

2005

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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>

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

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14

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

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

14

15 **Keywords**

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

18

19 **Introduction**

20 Lipids form a significant component of the fuel reserves in the muscles and
21 organs of adult molluscs (Suryanarayanan and Alexander 1971; Jarzebski et
22 al. 1986) and are thought to act as a storage product in the freely spawned
23 eggs of bivalve molluscs (Vassallo 1973; Bayne et al. 1978; Kluytmans et al.
24 1985). However, the composition and significance of lipids in spawn,
25 deposited as benthic egg masses and capsules by aquatic molluscs, remains
26 unclear. Early histochemical studies provide little evidence for the presence of

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

7

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

25

1 Both types of benthic egg mass are thought to provide protection to the
2 encapsulated embryos against predation and environmental stress (Pechenik
3 1979; 1983; Rawlings 1994; Przeslawski et al. 2004). Recently, extracts from
4 the benthic spawn of a broad range of aquatic molluscs have also been
5 shown to possess antimicrobial properties (Benkendorff et al. 2000a; 2001a).
6 Activity against a broad spectrum of marine and human pathogenic bacteria is
7 primarily concentrated in the lipophilic layer of solvent extracts of the spawn.
8 Preliminary GC/MS analyses indicate that the predominant secondary
9 metabolites in these extracts are free fatty acids and sterols (Benkendorff
10 1999).

11

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

23

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

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

11

12 **Methods**

13 *Collection and preparation of biological material*

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

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

5

6 The lipophilic extracts used in this study were the same samples as had been
7 used previously to uncover antimicrobial properties in molluscan egg masses
8 and capsules (Benkendorff et al. 2001a) and in the present study we aimed to
9 investigate whether fatty acids could be responsible for the observed activity.

10 These organic extracts were prepared in chloroform/methanol (1:1, v:v) and
11 concentrated under vacuum pressure, as previously described for recovering
12 marine fatty acids and sterols (e.g. Stoilov et al. 1984; Yamaguchi et al.
13 1992b; Carballeira et al. 1997; Carballeira et al. 2001). Polar components
14 were removed from the extracts via the water/methanol fraction. Solvent
15 controls were prepared by evaporating chloroform/methanol (1:1, v:v, 200ml).

16

17 *Gas Chromatography-Mass Spectrometry*

18 The volatile organic components in the egg masses and capsules were
19 examined using a GC-17A (Shimadzu) gas chromatograph coupled to a QP-
20 5000 (Shimadzu) mass spectrometer, using a DB-5MS column (30m x
21 0.32mm with 0.25 micron film, J&W Scientific). The samples were run in
22 splitless mode and, in general, 50 µl of sample dissolved in dichloromethane
23 (DCM) was injected, at an estimated concentration of 10mg/ml. The injector
24 temperature was set at 260°C. The oven temperature was held at 40°C for 2
25 min then increased to 290°C at a rate of 4°C per min. The final oven

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

9

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

22

23 *Statistical analyses*

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

1 content between leathery egg capsules and gelatinous egg masses were
2 analysed using independent *t*-tests on the SPSS statistical package. Prior to
3 analysis, the data were examined visually for normal distribution and
4 Cochran's C test was used to check for homogeneity of variances (Winer
5 1971).

6
7 Multivariate statistical techniques were used to examine phylogenetic
8 similarities in the free lipid composition of gastropod egg masses and
9 capsules using the PRIMER software package (Plymouth Marine
10 Laboratories, UK). Bray-Curtis measures of similarity were used to examine
11 the data matrices (concentration of each free lipid as a proportion of total
12 extract) which were fourth-root transformed to reduce weighting given to
13 abundant fatty acids (Clarke and Warwick, 1994). Two analyses were
14 undertaken to assess taxonomic patterns in the free lipid composition. In the
15 first analysis samples were grouped according to Superorder
16 (Caenogastropoda & Heterobranchia), whereas in the second analysis
17 samples were grouped according to Order or Infraorder (Anaspidea,
18 Basommatophora, Littorinimorpha and Neogastropoda). Non-metric multi-
19 dimensional scaling (nMDS) ordinations were constructed to graphically
20 illustrate relationships between groups. The significance of any apparent
21 differences among taxa was determined using one-way analysis of similarity
22 (ANOSIM) tests (Clarke and Warwick 1994).

