Coral Reefs (1993) 12:71–75

Coral Reefs
© Springer-Verlag 1993

Lipid composition of positively buoyant eggs of reef building corals

Takayuki Arai [1], Misako Kato [1], Andrew Heyward [2], Yutaka Ikeda [1], Tokio Iizuka [1] and Tadashi Maruyama [1]

[1] Marine Biotechnology Institute, Sodeshi 1900, Shimizu-shi, Shizuoka 424, Japan
[2] Department of Obstetrics and Gynecology, The University of Adelaide, G.P.O. Box 498, Adelaide SA, Australia

Accepted 16 September 1992

Abstract. Lipid composition of the eggs of three reef building corals, *Acropora millepora, A. tenuis* and *Montipora digitata,* were determined. Sixty to 70% of the egg dry weight was lipid, which consisted of wax esters (69.5–81.8%), triacylglycerols (1.1–8.4%) and polar lipids c/ mainly phospholipids) (11.9–13.2%). *Montipora digitata* also contained some polar lipids typical of the thylakoid membrane in chloroplasts, probably due to the presence of symbiotic zooxanthellae in the eggs. The wax esters appeared to be the major contributor to positive buoyancy of the eggs, and specific gravity of wax esters in *A. millepora* was estimated to be 0.92. Among the fatty acids of the wax esters, 34.9–51.3% was hexadecanoic acid (16:0) while the major fatty acids in polar lipids were octadecenoic acid (18:1), hexadecanoic acid (16:0), eicosapentaenoic acid (20:5) and eicosatetraenoic acid (20:4). The wax ester appears to be the main component of the 4.5–6.0 μm diameter lipid droplets which fill most of the central mass of the coral eggs.

Introduction

The majority of reef building coral species studied from the Great Barrier Reef region has been observed to release their eggs synchronously during brief annual spawning events associated with full or new moon periods during late spring or summer (Harrison et al. 1984; Babcock et al. 1986). In hermaphroditic species, the eggs are commonly spawned as buoyant bundles of eggs and sperm packed together. The bundle breaks apart at the sea surface, releasing individual eggs and sperm. Following fertilization, embryogenesis proceeds rapidly to an early spherical planula larval form in the first 24 h, and these larvae first settle on substrata 3–5 days after the spawning event (Babccock and Heyward 1986). Duration of the floating stage of planula is variable and is an important aspect of coral dispersal and recruitment (Harrison and Wallace 1990).

The buoyancy of coral larvae during early development is important while they remain planktonic. Reduced buoyancy could be a significant factor influencing searching behavior and settlement as the larvae age. Light microscopic observation reveals that coral eggs have numerous oil droplets occupying most of the egg volume (Babcock and Heyward 1986). While the contribution of these lipids to larval buoyancy and energy reserves is likely to be important, the lipids of coral eggs have not been analyzed in any detail. This paper describes the lipid composition of eggs from the hermatypic corals, *Acropora millepora, A. tenuis* and *Montipora digitata* in relation to the buoyancy of the eggs. The lipid composition and the role of lipid in coral larval dispersal is discussed.

Correspondence to: T. Maruyama

Materials and methods

Collection of the eggs and egg bundles

A few days prior to anticipated spawning in November 1989, colonies of *A. millepora, A. tenuis* and *M. digitata* were collected from Pioneer Bay, Orpheus Island, 70 km northeast of Townsville, Australia. The corals were transported to outdoor aquaria at Orpheus Island Research Station and mono-specific groups of corals were isolated and maintained in a flow-through seaweater system until spawning. Following the natural spawning, released egg-sperm bundles of three species were skimmed from the water surface of the holding tanks with a 500 mL beaker and poured into a 1L volumetric cylinder. The cylinder was allowed to stand for a few minutes until the eggs all accumulated in a layer at the surface, then the lower layer of seawater and sperm was removed. This process allowed substantial volumes, up to 500 mL, of packed eggs to be collected while at the same time removing most of the sperm. These samples were used for the further lipids analysis.

Size of eggs

Size of *A. millepora* egg was measured under a microscope using objective and ocular micrometer or calculated from photographs.

