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Lipid as an Index of Growth and Viability in Three Species of Bivalve Larvae

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ABSTRACT

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The larvae of three species of bivalve molluscs, *Crassostrea virginica* (Gmelin), *Ostrea edulis* (L.) and *Mercenaria mercenaria* (L.) were cultured under a variety of temperature and food species regimes in the laboratory. Data were collected on growth, survival, total lipid content and lipid class composition of both healthy and starved larvae. Subsamples were stained specifically for lipid content with the lipid-specific stain Oil Red O. Newly formed straight hinge larvae of all three species exhibited small lipid droplets dispersed throughout the tissues. These were resorbed within 4 days of the initiation of feeding while the digestive gland filled with exogenously supplied lipid. Embryogenesis took place at the expense of 69 and 71% of parentally derived total lipid, 92.8 and 68.3% of which was composed of triacylglycerols (*M. mercenaria* and *C. virginica*, respectively). After 8 days of feeding on *Isochrysis* aff. *galbana* (clone T-ISO), total lipid had increased 2.7-fold in larvae of *C. virginica* grown at 25°C, 37.6-fold in larvae of *M. mercenaria* grown at 22°C, and 1.7-fold in larvae of *O. edulis* grown at 25°C. Shell growth in larvae of *C. virginica* grown at 30°C was initially higher than that at 25°C, but lipid was accumulated 2.5 times slower and survival of pediveliger larvae through metamorphosis was considerably reduced at this elevated temperature. The lipid class composition of healthy larvae of *C. virginica* and *M. mercenaria* was dominated by triacylglycerols and phospholipids in approximately equal proportions; however, triacylglycerols were preferentially catabolized during starvation and were responsible for greater than 80% of the total lipid loss. Color photographs of larvae stained with Oil Red O are presented that illustrate lipid levels in developing larvae in both healthy and poor condition. From these, a visual index of lipid content was developed to allow comparisons between larval survival and lipid levels. In general, high survival was always accompanied by a high lipid index but a high lipid index did not guarantee good survival. We suggest that the lipid index may be used to document visually the lipid content in individual bivalve larvae as an indicator of physiological condition and potential for successful metamorphosis.

INTRODUCTION

Despite a growing knowledge of the effects of environmental condition, disease and genetic history on the performance of bivalve larvae in artificial cul-

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ture, the success of laboratory and commercial hatchery culture remains highly unpredictable (Lannan et al., 1980). This unpredictability necessitates the use of condition indices such as shell growth, mortality and tissue necrosis throughout larval development. Gallager and Mann (1981) pointed out that these indices are essentially *post facto* in nature, making rectification of diagnosed problems improbable. The latter authors presented a simple and inexpensive technique to assess visually the physiological condition of bivalve larvae in culture by staining stored energy reserves with the lipid-specific stain Sudan Black. Visible lipid reserves have also been used as a sensitive indicator of nutritional status in freshwater cladoceran populations (Goulden and Hornig, 1980; Tessier and Goulden, 1982). Holm and Shapiro (1984) have recently confirmed the usefulness of lipid as an index of potential reproductive capacity in these microcrustacea. Croll (1972a,b) used the lipid-specific stain Oil Red O in conjunction with scanning microdensitometry to quantify fluctuating lipid levels in infectious stages of nematodes.

Holland (1978) reviewed the literature on energy metabolism in many invertebrate larvae and concluded that neutral lipid, especially triacylglycerol, was most important as a storage medium for use during nutritional or environmental stress. The nutritional status of detrital organisms and their grazers was established by analysis of the triacylglycerol/phospholipid ratio (T/P) in healthy, fed specimens (high T/P ratio) and those that were starved (low T/P ratio) (Gehran and White, 1982). The authors concluded that the ratio illustrated a rapid response to imposed stress and environmental perturbation.

This study further examines the role of lipids in marine bivalve larvae and documents the use of Oil Red O to visualize normal/abnormal lipid accumulation/depletion in larvae of three commercially important species. Quantitative analytical techniques were employed to detect fluctuations in both gross total lipid content and lipid class levels of larvae corresponding to those stained with Oil Red O, thereby providing complementary quantitative and visual results. It is suggested that the photographs presented here, depicting lipid distribution under various conditions, may serve as a reference for other users of the staining technique. We further suggest that the lipid staining technique can be used as a sensitive, powerful tool for early detection of metabolic dysfunction when applied routinely to monitor cultures in the laboratory or commercial hatchery.

METHODS AND MATERIALS

Three species of adult bivalves, *Crassostrea virginica* (Gmelin), *Ostrea edulis* (L.) and *Mercenaria mercenaria* (L.) were obtained from Frank M. Flowers, Inc., Bayville, New York; Chester Brown, Walpole, Maine; and Bluepoints Company, Inc., West Sayville, New York, respectively. They were conditioned to spawn at 20°, 10° and 15°C, respectively, in flowing seawater of 30–32‰

salinity with a continuous algal supplement of *Isochrysis* aff. *galbana* (Clone T-ISO) for 6 weeks. Thermal stimulation induced spontaneous spawning and larvae were reared as described in detail in Mann and Gallager (1984). Briefly, replicate 50-l or 200-l Nalgene tubs containing 0.2 μm filtered seawater were held at 30° and 25°C for *C. virginica*, 25° and 15°C for *O. edulis* and 22 ± 1°C for *M. mercenaria* by thermostatically controlled water jackets. Filtered seawater was replaced every second or third day and a culture of *I. galbana* added to a final concentration of 5×10^4 cells·ml^{-1}. Additional replicate larval cultures of *C. virginica* were maintained at 25°C and fed the diatom *Phaeodactylum tricornutum* (Bohlin) (Clone Phaeo).

