g. Przeslawski, 2004). Thus, unsaturated fatty acids could be of greater functional importance for embryos contained in gelatinous egg masses and this warrants further investigation.

It is also potentially significant that all of the species that deposit leathery egg capsules are predatory molluscs, whereas most of the gelatinous egg masses are deposited by herbivores. Thus, the observed differences in the saturation of fatty acids in gelatinous egg masses and leathery egg capsules may be related to differences in the diets of the adult molluscs, rather than differences in the biochemical pathways. In filter-feeding bivalves most of the stored fatty acids appear to be diet-derived (Beninger and Stephan 1985; Kluytmans et al. 1985). Rakshit et al. (1997) also suggested that differences in the fatty acid content of the gastropod *Telescopium telescopium* from two different communities were the result of different dietary sources. However, the encapsulated gelatinous egg masses of one predatory gastropod (*Conuber sordidus*), as well as the spawn of a cephalopod (also a carnivore), were found to contain some unsaturated fatty acids (see Table 3b). Consequently, at the very least, there does appear to be a difference in types of fatty acids

that predatory molluscs allocate towards leathery versus gelatinous egg masses.

The composition of free fatty acids and sterols may provide useful chemical markers for inclusion in future taxonomic studies. The analysis of metabolites such as fatty acids has been widely applied to the taxonomy of microorganisms (e.g. Brondz and Olsen 1986), but is underutilized in the taxonomy and systematics of invertebrates. Substantial differences in free lipid content were observed in the spawn of the molluscan taxa examined here. In particular, the samples from the Neogastropoda egg capsules formed a significantly distinct group in the MDS ordination (Figure 3). The detailed profiles of isomeric forms of unsaturated fatty acids may provide additional chemical indicators in the other molluscan taxa. Nevertheless, substantial inter-and intra-species variation was observed in the samples highlighting the need for replication and the importance of standardizing all procedures for processing and storing the extracts. Furthermore, the type of spawn (gelatinous egg mass vs. capsules), as well as the habitat and trophic niche of the molluscs should all be taken into consideration, since these factors may be even more important determinants of fatty acid composition than phylogeny. Future comparative investigations involving the identification and quantification of fatty acids and sterols could be conducted within conserved taxonomic groups for the determination of environmental effects. Further interesting insights into molluscan biochemical evolution might be obtained by phylogenetic comparisons within groups of ecologically similar molluscs and their spawn.

Overall, there are considerable differences in the lipid extracts from the benthic spawn of different molluscan species. The reasons for these large differences are not presently clear and deserve further investigation. Nevertheless, it is apparent from this study that the free unsaturated fatty acids found in some molluscan spawn can inhibit the growth of marine pathogenic bacteria. Thus, among other potential functions, these metabolites could play a role in protection against infection, if shown to be bioavailable in sufficient quantities on the surface or in the extracellular matrix of gelatinous egg masses.

Acknowledgements

We are grateful to Dr John Korth (University of Wollongong) for technical assistance with the GC/MS. We are also grateful to Dr Manuel Ballesteros (University of Barcelona) for assistance in the collection and identification of the egg masses from the Mediterranean. The marine pathogenic bacteria were kindly provided by Dr Jeremy Carson from the Fish Health Unit, Department of Primary Industry and Fisheries, Tasmania. Comments on the manuscript by the marine biology discussion group at Flinders University and two anonymous reviewers are appreciated. We thank the University of Wollongong for financial support for this project. Specimens were collected under scientific permit no. F95/269, in accordance with the provisions of Section 37 of the N.S.W. Fisheries Management Act 1994.

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Table 1: Species of mollusc used for lipid analysis by GC/MS. The species were collected from marine (M), estuarine (E) and freshwater (F) habitats along the Illawarra Coast, N.S.W. Australia. The type of egg mass deposited by the species is provided (i.e. leathery egg capsules or gelatinous egg mass). Number of samples refers to the number of extracts that were prepared from independent collections of the egg masses.