Density of spawned eggs and larvae

Collected eggs of *A. millepora* and *M. digitata* were centrifuged at 450 × g for 5 min and the lower aqueous layer removed using a Pasteur pipette. Packed volumes of the eggs remaining in the centrifuge tubes were measured at the meniscus using the volumetric scale of the tubes. Then the correct volumes were later calibrated by weighing the tubes filled with distilled water at the same meniscus. The packed wet weight of the eggs in the pre-weighed centrifuge tubes were determined with a precision balance by difference. The density of eggs was then calculated by dividing the packed wet weight by the packed volume.

Densities of intact eggs of *A. millepora* and *M. digitata* and of 4 day larvae of *A. millepora* were estimated in both seawater and 0.5M LiCl by observing their floating or sinking behavior in the solutions after centrifugation at 450 × g for 5 min at room temperature (28–32° C). Specific gravity of each solution was measured with a hydrometer at the same temperature.

Electron microscopy

Eggs of *A. millepora* were fixed with about 4% glutaraldehyde in natural unbuffered sea water and kept at room temperature. For scanning electron microscopy (SEM), the eggs were washed serially with aqueous 3% NaCl, 1% NaCl, distilled water, and dehydrated through ethanol series, acetone and isoamyl acetate and then dried with a Hitachi HCP-2 critical point dryer. Dried specimens were coated with Pt–Pd by a Hitachi E102 ioncoater and observed in a Hitachi S 2500 scanning electron microscope.

In preparation for the transmission electron microscopy, the eggs were post-fixed in 0.1M phosphate (pH 7.2) buffered aqueous 1% OsO_4 and dehydrated through an ethanol series, QY-2 and embedded in Quetol 812. Thin sections were cut with a Reichert ultramicrotome using glass knives. Silver sections were stained with lead citrate (Reynolds 1963) and then observed with a Hitachi H7000 electron microscope operated at 75 kV.

Larval culture

Fertilized eggs of *A. millepora* and *A. tenuis* were incubated in natural sea water in 1L plastic bottles, fitted with gauze mesh for water exchange, agitated randomly in ambient sea water during embryogenesis (after Babcock and Heyward 1986). Duplicate samples of about 100 fertilized eggs and larvae at the stage of morula and gastrula were sampled from both cultures for lipid analysis.

Lipid analysis

Lipids were extracted from 0.5 g of lyophilized eggs of three species by the method of Bligh and Dyer (Bligh and Dyer 1959). The resulting chloroform-methanol fraction was concentrated *in vacuo*, then weighed by precision balance and dissolved in 1 mL of chloroform-methanol (2:1). This fraction was used for further analysis as the crude lipid fraction. The crude lipid fraction was methylated at 90° C for 2 h with 2 mL of 5% (w/v) HCl in methanol in order to obtain fatty acid methyl esters. The resulting fatty acid methyl esters were extracted twice with 2 mL of n-hexane, concentrated in vacuo and dissolved in a minimal volume of n-hexane. The methyl esters were then analyzed with a gas chromatograph (Shimadzu GC-14A equipped with chromatopack C-R4A) using a silica capillary column, Chrompack Cp-Sil 88 (50 m × 0.25 mm). Lipid classes were separated by silica gel thin layer chromatography. Neutral lipids and polar lipids were developed with petroleum ether/ethyl ether/acetic acid (85:15:1, v/v/v). Lipid spots were located under UV light after spraying the plate with 0.01% (w/v) primulin in 80% (v/v) acetone. Wax esters and triacylglycerol were identified by comparing the *Rf* values of authentic samples, bees wax and triparmitin, respectively. For the analysis of the fatty acid moieties of each lipid, the separated lipid spots were scraped off and the methyl esters were obtained and analyzed as described above.