At 2- to 5-day intervals, subsamples were removed from each culture and placed in separate containers at the corresponding temperature without food addition for a 3- to 4-day starvation period. Subsamples removed from all cultures at 2- to 3-day intervals were split into two fractions and processed as follows: (1) 100–1000 larvae were narcotized, fixed and stained specifically for lipid by a modification of the procedure outlined in Gallager and Mann (1981, described below) and stored in ethylene glycol for subsequent visual examination, photography, measurements of length (anterior-posterior) and height (dorso-ventral) on a minimum of 30 individuals and estimate of mortality indicated by the number of empty shells; (2) >5000 larvae were rinsed with isoosmotic ammonium formate (3% *w/v*), frozen at −20°C, lyophilized and stored in a desiccator to await total lipid and lipid class analysis.

Cultures of larvae that attained metamorphic competence were allowed to set on ground oyster shell cultch (*Crassostrea virginica* and *Ostrea edulis*) or on the sides of the containers (*Mercenaria mercenaria*) while growth and mortality were followed during a week post-set period.

Modifications to the Gallager and Mann (1981) lipid staining technique included substituting the lipid-specific stain Oil Red O (C.I. 26125) for Sudan Black, and dimethyl-sulfoxide (DMSO) for propylene glycol as the carrier solvent. The intense red color of Oil Red O (ORO) proved superior to other lipid stains, while DMSO reduced the staining time requirement from >30 min to 3–5 min depending on larval stage. ORO is an ortho-naphthol diazo stain that is insoluble in oleic and stearic acids and very soluble in acetone, benzene and toluene. Although its chemical structure suggests neutral lipid specificity, this has not been unequivocally documented. Therefore, we shall consider the material stained by ORO to be any lipid in which ORO is soluble; hereafter designated by the term ORO lipid.

Individual larvae visually representing the average ORO lipid content and distribution of a sample were photographed at 250× on a Leitz compound microscope with 200 ASA daylight Ektachrome film. Color correction was provided by a Kodak blue 80b filter. Low magnification photographs (100×) were also taken to illustrate the degree of individual variability in shell size and ORO lipid content within a population.

Larval survival was estimated during two developmental periods: percentage ova surviving to metamorphically competent pediveligers and percentage competent veligers surviving metamorphosis to sedentary spat one week post-set.

A subjective ORO lipid index was developed to grade stained larvae. The qualitative estimates were: 0, no stainable lipid detected; 1 and 2, indicating relatively greater lipid content; and (3), indicative of the greatest lipid level observed in healthy larvae.

Total lipid was extracted and analyzed by the technique of Mann and Gallager (1985). Following sonic homogenization, sequential double extraction with first 1:2 *v/v* and then 2:1 *v/v* chloroform— methanol, and purification by 0.7% NaCl, lipid was quantified gravimetrically on a CAHN Model 29 electrobalance. Individual larval dry weights were calculated from the weight of groups of 10–50 larvae on an electrobalance.

Analysis of the lipid class composition of selected samples was performed as described by Sasaki and Capuzzo (1984) by separation on silica-coated chromarods and quantified on an Iatroscan TH-10 Mark 3. Neutral lipids were separated in a solvent system containing dichloroethane:chloroform:acetic acid:isopropanol in a ratio of 92:8:0.1:0.15. This allowed complete separation of sterylesters (SE), triacylglycerols (TRI), free fatty acids (FFA), sterols (STER), 1-2 diacylglycerol (1-2 Di), while leaving polar phospholipids (PHOSPHO), as an undifferentiated spot. The response of the Iatroscan flame ionization detector as each lipid class was detected was recorded and integrated. Standards allowed peak identification.

RESULTS

Plate IA illustrates the distribution of lipid in developing larvae of *Crassostrea virginica* reared at 25°C and stained with Oil Red O. Twenty-four hours after fertilization and prior to initiation of feeding, initial straight hinge veligers (D-larvae) exhibited many small lipid droplets (e.g., 2–5 μm in diameter) dispersed throughout the tissues. A low magnification photograph of the larval population (Plate IB) reveals a high degree of individual variability in lipid content between veligers. Stain intensity ranged from very pale, ORO lipid index 0–1, to individuals that were heavily stained and appeared very dark, ORO lipid index 3. The parentally derived endogenous lipid content decreased precipitously during embryonic development (i.e., that period between fertilization and prodissoconch I formation) from 23% of the dry weight in the egg to 4% in the first shelled veliger (Table 1), corresponding to a loss of 6.7 ng lipid per embryo (Table 2).

By day 4, all of the stainable material in peripheral tissues had presumably been catabolized while the digestive gland began filling with lipid droplets ranging in diameter from 2 to 10 μm. Plate IC illustrates the high individual variability associated with ORO lipid content in these healthy larvae. Shell

TABLE 1

Lipid and dry weight content of healthy and starved *Crassostrea virginica* larvae cultured at two temperatures