Order/ Infraorder	**Family**	**Species**	**Habitat**	**Type of egg mass**	**No. samples**
CAENOGASTRPODA					
Littorinimorpha	Littorinidae	*Bembicium nanum*	M	Gelatinous	2
	Naticidae	*Conuber* c.f. *sordidus*	E	Gelatinous	3
	Ranellidae	*Cabestana spengleri*	M	Leathery	1
Neogastropoda	Muricidae	*Agnewia tritoniformis*	M	Leathery	2
		Dicathais orbita	M	Leathery	3
		Lepsiella reticularis	M	Leathery	1
		Morula marginalba	M	Leathery	2
	Conidae	*Conus papilliferus*	M	Leathery	1
HETEROBRANCHIA – PULMONATA					
Basommatophora	Amphibolidae	*Salinator fragilis*	E	Gelatinous	2
		Salinator solida	E	Gelatinous	1
	Siphonariidae	*Siphonaria denticulata*	M	Gelatinous	2
		Siphonaria zelandica	M	Gelatinous	1
Eupulmonata	Planorbidae	*Isidorella hainesi*	F	Gelatinous	1
HETEROBRANCHIA – OPISTHOBRANCHIA					
Anaspidea	Aplysiidae	*Aplysia juliana*	M	Gelatinous	1
		Stylocheilus longicauda	M	Gelatinous	1
Cephalaspidea	Philinidae	*Philine angasi*	M	Gelatinous	1
CEPHALOPODA					
Teuthoidea	Loliginidae	*Sepioteuthis australis*	M	Tough capsules	1

Table 2: Average lipid composition in egg masses for five major groups of molluscs and corresponding artificial mixtures modeled on these (=equiv.). The lipid mixtures are made up entirely from 5 saturated fatty acids, 5 unsaturated acids and cholesterol, although a greater range of lipids is found in the egg mass extracts. Consequently, all sterols have been pooled together and some fatty acids of similar chain length and degree of saturation have been pooled as follows: C:15 = <14:0 + 15:0; C:16:0 = 14:0 + 16:0; C:17:0 = 17:0 + 19:0; C20:0 = 20:0 + 22:0; C18:1 = 16:1 + 17:1 + 18:1 + 19:1; C18:4 = 18:2 + 18:3 + 18:4; C20:4 = 20:1 + 20:2 + 20:3 + 20:4; C20:5 = 20:5 + 22:5; C22:4 = 22:1 + 22:2 + 22:3 + 22:4. The mean proportion ± standard error is provided for each of these lipid groups, as well as the total percentage of saturated fatty acids (sat.), unsaturated fatty acids (unsat.) and sterols from both the freshly extracted egg masses and capsules (Fresh) used in this study (see Table 1), as well as those taken from a greater pool of samples (All) including extracts from freeze-dried egg masses and derivatised extracts that have previously been tested and found to have antimicrobial activity.

Taxa	**Sample**	**C15:0**	**C16:0**	**C17:0**	**C18:0**	**C20:0**	**C18:1**	**C18:4**	**C20:4**	**C20:5**	**C22:4**	**Total sat.**	**Total Unsat.**	**Sterol**
Aplysiidae	Fresh [1]	3±0.3	18±6	15±2	16±4	0.4±.0.4	16±8	0	13±6	2±2	8±6	**52±8**	**40±9**	**8±0.7**
	All [2]	2±0.3	17±7	17±14	10±6	0.2±.4	14±7	5±7	19±10	4±5	7±5	**47±10**	**50±12**	**4±3**
	Equiv.	2	15	15	14	1	16	6	16	6	6	**47**	**50**	**3**
Basommatophora	Fresh [3]	4±1	32±6	3±1	6±2	0.4±0.4	23±4	1±1	2±1	0	0	**45±6**	**26±5**	**29±7**
	All [4]	3±3	32±13	3±3	6 ± 6	0.4± 1	23±14	1±2	2±2	0	0	**44±15**	**27±18**	**30±18**
	Equiv.	4	30	4	9	2	20	1	4	0	0	**49**	**25**	**26**
Littorinimorpha	Fresh [5]	7±5	35±8	6±2	10±2	2± 2	8±3	2±1	5±2	0	2±2	**61±3**	**17±6**	**22±5**
	All [6]	4±5	30±19	6±3	10±7	2± 3	10±11	3±3	5±7	0	1±3	**52±15**	**19±17**	**32±23**
	Equiv.	5	25	6	10	2	10	2	5	0	1	**48**	**18**	**34**
Neogastropoda	Fresh [7]	0.3±0	24±9	0	13±6	0	0	0	0	0	0	**38±14**	**0**	**62±14**
	All [8]	0	22±12	0	11±13	0	0	0	0	0	0	**33±34**	**0**	**67±34**
	Equiv.	0	23	0	10	0	0	0	0	0	0	**33**	**0**	**67**
Sepioteuthis	Fresh [9]	7	33	4	19	0	12	0	0	0	0	**64**	**12**	**24**
	All [10]	5± 1	32±3	4± 1	12±10	0	7± 6	0	0	0	0	**54±14**	**7±6**	**39±21**
	Equiv.	5	30	4	15	0	8	0	0	0	0	**54**	**8**	**38**