Each lipid was quantified by TLC/FID analyzer (Iatroscan, Iatron Laboratories Inc., Japan). Crude lipids were separated by chromarod-SIII using the following solvent system; n-hexane/diethyl ether/formic acid (50:20:0.3, v/v/v) in the first development and with n-hexane/benzene (1:1, v/v) in the second. The separated lipid bands were quantified with an Iatroscan TH-10 equipped with an Iatrocorder. Relative ratio of wax esters to polar lipids was determined by this system. To identify unknown fatty acids in wax esters, analysis was performed by gas chromatography-mass spectrometry. Mass spectra were recorded with a JEOL JMS-SX102 mass spectrometer-Hewlett Packard 5890 series II gas chromatograph equipped with a silica capillary column (Chrompack Cp-Sil 88, 50 m × 0.25 mm) at 70 eV ionization potential. Frozen rather than lyophilized samples were used for the lipid classes analysis of cultured larvae. Extraction and quantitative analysis were carried out as described above.

Density of the lipid droplets

One gram of frozen *A. millepora* eggs were homogenized by an ultrasonic-disintegrater with 5 mL of water at 4° C for 5 min. After purging with nitrogen, the homogenized sample was heated in a water bath at 95° C in order to melt the lipid droplets. The melted lipid droplets were isolated by centrifugation at 22,000 × g for 15 min at 45° C. The top lipid layer was then transferred to a 5 mL picnometer with a pipette, dissolved in chloroform, and purged with nitrogen. This procedure was repeated until most of the lipid was transferred. The chloroform was removed by evaporation, and the picnometer was filled with distilled water and weighed with a precision balance at 20° C. The specific gravity of the droplets was then calculated.

Results

Morphology of the eggs and egg bundles

Eggs were not spherical immediately after bundle breakdown, but generally became even and spherical during the first hour of incubation in seawater. The mean diameter of *A. millepora* egg was 522 ± 24 μm (mean ± SD, n = 24). In SEM micrographs of razor blade sectioned *A. millepora* eggs (Figs. 1 a, b), most of the sectioned surface was occupied by 4.5–6.0 μm diameter empty spherical spaces except for the peripheral layer of about 5 μm. Dehydration and acetone treatment of eggs prepared for SEM resulted in the spherical spaces being empty, but TEM micrographs of the egg section, where lipid was fixed during treatment with OsO_4, indicate that they were filled with lipid (Fig. 1 c). The peripheral area (cortex) of the eggs was occupied by yolk-like granules.

Density of the spawned eggs

Density of spawned eggs was 0.98 in *A. millepora* and 0.99 in *M. digitata*. All of the spawned eggs of *A. millepora* floated in both seawater (d = 1.024 at 32° C) and 0.5M LiCl solution (d = 1.010 at 28° C). However, 4-days-old larvae of *A. millepora* behaved differently. Five of the 12 larvae in sea water sank to the bottom, 5 floated and 2