	Days age	Days fed	Days starved	Length (μm)	Height (μm)	Dry weight ($\mu g \cdot larva^{-1}$)	% Lipid of dry weight	Lipid content ($ng \cdot larva^{-1}$)
30°C	0	eggs	0	46	—	0.052	13.5	7.0
	1	1	0	77	61	0.094	4.9	4.6
	4	4	0	84	80	0.201	1.5	3.0
	8	8	0	111	119	0.613	1.3	8.0
	12	12	0	150	159	0.940	2.1	19.7
	16	16	0	168	171	1.660	1.3	21.6
	18	18	0	184	192	1.860	0.9	16.7
	21	21	0	210	204	2.010	1.3	26.1
	23	23	0	269	262	2.460	1.1	27.1
	12	9	3	128	130	0.580	0.7	4.1
	19	16	3	147	153	1.400	0.6	8.4
	21	18	3	180	193	1.540	0.5	7.7
25°C	0	eggs	0	40	—	0.040	23.0	9.2
	1	1	0	75	64	0.062	4.0	2.5
	2	2	0	75	55	0.148	5.7	8.4
	4	4	0	88	81	0.472	2.9	13.7
	8	8	0	99	101	0.847	2.9	24.6
	9	9	0	110	118	0.880	3.3	29.0
	12	12	0	123	125	0.910	3.5	31.9
	16	16	0	155	144	1.008	3.2	32.3
	18	18	0	228	224	1.290	3.7	47.7
	20	20	0	277	269	1.900	3.6	68.4
	44	44	0	280	260	2.600	2.9	75.4
	4	1	3	75	66	0.034	2.3	0.7
	11	8	3	98	102	0.384	2.5	9.6
	12	9	3	109	114	0.301	1.9	5.7
	15	12	3	123	125	0.489	2.7	13.2
	19	16	3	161	148	0.519	2.3	11.9
	23	20	3	202	198	1.400	3.0	42.0
	47	44	3	280	260	2.070	2.3	47.6

size was not necessarily coupled with lipid content, although the ORO lipid index of most large veligers was high, i.e., 2–3. Micro-dissections of larvae showed that ORO lipid droplets appearing in the intestines were not stained luminal contents; rather, they were true droplets of lipid deposited in the intestinal wall.

After 8 days in culture at 25°C, total lipid content had increased 10-fold to 24.6 $ng \cdot larva^{-1}$ (Table 1), and was largely contained in the digestive gland. Prior to development of a foot at day 16, the digestive gland, intestines and

TABLE 2

Lipid and dry weight losses during starvation of three species of bivalve larvae

Initial age	Days starved	Lipid		Dry weight	
		(ng)	% of initial	(ng)	% of initial
Crassostrea virginica					
30°C					
9	3	9.4	69.6	170	22.7
16	3	13.2	61.1	260	15.7
18	3	9.0	53.9	320	17.2
25°C					
0	1	6.7	72.8	+22	—
1	3	1.7	68.8	28	45.2
8	3	15.0	61.0	463	54.7
12	3	18.7	58.6	421	46.3
16	3	20.4	63.2	489	48.5
20	3	26.4	62.9	500	26.3
44	3	27.8	36.9	530	20.4
Ostrea edulis					
25°C					
0	3	4.4	14.4	+480	—
8	3	15.4	29.9	90	5.4
15	3	53.8	50.5	400	15.4
20	3	80.7	47.1	692	20.6
27	3	186.5	64.9	1034	24.1
35	3	149.2	30.9	1570	26.3
15°C					
0	3	4.9	13.3	100	11.1
10	4	13.7	25.9	270	22.5
19	4	16.8	16.0	380	20.2
Mercenaria mercenaria					
22°C					
0	3	3.1	81.6	+22	—
1	3	4.9	86.0	66	29.9
5	3	28.3	78.8	143	15.9
9	3	59.2	54.2	247	14.0
11	3	79.0	55.3	156	8.3

(+) indicates dry weight gain due to shell growth.

velum became darkly stained and contained a mean of 32.3 ng total lipid per larva. Cultures with pediveligers exhibiting ORO lipid indices of 2–3 at day 16 consistently showed 70–80% survival through metamorphosis, although the range extended from 10 to 92% (Table 3).

Plate IIA illustrates the effect of 3-day starvation periods on the ORO lipid content of *Crassostrea virginica* veligers. Straight hinge larvae rapidly catabol-

TABLE 3

Combined survival data from 20 laboratory cultures of *Crassostrea virginica* and *Mercenaria mercenaria* larvae reared at 25°C and 22°C, respectively, and fed *Isochrysis* aff. *galbana* (T-ISO)

Average lipid index of culture	Number of cultures	Percentage survival: egg to pediveliger (range)	Percentage competent larvae completing metamorphosis (range)	Total survival: egg through one week post-set (range)
0 (poor)	5	0–0.07	0–3	0–0.002
1 (mediocre)	3	0–0.2	0–9	0–0.02
2 (good)	8	1–6	10–92	0.1–6
3 (excellent)	4	5–18	8–93	0.4–17

ized more than 73% of the parentally derived lipid (Table 2) and appeared devoid of ORO lipid after 3 days of starvation. Clear shells in Plate IIB indicated moribund or dead larvae.

After starvation, the digestive gland of older larvae, 7 to 19 days, retained low levels of stained lipid indicating incomplete catabolism of storage reserves at these older stages. Total lipid utilization during 3-day starvation periods, expressed as a percentage of initial lipid content, decreased from 72.8 to 36.9% for 44-day-old larvae (settlement had been delayed 24 days in the latter group, Table 2).

The effect of elevated temperature on ORO lipid distribution and accumulation in *Crassostrea virginica* larvae is shown in Plate III. Although shell growth (i.e., length) at 30°C was initially high (8.6 $\mu m \cdot day^{-1}$), accumulation of lipid was low and quite variable (Plate IIIB). Unlike the pattern seen at 25°C, lipid was not accumulated in the digestive gland but deposited in the intestinal wall. Loss of lipid from the intestines upon starvation was comparable to that at 25°C (Table 2). The ORO lipid index of these groups never exceeded 1, and total survival was less than 2% (Table 3).