[1] N = 2 extracts from 2 species; [2] N = 6 extracts from 4 species; [3] N = 6 extracts from 4 species; [4] N = 7 extracts from 4 species; [5] N = 5 extracts from 2 species with gelatinous egg masses; [6] N = 7 extracts from 2 species; [7] N = 8 extracts from 5 species; [8] N = 12 extracts from 7 species; [9] N = 1 extract from *Sepioteuthis australis*; [10] n = 2 extracts from *Sepioteuthis australis*.

Table 3: The percent of; a) saturated fatty acids; b) unsaturated fatty acids and c) sterols, in the volatile organic compounds found in extracts from the egg masses and capsules of 17 species of molluscs from one Cephalopoda and six orders of Gastropoda. Means ± standard deviations used where replicate samples from the same species have been analysed. The length of the carbon chain (C) is presented for each fatty acid, followed by the number of double bonds (n) for each unsaturated acid. The molecular ion (M+) is provided for the sterols.

a)

Species	% of volatile compounds represented by saturated fatty acids									
	< C14	C14	C15	C16	C17	C18	C19	C20	C22	Total
Aplysia juliana	0.79	1.75	0.68	9.93	6.16	9.31	0	0	0	28.62
Stylocheilus longicauda	0.26	1.5	1.26	6.14	10.13	7.25	0	0.32	0.2	27.06
Philine angasi	0.95	3.86	1.96	13.3	3.03	5.24	0	0.65	0	28.99
Siphonaria denticulata	0.16 ±0.2	1.46 ±0.9	1.51 ±1.2	9.01 ±2.5	0.63 ±0.3	3.39 ±2.3	0	0.44 ±0.6	0	16.59 ±5.6
Siphonaria zelandica	1.24	2.36	1.31	10.95	3.49	5.62	0	0	0	24.97
Salinator fragilis	0.07 ±0.1	0.44 ±0.6	0.37 ±0.5	21.36 ±17.7	0	0.44 ±0.6	0	0	0	22.67 ±15.8
Salinator solida	0.34	4.9	3.27	14.16	2.99	0.75	0	0	0	26.41
Isidorella hainesi	0	0.3	0	3.08	0	1.22	0	0	0	4.6
Bembicium nanum	0.71 ±0.3	7.56 ±4.9	1.47 ±0.8	18.85 ±7.6	4.42 ±4.4	8.1 ±5.38	0.22 ±0.3	3.96 ±3.5	0	45.28 ±4.1
Conuber c.f. *sordidus*	5.56 ± 8.1	4.4 ±3.1	0.77 ±0.9	16.35 ±13.2	3.75 ±2.6	5.96 ±3.1	0	0.18 ±0.3	0	36.96 ±4.3
Cabestana spengleri	0.5	0	0.47	0.87	2.18	3.07	0	0.37	0	7.46
Conus papilliferus	0	0	0	2.59	0	0	0	0	0	2.59
Agnewia tritoniformis	0	0	0	1.24	0	0	0	0	0	1.24
Dicathais orbita	0.24 ±0.4	0	0	3.06 ±1.7	0	1.93 ±3.3	0	0	0	5.22 ±5.4
Lepsiella reticularis	0	0	0	12.8	0	8.23	0	0	0	21.03
Morula marginabla	0	0	0	16.97 ±0.2	0	9.26 ±1.6	0	0	0	26.23 ±1.4
Sepioteuthis australis	1.53	2.3	3.08	19.78	2.99	12.61	0	0	0	42.29

b)