23

24 *Model lipid mixtures*

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

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

23

24 *Antimicrobial assays*

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

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

14

15 **Results**

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

25

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

8
9 Extracts from the spawn of all the molluscs examined in this study contained
10 a substantial amount of sterol lipid (Table 3c). Cholesterol (M^+ 386) was the
11 only sterol recorded in the egg mass/capsules of every species and was the
12 dominant sterol in all species (Table 3c). Some of the other sterols tentatively
13 identified include; cholestanol (M^+ 388), cholesta-3,5-diene (M^+ 368),
14 cholesta-4,6-dien-3-ol (M^+ 384), methylcholestenol (M^+ 400),
15 methylcholestadienol (M^+ 398) and stigmast-4-en-3-one (M^+ 412). However,
16 most sterols remained inconclusively identified due to the very small amounts
17 that were present.

18

19 *Leathery capsules vs. Gelatinous egg masses*

20 The lipid composition of extracts from molluscan spawn differed substantially
21 according to the structural type (Figure 1). Fatty acids were minor
22 components in the encapsulated leathery egg capsules of gastropods,
23 whereas a high concentration of fatty acids occurred in encapsulated
24 gelatinous egg masses (Figure 1a): *t*-tests revealed a significant difference in
25 both the number of fatty acids (equal variances not assumed; $t = 6.48$, $DF =$
26 16.63 , $p < 0.001$) and the relative amount of fatty acids, as a proportion of all

1 volatile compounds found in the lipid extract ($t = 5.63$, $DF = 21$, $p < 0.001$)
2 from these different types of egg mass. The most notable difference between
3 leathery capsules and gelatinous masses was the complete absence of
4 unsaturated fatty acids in the leathery capsules (Figure 1a). The proportion of
5 saturated fatty acids is also significantly higher in extracts from gelatinous egg
6 masses compared to leathery egg capsules (Figure 1a; $t = 4.09$, $DF = 21$, p
7 $= 0.001$).

8
9 On average the proportion of sterols was higher in encapsulated leathery egg
10 capsules than gelatinous egg masses, although there was substantial
11 variation within the extracts from leathery egg capsules (Figure 1b). A
12 nonparametric t -test revealed no significance difference in the amount of
13 sterols in capsules or egg masses ($t = 1.78$, $DF = 12.36$, $p = 0.10$).
14 Cholesterol, the primary sterol present in both types of egg mass, appears to
15 be the major contributing factor to the differences in sterol concentration
16 (Figure 1b).

17

18 *Major taxonomic differences in lipid composition*

19 The fatty acid composition in extracts from molluscan spawn varied between
20 broad taxonomic groupings (Figure 2). MDS ordination analysis revealed
21 significantly different groupings for samples taken from the two gastropod
22 superorders, Heterobranchia and Caenogastropoda (Global $R = 0.168$; $p =$
23 0.046). In general, the encapsulated heterobranch egg masses contain a
24 wider range of both saturated and unsaturated fatty acids and the lowest
25 proportion of sterols in their extracts (Table 3). Nevertheless, there was
26 overlap between these two groupings, with greater variation occurring in the

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

3

4 At a finer taxonomic level, significant differences were observed between the
5 samples taken from different orders/infraorders (Figure 2b; Global $R = 0.0482$;
6 $p = 0.001$). In particular, the neogastropods were found to cluster separately
7 from all other taxa (from Anaspidea $R = 0.918$, $p = 0.022$; Basommatophora R
8 $= 0.575$, $p = 0.003$; Littorinimorpha $R = 0.575$, $p = 0.003$). The neogastropods
9 contain relatively small amounts of exclusively even chain length, saturated
10 fatty acids, and high concentrations of sterol (Table 2, 3). All other taxonomic
11 groups contain at least some species with unsaturated fatty acids (Table 3).
12 No significant differences were observed between pairs for the other
13 groupings at this taxonomic level (Anaspidea - Basommatophora $R = 0.167$, p
14 $= 0.25$; Anaspidea - Littorinimorpha, $R = 0.509$, $p = 0.095$; Basommatophora
15 - Littorinimorpha, $R = 0.048$, $p = 0.284$).