The photographs in Plate IV illustrate the effect of a diet composed of the diatom *Phaeodactylum tricornutum*. The high endogenous ORO lipid content seen in newly formed straight hinge veligers was rapidly catabolized within 3 days. Very little additional lipid was accumulated from the exogenous dietary source. Shell growth was poor but uniform, whereas lipid content between individuals was extremely variable (Plate IVD). The ORO lipid index ranged from 0 to 2, depending on larval stage, and the mean percentage survival to pediveliger was 17%. No larvae completed metamorphosis under this dietary regime.

Oil Red O lipid distribution in larvae of *Mercenaria mercenaria* is shown in Plate VA. Endogenous lipid was dispersed in tissues of 24-h straight hinge veligers similar to the pattern seen in *Crassostrea virginica*. The dispersed droplets disappeared within 2 days while the digestive gland filled with lipid

PLATE I
Larvae of *Crassostrea virginica* reared at 25°C, fed *Isochrysis* aff. *galbana* (T-ISO) and stained with Oil Red O.
A. Lipid content of healthy larvae throughout development (250×).
B. Low magnification (100×) photograph of 1-day-old straight hinge larvae.
C. 100× photograph of 8-day-old larvae. Note the variability in lipid content between individuals.
D. 100× photograph of 16-day-old pediveliger larvae ready to metamorphose.

PLATE II
Larvae of *Crassostrea virginica* reared as those in Plate I up to days 1, 4, 8, 12 and 16 and then each stage starved for 3 days.
A. Throughout development (250×).
B. 100× photograph of larvae fed 1 day then starved 3 days.
C. 100× photograph of larvae fed 8 days and then starved 3 days.
D. 100× photograph of larvae fed 16 days and starved 3 days.

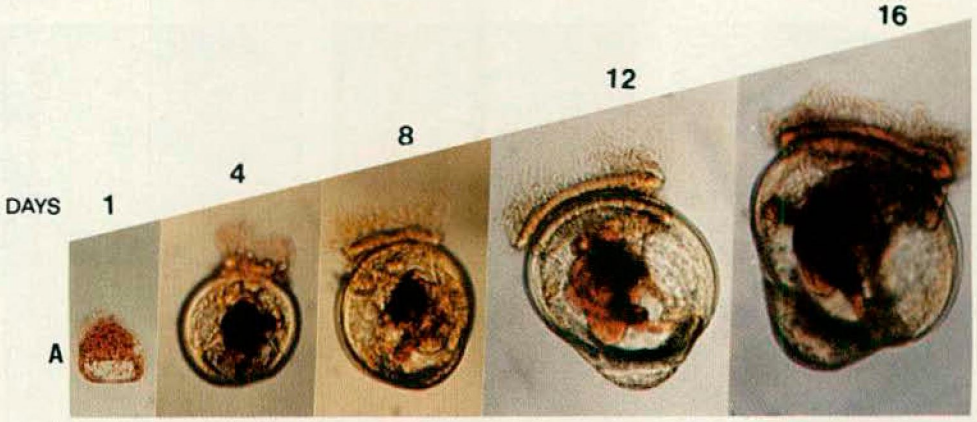
PLATE III
Larvae of *Crassostrea virginica* reared at 30°C and fed *Isochrysis* aff. *galbana* (T-ISO).
A. Lipid distribution throughout development. Note lack of lipid in digestive gland but some accumulation in the intestines.
B. 100× photograph of 1-day-old larvae. Note high individual variability.
C. 100× photograph of 8-day-old larvae.
D. 100× photograph of 16-day-old larvae.

PLATE IV
Larvae of *Crassostrea virginica* reared at 25°C and fed *Phaeodactylum tricornutum* (Phaeo).
A. Lipid distribution throughout development.
B. 100× photograph of 1-day-old larvae.
C. 100× photograph of 8-day-old larvae.
D. 100× photograph of 16-day-old larvae.

PLATE V
Larvae of *Mercenaria mercenaria* reared at 22°C and fed *Isochrysis* aff. *galbana* (T-ISO).
A. Lipid distribution in healthy larvae throughout development.
B. 100× photograph of 1-day-old larvae.
C. 100× photograph of 5-day-old larvae.
D. 100× photograph of 9-day-old pediveliger larvae.
E. 100× photograph of 11-day-old pediveliger larvae.

PLATE VI
Larvae of *Mercenaria mercenaria* reared as in Plate V up to days 1, 5, 9 and 11 and then starved for 3 days.
A. Lipid distribution of larvae starved 3 days.
B. 100× photograph of larvae fed 1 day and starved 3 days.
C. 100× photograph of larvae fed 5 days and starved 3 days.
D. 100× photograph of larvae fed 11 days and starved 3 days.