TAXON	% of volatile compounds represented by unsaturated fatty acids (C:n)																		
Species	14:1	16:1	17:1	18:1	18:2	18:3	19:1	20:1	20:2	20:3	20:4	20:5	21:2	21:3	22:1	22:2	22.3	22:4	22:5	Tot
A. juliana	0	1.1	0	2.9	0	0	0	1.9	1.3	0.4	0.2	0	3.8	2.6	0	0.1	0	0.3	0	14.7
S. longicauda	0	1.1	4.4	9.6	0	0	0	3.8	1.9	1.6	3.3	1.9	0	0	0	0.3	0.3	0.7	0.6	29.6
P. angasi	0	1.1	0	4.5	0	0	0	1.2	0.7	0	0.9	0	0	0	0.1	0	0	0	0	8.4
S. denticulata	0	0.6	0.4	4.0	0	0	0	0.4	0	0.9	0	0	0	0	0	0	0	0	0	5.9
		±0.0	±0.6	±1.9				±0.6		±0.5										±3.6
S. zelandica	0	0	0	6.7	0	0	0	1.47	0	0.6	0	0	0	0	0	0	0	0	0	8.7
S. fragilils	0	14.0	0.4	2.5	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	17.0
		±4.6	±0.6	±3.5						±0.2										±0.3
S. solida	0.2	14.4	1.2	5.9	2.7	0	2.3	0.4	0.4	1.4	0.6	0	0	0	0	0	0	0	0	29.4
I. hanesi	0	0	0	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6
B. nanum	0	4.1	0	3.9	2.7	0.1	0	4.4	2.3	1.2	0	0	0	0	2.2	0.7	0.2	0	0	21.7
		±3.4		±3.5	±3.8	±0.2		±4.0	±3.2	±1.7					±3.1	±0.9	±0.2			±2.2
Conuber sp.	0	1.9	0	2.1	0.9	0	0	0.6	0.1	0.2	0	0	0	0	0	0	0	0	0	5.8
		±3.3		±2.1	±0.4			±0.5	±0.2	±0.3										±6.3
C. spengleri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. papilliferus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A. tritoniformis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D. orbita	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. reticularis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M. marginalba	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. australis	0	2.6	0	5.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.9

1 c)

TAXON Species	Cholesta-3,5-diene M+ 368	Cholesta dienol M+ 384	Cholestanol M+ 388	Cholesterol M+ 386	Methyl cholesta-dienol M+ 398	Methyl cholestenol M+ 400	Stigmast-4-en-3-one M+ 412	Other sterols	Total
A. juliana	0	0.2	0	1.8	0	0	0	2.2	4.2
S. longicauda	0.2	1.0	0	2.2	0	0.2	0	0.9	4.5
P. angasi	0	2.3	1.2	26.5	7.8	1.4	1.9	3.7	44.8
S. denticulata	0.2 ± 0.3	0.3 ± 0.4	0	12.8 ± 5.9	0	0	0	0.7 ± 1.1	14 ± 4.7
S. zelandica	0	0	0	8.4	0.2	0	0	0.4	9.0
S. fragilis	0	2.4 ± 3.4	0	10.7 ± 5.3	2.4 ± 3.3	0.8 ± 1.2	0.3 ± 0.5	1.7 ± 2.3	18.3 ± 16
S. solida	0	0.5	0	3.4	0.4	0	0.5	3.4	8.2
I. hanesi	0	0	0	23.8	1.1	2.5	3.3	3.3	33.9
B. nanum	0.1 ± 0.2	0	0	8.7± 7.6	0	0	0	1.3 ± 1.1	10.0 ± 8.9
Conuber sp.	0	1.3 ± 0.7	0	10.8 ± 4.5	1.7 ± 0.7	0.7 ± 0.6	0.1 ± 0.2	1.9 ± 0.4	16.5 ± 5.2
C. spengleri	0	1.2	0	21.1	6.5	1.5	0.7	4.1	35.1
C. papilliferus	0	0	3.7	36.2	5.2	0	0	0	45
A. tritoniformis	0	5.8	1.5	31.4	0.9	0.8	0.6	2.4	43.4
D. orbita	0	1.6 ± 0.3	1.7 ± 0.6	24.4 ± 8.1	2.3 ± 2.0	0.4 ± 0.4	0	3.0 ± 3.5	33.5 ± 8.4
L. reticularis	0	0	0	2.4	0	0	0	0	2.4
M. marginalba	0	0	0	5.1 ± 0.5	0	0	0.4 ± 0.6	0	5.5 ± 1.0
S. australis	0	0	0	15.8	0	0	0	0	15.8

Table 4: Bactericidal activity of 11 long-chain fatty acids (>98% purity, Sigma) with various degrees of unsaturation, at three concentrations (mg/ml), against four marine pathogenic bacteria: *Lactococcus garvieae*, *Vibrio anguillarium*, *Vibrio harveyi* and *Vibrio alginocolyticus*.