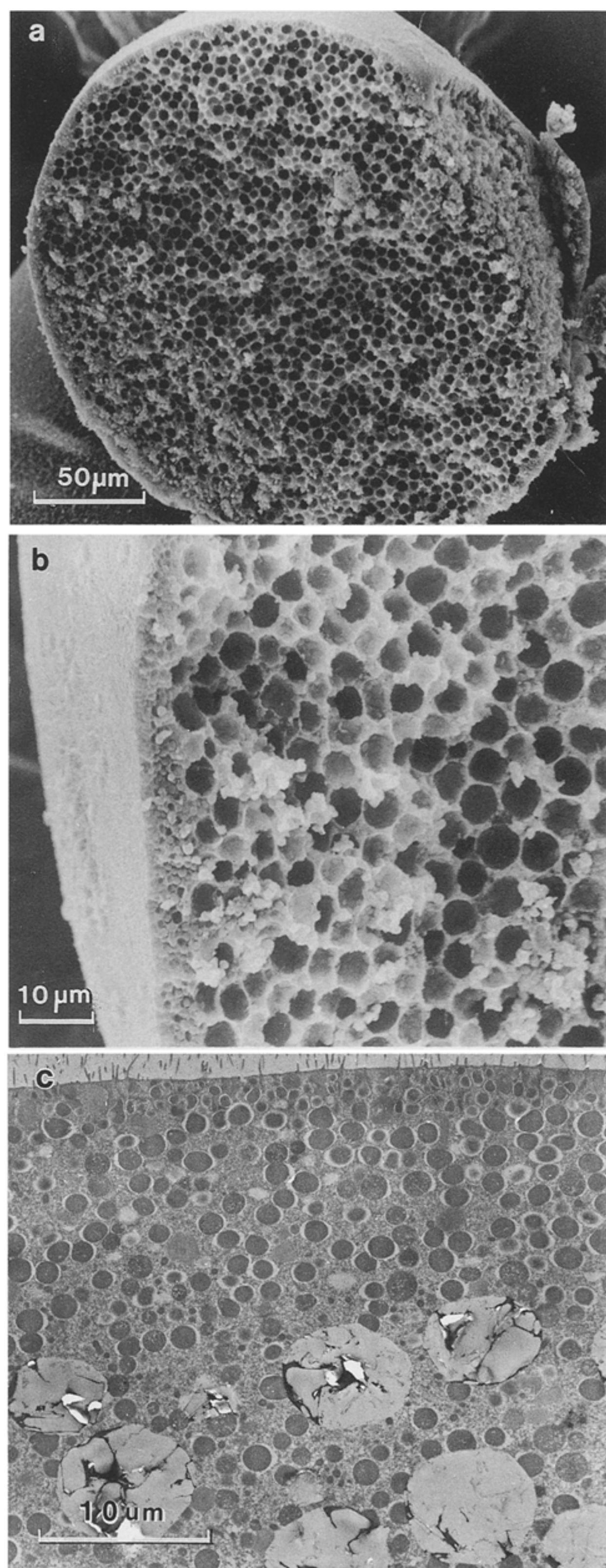

Fig. 1 a–c. Electron micrographs of the egg of *Acropora millepora*. **a, b** Scanning electron micrographs of a razor blade sectioned egg which was fixed with glutaraldehyde alone. **a** Whole section; **b** cortical portion of the egg. **c** Transmission electron micrograph of a thin sectioned egg of *A. millepora* which was postfixed with OsO_4. Note that the empty spherical spaces (possible artifacts of fixation) were observed in **a** and **b**, but not in **c**. Many lipid droplets were present in **c**

stayed in the middle of the centrifuge tube. In the 0.5M LiCl solution, 2 of 14 larvae sank to the bottom, 10 floated and 2 seemed to be neutral. These results suggest that after 4 days, larvae of *A. millepora* were becoming less buoyant.

Lipid composition

Lipid contributed 69.1%, 62.5% and 67.4% of egg dry weight in *A. millepora, A. tenuis* and *M. digitata* respectively. Neutral lipids dominated and polar lipids c/mainly phospholipids) were minor components in all three coral species. The major neutral lipids in the eggs of all species were wax esters, which represented 69.5–81.8% of total lipids (Table 1). Triacylglycerols were 1.1 and 1.9% of total lipids in *A. millepora* and *A. tenuis* but 8.4% in *M. digitata.* Triacylglycerols/wax esters ratios were 0.013 and 0.028 for *A. millepora* and *A. tenuis* but 0.121 for *M. digitata.* Polar lipids constituted 11.9–13.2% of the total lipids.

Interspecific differences were detected in the polar lipid components. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were detected in all species but monogalactocyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) were detected only in *M. digitata* eggs. No hydrocarbon was detected in any of the eggs.

In lipid droplets isolated by hot water extraction from an egg homogenate of *A. millepora,* wax ester constituted 92.4% of total lipid in the droplet fraction and polar lipid only 1.6%. The specific gravity of the droplets was 0.92.

Lipid profile during early development

The fertilized egg/morula/gastrula percentages for wax ester in the total lipids were 79.3/80.5/79.3 in *A. millepora* and 79.7/73.0/76.9 in *A. tenuis*. The equivalent polar lipid ratios were 12.7/13.5/12.3 in *A. millepora* and 13.1/17.4/15.0 in *A. tenuis.* A preliminary lipid analysis of early embryos revealed that the relative wax ester component changed very little within 10.5 (*A. millepora*) or 11.5 (*A. tenuis*) h after fertilization, by which time embryos had reached the stage of gastrulation.