Crassostrea virginica



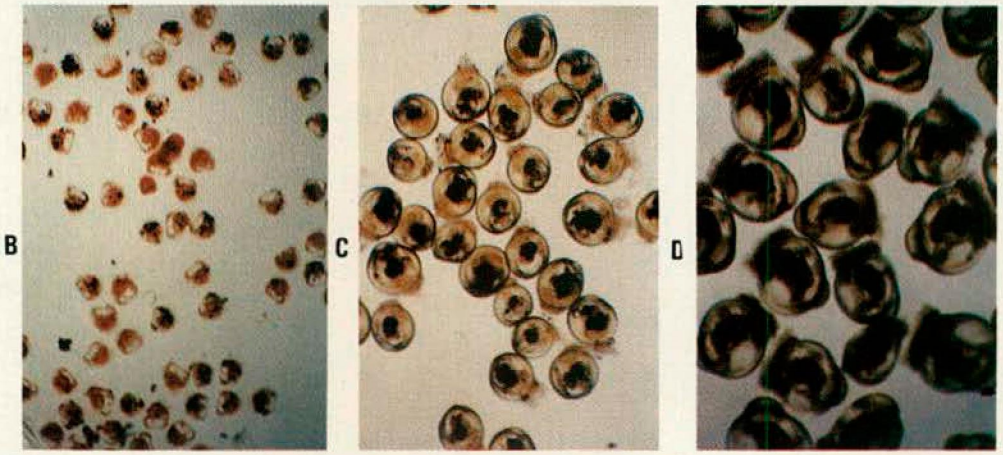
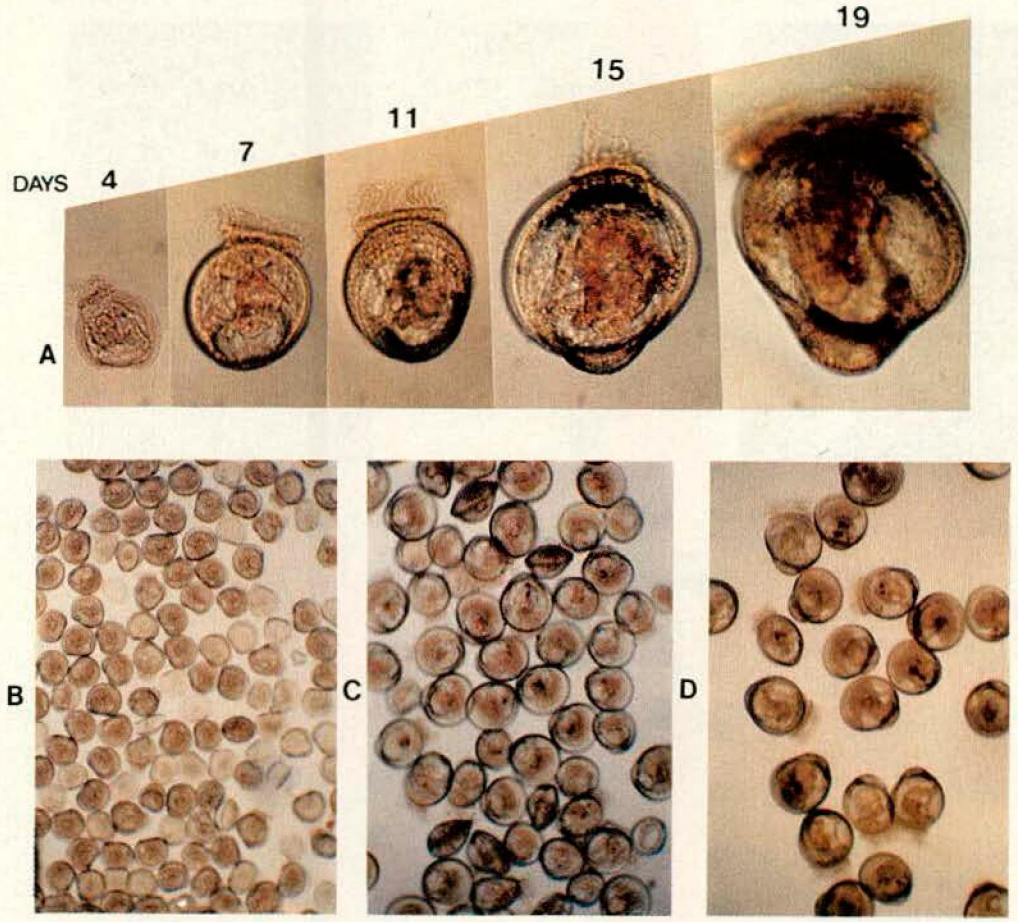


PLATE I (for legend see p. 88)



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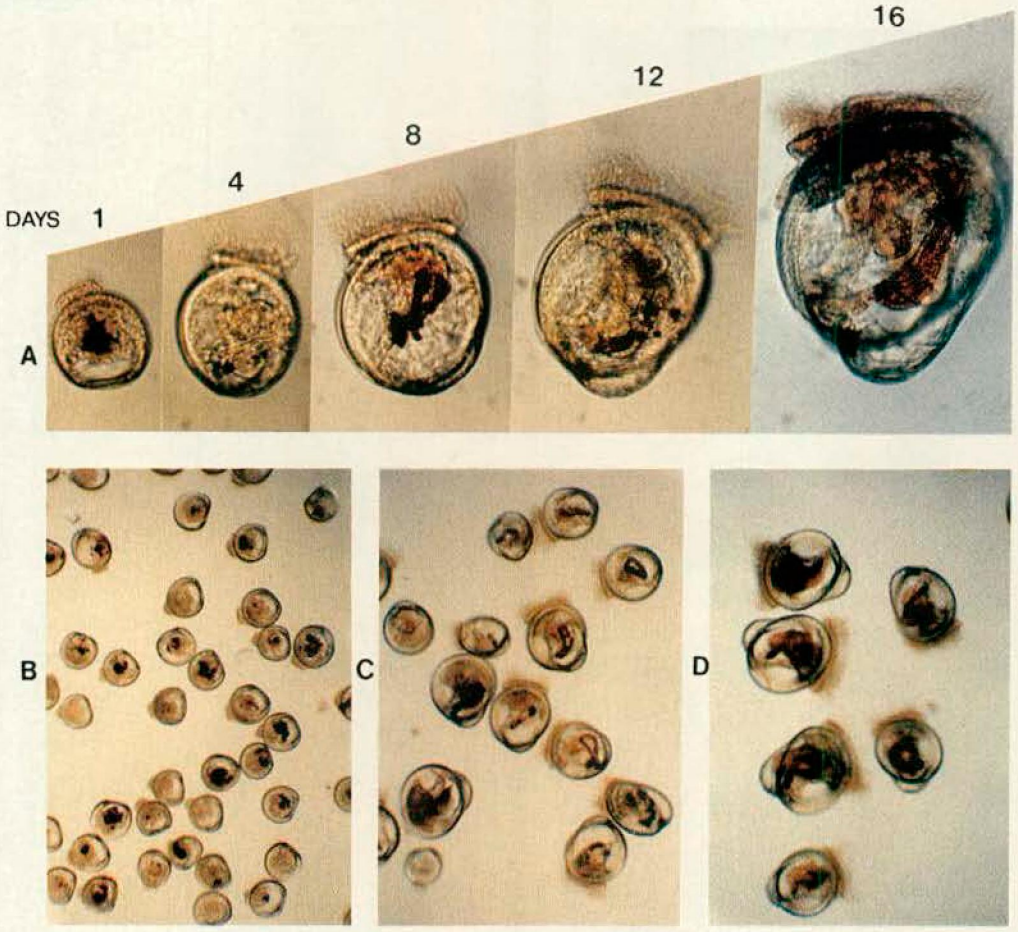