16

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

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

3

4 The lipid composition in the encapsulated egg masses from pulmonates
5 revealed striking differences between three families that occur in different
6 aquatic habitats (Table 1, 3). The freshwater species *Isidorella hainesi* was
7 found to contain much lower concentrations of fatty acids, and much higher
8 concentrations of sterol compared to the marine and estuarine species (Table
9 3). The two estuarine (*Salinator*) species appear to have a greater content of
10 unsaturated fatty acids than the two marine (Siphonariidae) species (Table
11 3c). Most of the variation in lipid composition observed within the marine and
12 estuarine families was due to intraspecific variation rather than any apparent
13 differences between the species. One pulmonate sample (Basommatophora,
14 Heterobranchia) appears to be an outlier in the ordination analysis (Figure 2).
15 This was a sample taken from *Salinator fragilis* egg masses and was found to
16 contain high concentrations of molecular sulfur (S_6 and S_8) (Benkendorff
17 1999). This affected the total concentration of fatty acids recorded in the egg
18 masses, which varied from 28% in the first sample to 51% in the second
19 sample (Table 3).

20

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

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

3

4 *Antimicrobial activity of fatty acids and lipid mixtures*

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

13

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

24

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

1 Aplysiidae-equivalent mixture caused high levels of cell death against all three
2 marine bacteria (Table 5). This mixture showed greater activity against *L.*
3 *garvieae* than any of the individual fatty acids (Table 4), with less than 1%
4 viability remaining after incubation with 0.0001mg/ml of the mixture (Table 5).
5 The remaining lipid mixtures showed relatively low levels of activity, with
6 significant cell death only occurring at the maximum test concentration
7 (0.1mg/ml) against one or two species of bacteria (Table 5). The
8 neogastropod-equivalent mixture, containing only saturated acids and sterols,
9 showed the least activity with the majority of the cell cultures remaining viable
10 after incubation with the lipid mixture at 0.1mg/ml.

11

12 **Discussion**

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

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

8

9 The composition of free fatty acids and sterols found in molluscan spawn
10 varied according to both structural type (leathery capsules vs. gelatinous
11 masses, Figure 1) and taxonomic origin of the species (Figure 2). Palmitic
12 (16:0) and stearic (18:0) acids were the dominant fatty acids found in all the
13 spawn extracts, which is consistent with previous studies on the lipids of adult
14 molluscs (Tibaldi 1966; Beninger and Stephan 1985; Yamaguchi et al. 1992a
15 b; Rakshit et al. 1997; Carballeira et al. 2001). *De novo* synthesis of these two
16 saturated fatty acids has been reported to precede spawning in a bivalve
17 mollusc (Kluytmans et al. 1985) and this may also be the case in other
18 molluscan taxa. Saturated fatty acids with an uneven chain length
19 (heptadecanoic and pentadecanoic acids) were detected in the extracts of
20 several molluscan taxa (Table 3a) and likewise have been previously reported
21 from molluscan tissue (Beninger and Stephan 1985; Rakshit et al. 1997).
22 Notably, these uneven length acids were entirely absent from the
23 Neogastropoda spawn, although they were detected in the encapsulated
24 leathery egg masses of a Littorinimorpha (Table 3). This suggests that the
25 biosynthetic pathways for producing these fatty acids and/or allocating them
26 to neogastropod spawn may have been lost in this relatively recent Infraorder.