Fatty acids

The saturated fatty acid fractions of total lipids were 49.4% (*A. millepora*), 61.3% (*A. tenuis*) and 43.5% (*M. digitata* Table 2 lists the fatty acids detected and their distribution in wax esters and polar lipids. The saturated fatty acid component in wax esters ranged between 46.6% in *M. digitata* and 64.0% in *A. tenuis* but was less in the polar lipids, and 32.8% in *M. digitata* being 24.0% in *A. tenuis.* The major fatty acids in wax esters, dominated by hexadecanoic acid (16:0) (34.9–51.3%), included tetradecanoic acid (14:0), hexadecenoic acid (16:1) and octadecenoic acid (18:1) for all species. Major fatty acids in polar lipids were 16:0, 18:1, eicosate-

Table 1. Lipid class composition of eggs of three hermatypic corals. *TG/WX,* triacylglycerols/wax esters ratio

Coral species	Percentage of lipid classes in total lipids				TG/WX
	Wax esters	Triacylglycerols	Unidentified lipids	Polar lipids	
A. millepora	81.8	1.1	4.9	11.9	0.013
A. tenuis	79.8	1.9	5.2	13.2	0.028
M. digitata	69.5	8.4	9.9	12.2	0.121

Table 2. Fatty acid composition of wax esters and polar lipids in eggs of three hermatypic corals. wax, wax esters; *PL,* polar lipids; *Tr,* trace amount

Fatty acid	Percentage of fatty acid in each lipid class					
	A. millepora		*A. tenuis*		*M. digitata*	
	wax	PL	wax	PL	wax	PL
14:0	11.3	2.0	10.8	1.0	10.5	1.9
16:0	40.8	19.7	51.3	15.5	34.9	27.2
16:1[a]	11.7	4.3	6.8	3.4	22.9	2.6
16:2	0.0	0.0	0.0	2.5	0.0	0.7
16:3	0.5	0.0	0.3	0.0	0.8	0.0
18:0	Tr	5.9	1.9	7.5	1.2	3.7
18:1[a]	11.1	29.8	8.0	25.1	14.6	11.6
18:2	2.7	0.8	3.3	4.0	3.3	4.7
18:3[a]	11.5	1.9	7.8	5.9	3.4	4.5
18:4	1.6	0.6	0.8	0.6	1.4	2.1
20:1	1.3	0.0	2.1	1.8	0.0	0.0
20:2	0.0	0.0	0.3	0.5	0.0	1.8
20:3	0.0	0.0	1.2	1.1	1.1	1.1
20:4	0.0	10.3	0.7	12.2	1.6	9.2
20:5	2.5	18.4	1.1	13.8	0.6	2.2
22:3	0.0	0.0	0.2	0.0	0.0	0.0
22:4	Tr	3.0	0.3	3.4	0.7	11.7
22:5	4.2	2.1	2.9	1.7	3.1	8.2
22:6	0.5	1.3	0.3	0.9	0.0	0.7
Unidentified	0.0	0.0	0.0	0.0	0.0	6.3
Saturated[b]	52.1	27.6	64.0	24.0	46.6	32.8
Unsaturated[c]	47.6	72.5	36.1	76.9	53.5	61.1
Total	99.7	100.1	100.1	100.9	100.1	100.2

[a] Isomers were not distinguished
[b] Percentage of saturated fatty acids
[c] Percentage of unsaturated fatty acids

traenoic acid (20:4), and eicosapentaenoic acid (20:5) in *A. millepora* and *A. tenuis,* but 16:0, 18:1, 20:4, docosatetraenoic acid (22:4), and docosapentaenoic acid (22:5) in *M. digitata.* In the polar lipid fraction, fatty acid 18:1 was dominant for *A. millepora* (29.8%) and *A. tenuis* (25.1%), while 16:0 was dominant in *M. digitata* (27.2%). In addition, *M. digitata* had significant amounts of 22:4 and 22:5 while the two *Acropora* species did not.