PLATE III (for legend see p. 88)

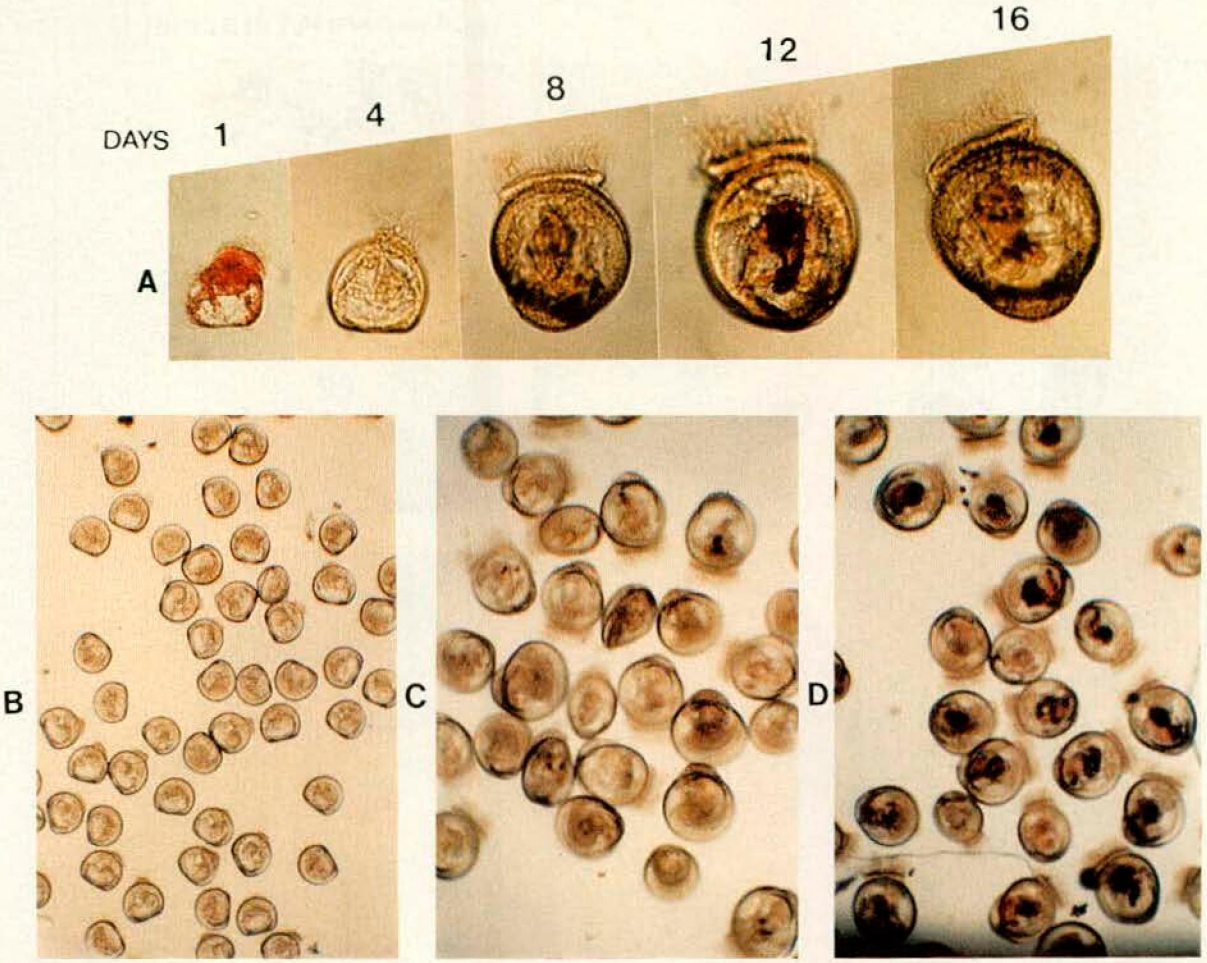

PLATE IV (for legend see p. 88)