Fatty acid	**Conc.**	**L. gar.**	**V. ang.**	**V. har**	**V. alg**
C15:0	0.1	++++	++++	++++	++++
	0.01	++	+	++++	++++
	0.001	-	-	++++	-
C16:0	0.1	+	++++	++++	++++
	0.01	-	-	++++	++++
	0.001	-	-	+	-
C17:0	0.1	-	++++	++++	++
	0.01	-	-	+++	-
	0.001	-	-	+++	-
C18:0	0.1	-	+++	++++	+++
	0.01	-	-	++	-
	0.001	-	-	-	-
C18:1	0.1	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
	0.001	++	++++	++	-
C18:4	0.1	++++	++++	++++	++++
	0.01	-	+++	++++	++++
	0.001	-	-	++	+
C20:4	0.1	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
	0.001	-	-	+++	-
C20:5	0.1	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
	0.001	-	-	+++	-
C22:0	0.1	++++	++++	++++	++++
	0.01	-	-	+++	+
	0.001	-`	-	+	-
C22:4	0.1	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
	0.001	-	-	++	+
C22:5	0.1	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
	0.001	++	-	+++	-

-	100% viability
+	> 50% viability
++	10 – 50% viability
+++	1 – 10% viability
++++	<1% viability

Table 5: Bactericidal activity of five lipid mixtures modeled (equiv.) on the fatty acid and sterol content of different molluscan taxa (see Table 2), at four concentrations (mg/ml) against three marine pathogenic bacteria: *Lactococcus garvieae*, *Vibrio harveyi* and *Vibrio alginocolyticus*.

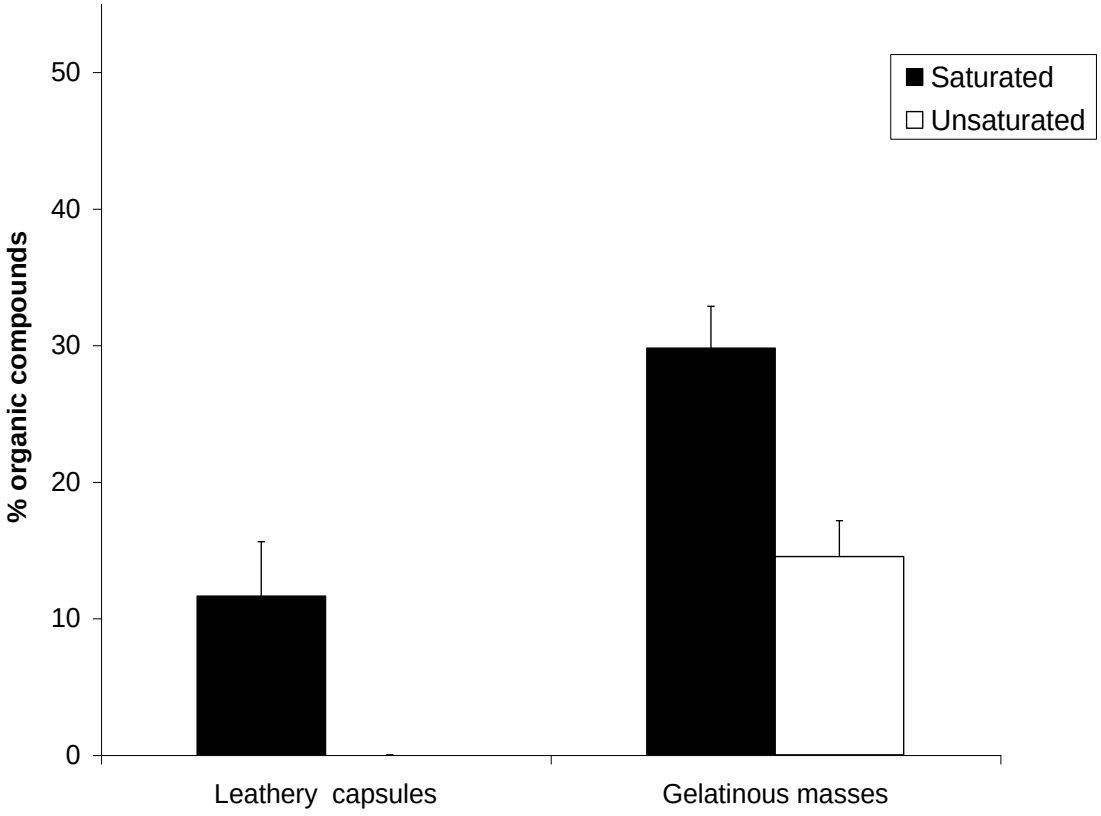
Lipid mixture	**Conc.**	**L. gar**	**V. har**	**V. alg**
Aplysiidae – equiv.	0.1	++++	++++	++++
	0.01	++++	++++	++++
	0.001	++++	++	+
	0.0001	++++	++	-
Basommatophora - equiv.	0.1	+	++	++++
	0.01	-	+	++
	0.001	-	+	-
	0.0001	-	-	-
Littorinimorpha – equiv.	0.1	++	++++	++
	0.01	-	++	++
	0.001	-	-	-
	0.0001	-	-	-
Neogastropoda – equiv.	0.1	+	+	++
	0.01	-	-	-
	0.001	-	-	-
	0.0001	-	-	-
Sepioteuthis – equiv.	0.1	++++	++	+
	0.01	-	++	-
	0.001	-	+	-
	0.0001	-	-	-