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

3

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

19

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

1 intracapsular fluid for lipid extraction in this study. Future studies should
2 endeavor to address whether or not unsaturated fatty acids are bioavailable in
3 the extra-embryonic material, thus providing a clear mechanism for defending
4 the encapsulated embryos against aquatic pathogens. Nevertheless, it is
5 apparent from our study that the whole composition of a lipid mixture will
6 influence the level of bacteriolytic activity against a suite of aquatic bacteria
7 (Table 4, 5). Bioactive unsaturated fatty acids were not present in sufficiently
8 high concentrations in the whole spawn extracts of most molluscan taxa to
9 provide any potential sterilization. Indeed of the five lipid mixtures modeled on
10 extracts from different molluscan taxa (see Table 2) only one, the Aplysiidae-
11 equivalent mixture, was active at sufficiently low concentrations to account for
12 all the previously recorded antimicrobial activity (Table 5; Benkendorff et al.
13 2001a). This suggests that activity in the extracts from the other taxa must be
14 mediated by alternative or additional components. Notably, a number of
15 bioactive brominated alkaloids have been identified in the spawn of Muricidae
16 (Benkendorff et al. 2000b; 2001b; 2004). The capsule wall could also provide
17 a barrier against microbial invasion in leathery egg capsules of
18 caenogastropods, as suggested by Lord (1986). Preliminary analysis of all the
19 gelatinous spawn extracts studied here have revealed mixtures of secondary
20 metabolites (Benkendorff 1999), which may act synergistically with fatty acids
21 and/or complement their activity.

22

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

1 Cholesterol was the most abundant sterol and the only sterol found in the
2 spawn of every species examined (see Table 3c), which is consistent with
3 previous studies on sterols in adult gastropods and cephalopods (Bergmann
4 1949; Idler and Wiseman 1972). With the exception of *Sepioteuthis australis*,
5 all species contained a mixture of sterols in their encapsulated spawn (see
6 Table 3c). Previous studies indicate that cholesterol is the only sterol
7 occurring in cephalopods (Idler and Wiseman 1972). Numerous other sterols
8 were detected in the gastropod spawn (see Table 3c), but in such small
9 quantities that most remain unidentified.

10

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

1 However, all species were collected from the same temperate location in this
2 study so climatic differences cannot directly explain the fact that these
3 metabolites are present in encapsulated gelatinous egg masses but not
4 leathery capsules. It remains possible that the caenogastropods first evolved
5 leathery egg capsules in warmer climates with fewer requirements for
6 unsaturated fatty acids. Alternatively, the wall of fibrous proteins found in
7 leathery egg capsules (Flower et al., 1969; Hunt, 1971) may effectively buffer
8 the encapsulated embryos from environmental fluctuations (e.g. Przeslawski,
9 2004). Thus, unsaturated fatty acids could be of greater functional importance
10 for embryos contained in gelatinous egg masses and this warrants further
11 investigation.

12

13 It is also potentially significant that all of the species that deposit leathery egg
14 capsules are predatory molluscs, whereas most of the gelatinous egg masses
15 are deposited by herbivores. Thus, the observed differences in the saturation
16 of fatty acids in gelatinous egg masses and leathery egg capsules may be
17 related to differences in the diets of the adult molluscs, rather than differences
18 in the biochemical pathways. In filter-feeding bivalves most of the stored fatty
19 acids appear to be diet-derived (Beninger and Stephan 1985; Kluytmans et al.
20 1985). Rakshit et al. (1997) also suggested that differences in the fatty acid
21 content of the gastropod *Telescopium telescopium* from two different
22 communities were the result of different dietary sources. However, the
23 encapsulated gelatinous egg masses of one predatory gastropod (*Conuber*
24 *sordidus*), as well as the spawn of a cephalopod (also a carnivore), were
25 found to contain some unsaturated fatty acids (see Table 3b). Consequently,
26 at the very least, there does appear to be a difference in types of fatty acids

1 that predatory molluscs allocate towards leathery versus gelatinous egg
2 masses.

3

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

26

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

10

11 **Acknowledgements**

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

23

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- 9

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

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

1 *Table 2: Average lipid composition in egg masses for five major groups of molluscs and corresponding artificial mixtures modeled on*
 2 *these (=equiv.). The lipid mixtures are made up entirely from 5 saturated fatty acids, 5 unsaturated acids and cholesterol, although a*
 3 *greater range of lipids is found in the egg mass extracts. Consequently, all sterols have been pooled together and some fatty acids*
 4 *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 =*
 5 *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;*
 6 *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*
 7 *groups, as well as the total percentage of saturated fatty acids (sat.), unsaturated fatty acids (unsat.) and sterols from both the*
 8 *freshly extracted egg masses and capsules (Fresh) used in this study (see Table 1), as well as those taken from a greater pool of*
 9 *samples (All) including extracts from freeze-dried egg masses and derivatised extracts that have previously been tested and found to*
 10 *have antimicrobial activity.*

11

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

12 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
 13 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*
 14 *australis*; ¹⁰ n = 2 extracts from *Sepioteuthis australis*.