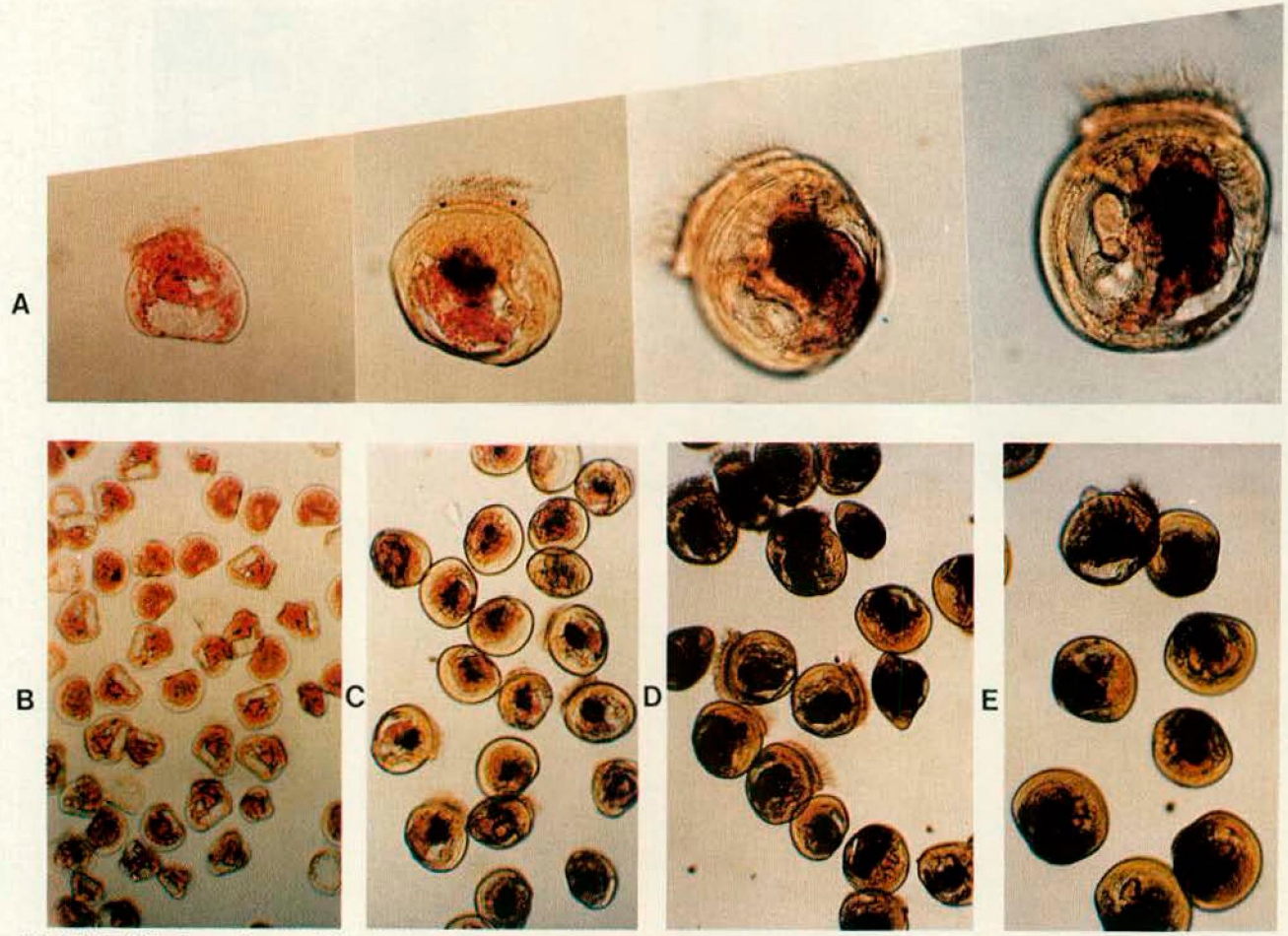

PLATE V (for legend see p. 88)