-	100% viability
+	> 50% viability
++	10 – 50% viability
+++	1 – 10% viability
++++	<1% viability

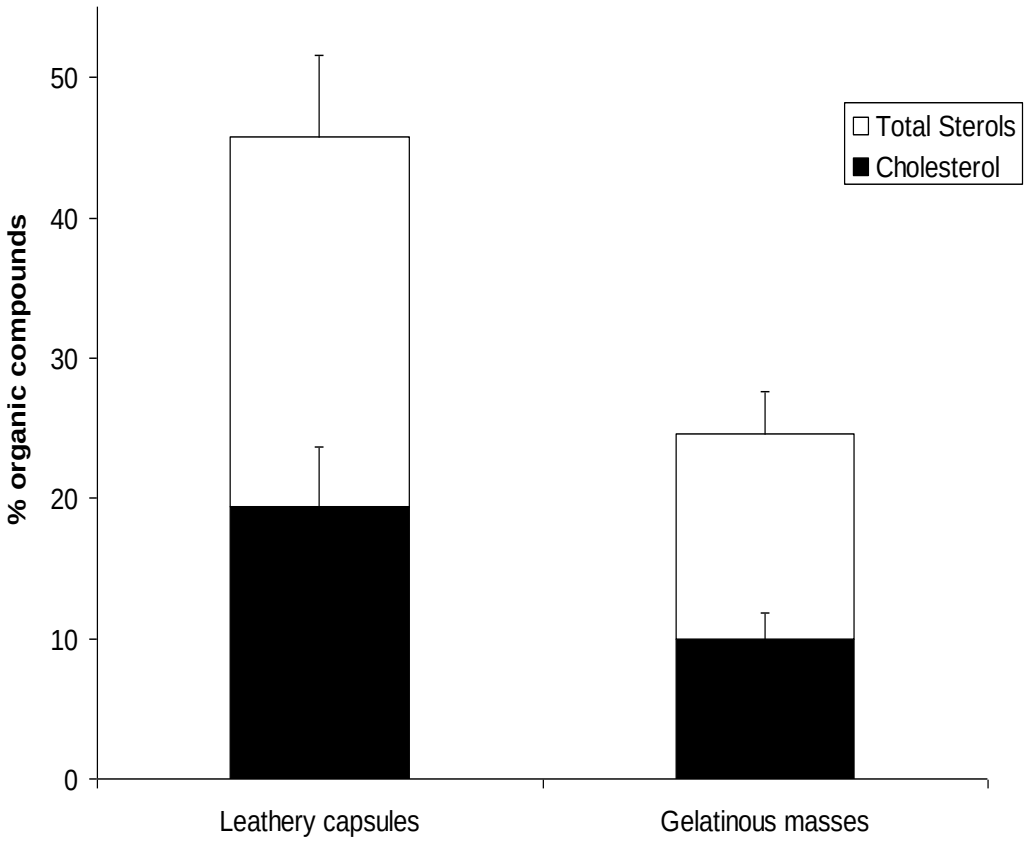
Figure legends

Figure 1: A comparison of the lipid composition found in the leathery egg capsules (N = 9 extracts from 6 species) and gelatinous egg masses (N = 14 extracts from 9 species) deposited by intertidal gastropods; a) the relative proportion of saturated and unsaturated fatty acids or their methyl esters and; b) the relative proportion of cholesterol and other sterols. The proportion of each lipid class is calculated as a mean percent (+ standard error) of the combined concentration of all organic compounds in the extracts, estimated from the relative intensities of each peak detected using gas chromatography/ mass spectrometry.