Discussion

Electron microscopic observation shows that the ultrastructure of *A. millepora* is similar to the red eggs of *Galaxea fascicularis,* which possess large numbers of lipid droplets in the egg interior (Harrison 1988). The present study indicates that wax esters of *A. millepora,* with a specific gravity of 0.92 present as lipid droplets throughout the cytoplasm, confer buoyancy to the egg. The major lipid class found in adult corals is wax esters (Benson and Muscatine 1974), so it is not surprising that wax esters are the dominant lipid found in the eggs. In adult colonies of *Pocillopora capitata,* lipid values of 30–40% tissue dry weight have been documented (Patton et al. 1977). In this study, eggs of *Acropora* and *Montipora* contained 62–70% egg dry weight of lipid, almost twice the value reported for polyp tissue samples from adult colonies.

Wax esters are known to work as buoyant substances and energy reserves in crustacean zooplankton and other marine organisms (Lewis 1970; Nevenzel 1970; Takama 1982). The present study indicates they may have a similar function in corals. Our experiment did not reveal any qualitative change in lipid composition during the first 10–11 h of embryogenesis of *Acropora.* It was noted that during the first 10–12 h of *Acropora* development, most changes related to increasing cell numbers, and little cellular differentiation was detected using standard histology and light microscopy. Histological observation of developing larvae in a previous study with *M. digitata* and *Acropora* species (Heyward 1987) suggests rapid utilization of maternal lipid during later development as planulae differentiate and become more mobile. It is noteworthy that positively buoyant, newly released planulae from the brooder *Pocillopora damicornis* contain 70% lipid, 17% more lipid than larvae searching the substratum prior to settlement (Richmond 1987).

The major fatty acid in wax esters of the eggs was 16:0 as reported in adult colonies in many coral species (Latyshev et al. 1991; Patton et al. 1977, 1983; Meyer et

al. 1974, 1978). In addition to 16:0, major fatty acids in total lipid of adult colonies of *Acropora* were reported to be 18:0, 18:3, 20:4, 20:5 and 22:6 (Latyshev et al. 1991). In the wax esters of eggs, 18:0, 20:4, 20:5, and 22:6 were minor, while 16:1 and 18:1 prevailed. Fatty acids 20:4 and 20:5 were predominant in polar lipids. It remains to be established whether fatty acid composition of eggs is different from these of adult tissues. While the eggs of all species were similar in most respects, reasonably large interspecific differences were detected in levels of saturated fatty acid moieties and the triacylglycerol component of *M. digitata* versus the *Acropora* species. There is a strong possibility that the observed lipid profiles were influenced by the presence or absence of algal symbionts in the eggs, particularly in the fatty acid composition of the polar lipid fraction. *M. digitata* inherits zooxanthellae maternally in the oocytes (Heyward and Collins 1985) and, at the time of spawning, each egg of various *Montipora* species may contain in the order of 10^2–10^3 zooxanthellae (Maruyama and Heyward, unpublished observations). These zooxanthellae undergo division during embryogenesis of the host and are known to be capable of translocating carbon compounds to the host during early development (McNeil, personal communication to Heyward). In contrast, the *Acropora* species release eggs which carry no symbiotic micro-algae, and development proceeds through to settlement without zooxanthellae. This difference between the genera is likely to account for the differences in triacylglycerol composition, although no attempt was made in the present study to separately analyze the zooxanthellae from *M. digitata* eggs. Recent comparison of lipids of zooxanthellae and its host the temperate anemone, *Anemonia viridis* (Hartland et al. 1991), suggests synthesis of triacylglycerol by zooxanthellae and translocation to the host. Similarly, that study indicated the presence of fatty acid 22:5 in the zooxanthellae, which is consistent with our observation that higher contents of 22:4 and 22:5 were detected in *M. digitata* compared with *Acropora* eggs. The utilization of storage lipids by corals during early development and the larval dispersal stages is likely to play a key role in coral distribution. Positive buoyancy conferred by the wax esters during the first few days of larval life would enhance passive dispersal by currents, while maximum competency intervals are dependent on available energy resources in the absence of inputs via symbionts or uptake of exogenous food. Although no qualitative change in lipid composition was observed during early embryogenesis, quantitative evaluation of lipid utilization in later development and the limits on dispersal imposed by lipid reserves remain to be evaluated. The observations of this study suggest that zooxanthellate and non-zooxanthellate embryos of broadcast spawning corals are good models for comparison of the role of micro-algal symbionts in coral energy budgets.