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

6
 7 a)

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

1 b)

2

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
<i>A. juliana</i>	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
<i>S. longicauda</i>	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
<i>P. angasi</i>	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
<i>S. denticulata</i>	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
<i>S. zelandica</i>	0	0	0	6.7	0	0	0	1.47	0	0.6	0	0	0	0	0	0	0	0	0	8.7
<i>S. fragilils</i>	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
<i>S. solida</i>	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>I. hanesi</i>	0	0	0	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6
<i>B. nanum</i>	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
<i>Conuber</i> 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
<i>C. spengleri</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. papilliferus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. tritoniformis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>D. orbita</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>L. reticularis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>M. marginalba</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. australis</i>	0	2.6	0	5.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.9

3

1 c)

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

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

5

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	++	-	+++	-

6

7

8

9

10

11

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

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

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	-	-	-

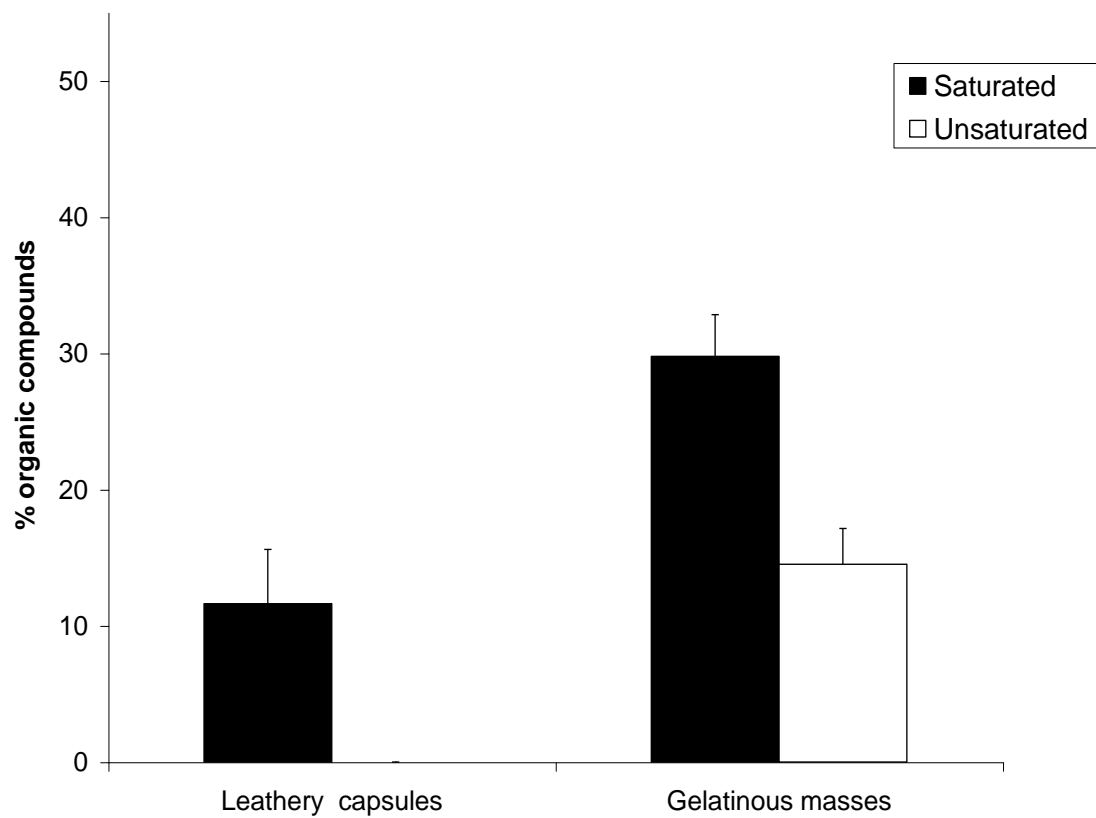
6 - 100% viability
 7 + > 50% viability
 8 ++ 10 – 50% viability
 9 +++ 1 – 10% viability
 10 ++++ <1% viability
 11

1 **Figure legends**

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

11

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



b)