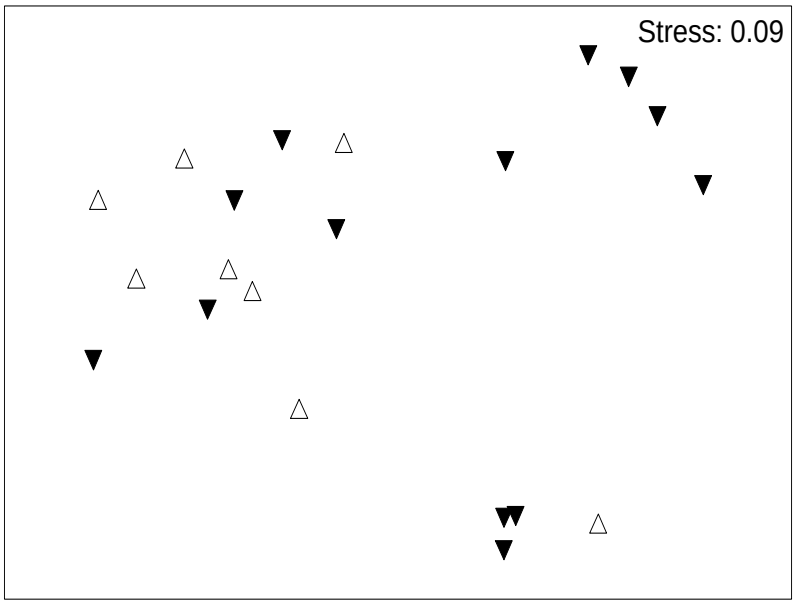
Figure 2: Non-metric multi-dimensional scaling (nMDS) ordination of the free fatty acid and sterol composition in the egg masses and capsules of marine gastropods grouped according to phylogeny: a) superorder; b) order/infraorder.


Saturated
Unsaturated
% organic compounds
50
40
30
20
10
0
Leathery capsules
Gelatinous masses

b)


Total Sterols
Cholesterol
% organic compounds
50
40
30
20
10
0
Leathery capsules
Gelatinous masses

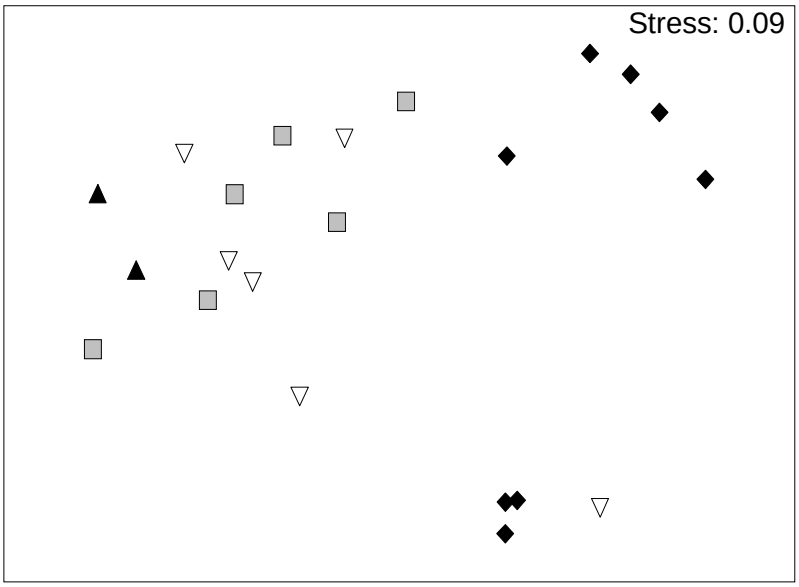
a)



△ Heterobranchia

▼ Caenogastropoda

b)



▲ Anaspidea

▽ Basommatophora

◻ Littorinimorpha

◆ Neogastropoda