Acknowledgements. We are grateful to Mr. Y. Kikuchi for electron microscopy and to Ms. N. Ohtaki for GC-Mass. Dr. A. A. Benson is acknowledged for his comments on the manuscript. We are also indebted to Drs. S. Miyachi and M. Endo for their interest in our research and for their encouragement.

References

Babcock RC, Bull GD, Harrison PL, Heyward AJ, Oliver JK, Wallace CC, Willis BL (1986) Synchronous spawning of 105 scleractinian coral species on the Great Barrier Reef. Mar Biol 90:379–394

Babcock RC, Heyward AJ (1986) Larval development of certain gamete-spawning scleractinian corals. Coral Reefs 5:111–116

Benson AA, Muscatine C (1974) Wax in coral mucus: energy transfer from corals to reef fishes. Limnol Oceanogr 19:810–814

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Harrison PL, Wallace CC (1990) Reproduction, dispersal and recruitment of scleractinian corals. In: Dubinsky Z (ed) Coral reefs. Elsevier, Amsterdam, pp 133–207

Harrison PL, Babcock RC, Bull GD, Oliver JK, Wallace CC, Willis BL (1984) Mass spawning in tropical reef corals. Science 223:1186–1189

Harrison PL (1988) Pseudo-gynodioecy: An unusual breeding system in the scleractinian coral *Galaxea fascicularis*. Proc 6th Int Coral Reef Symp, 2:699–698

Hartland AD, Fixter LM, Spencer Davies P, Anderson RA (1991) Distribution of lipids between the zooxanthellae and animal compartment in the symbiotic sea anemone *Anemonia viridis:* wax esters, triglycerides and fatty acids. Mar Biol 110:13–19

Heyward AJ (1987) Genetic systems and hereditary structures of reef corals. PhD thesis, James Cook University of North Queensland, p 116

Heyward AJ, Collins JD (1985) Growth and sexual reproduction in the scleractinian coral *Montipora digitata* (Dana). Aust J Mar Freshw Res 36:441–446

Latyshev NA, Naumenko NV, Svetashev VI, Latypow YY (1991) Fatty acids of reef-building corals. Mar Ecol Prog Ser 76:295–301

Lewis RW (1970) Densities of three classes of marine lipids in relation to their possible role as hydrostatic agents. Lipids 5:151–153

Meyer PA, Porter JW, Chad RL (1978) Depth analysis of fatty acids in two Caribbean reef corals. Mar Biol 49:197–202

Meyer PA, Quin JG, Marshal N (1974) A method for analysis of fatty acids in coral. Limnol Oceanogr 19:846–848

Nevenzel JC (1970) Occurrence, function, and biosynthesis of wax esters in marine organisms. Lipids 5:308–319

Patton JS, Abraham S, Benson AA (1977) Lipogenesis in the intact coral *Pocillopora capitata* and its isolated zooxanthellae: evidence for a light-driven carbon cycle between symbiont and host. Mar Biol 44:235–247

Patton JS, Battey JF, Rigler MW, Porter JW, Black CC, Burris JE (1983) A comparison of the metabolism of bicarbonate ^{14}C and acetate 1–^{14}C and the variability of species lipid compositions in reef corals. Mar Biol 75:121–130

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212

Richmond RH (1987) Energetics, competency, and long-distance dispersal of planula larvae of the coral *Pocillopora damicornis*. Mar Biol 93:527–533

Takama K (1982) Distribution of nonglyceride-lipids in zooplankton. In: Kayama M, Kaneda N, Takagi T, Mori M, Ito K (ed) Nonglyceride-lipids of marine animals. Japan Society of Scientific Fisheries. Kouseisha-Kouseikaku (in Japanese)