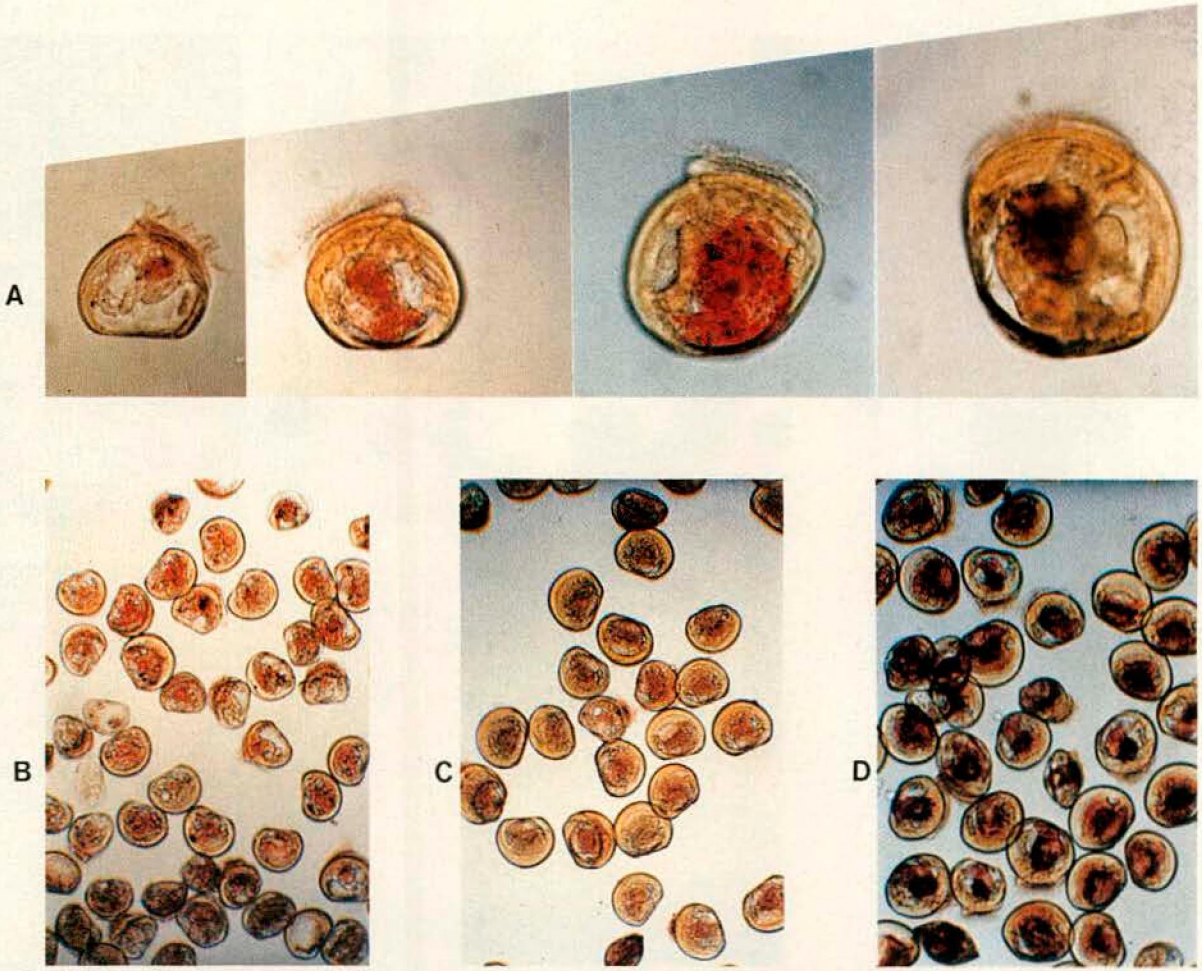

PLATE VI (for legend see p. 88)

TABLE 4

Lipid and dry weight content of healthy and starved *Mercenaria mercenaria* larvae cultured at 22°C

Age (days)	Days fed	Days starved	Length (μm)	Height (μm)	Dry weight ($\mu g \cdot larva^{-1}$)	% Lipid of dry weight	Lipid content ($ng \cdot larva^{-1}$)
0	egg	0	56	—	0.062	6.1	3.8
1	1	0	101	79	0.221	2.6	5.7
3	3	0	115	93	0.610	3.1	18.9
5	5	0	138	118	0.899	4.0	35.9
7	7	0	168	147	1.341	5.2	69.7
9	9	0	192	171	1.760	6.2	109.1
11	11	0	235	218	1.880	7.6	142.8
3	0	3	103	81	0.084	0.8	0.7
4	1	3	120	98	0.155	0.5	0.8
8	5	3	132	112	0.756	1.0	7.6
12	9	3	160	141	1.513	3.3	49.9
14	11	3	237	221	1.724	3.7	63.8

droplets 8 to 12 μm in diameter. The stomach, crystalline style sac and intestines also contained many lipid droplets. Total lipid increased 37.6-fold within 11 days at a rate of 13 $ng \cdot day^{-1} \cdot larva^{-1}$ and remained more uniform between individuals than in *C. virginica.*

Enforced 3-day starvation periods of *Mercenaria mercenaria* larvae produced the effects depicted in Plate VI. A catabolic loss of ORO lipid similar to that seen in *Crassostrea virginica* occurred in young larvae (4 and 8 days old). More lipid remained in 10- and 14-day-old larvae after starvation than in similar stages of *C. virginica* even though *M. mercenaria* utilized a greater percentage of the initial lipid content (i.e., 82 to 55%, Tables 2 and 4). This was apparently due to *M. mercenaria* larvae accumulating more than twice the total lipid content of *C. virginica* larvae during normal development.

Oil Red O lipid content and its distribution in larvae of *Ostrea edulis* was similar to that of *Mercenaria mercenaria* and is not illustrated here. Newly released brooded veligers of *O. edulis* did not contain lipid droplets dispersed in peripheral tissue, but lipid was heavily concentrated in the digestive gland and associated digestive tract. Total lipid increased 9.4-fold from release to pediveliger at 25°C (day 27) and 2.9-fold from release to a shell length of 193 μm at 15°C (Table 5, data were not collected beyond this size at 15°C). On a size-specific basis, accumulation rates were comparable at the two temperatures. Unlike both *Crassostrea virginica* and *Mercenaria mercenaria*, percentage total lipid utilized during 3-day starvation periods in *O. edulis* increased, rather than decreased, from 14 to 31% of initial content at 25°C and fluctuated between 13 and 26% at 15°C (Table 2).

TABLE 5

Lipid and dry weight content of healthy and starved *Ostrea edulis* larvae held at two temperatures

Age from release	Days fed	Days starved	Length (μm)	Height (μm)	Dry weight (μg·larva^{-1})	% Lipid of dry weight	Lipid content (ng·larva^{-1})
25°C							
0	0	0	173	154	0.900	3.4	30.6
3	3	0	183	164	1.590	2.8	44.5
8	8	0	187	169	1.660	3.1	51.5
10	10	0	197	173	1.900	3.8	72.2
15	15	0	225	203	2.600	4.1	106.6
20	20	0	243	216	3.360	5.1	171.4
27	27	0	258	229	4.290	6.7	287.4
35	35	0	294	263	5.960	8.1	482.8
3	0	3	182	162	1.380	1.9	26.2
11	8	3	182	169	1.570	2.3	36.1
18	15	3	230	210	2.200	2.4	52.8
23	20	3	251	222	2.668	3.4	90.7
30	27	3	260	229	3.256	3.1	100.9
38	35	3	291	258	4.390	7.6	333.6
15°C							
0	0	0	172	151	0.900	4.1	36.9
3	3	0	181	160	1.200	3.4	40.8
10	10	0	185	166	1.200	4.4	52.8
16	16	0	188	169	1.600	4.8	76.8
19	19	0	193	171	1.880	5.6	105.3
3	0	3	174	154	0.800	4.0	32.0
14	10	4	184	167	0.930	4.2	39.1
23	19	4	191	171	1.500	5.9	88.5

Combined larval survival data from 20 cultures of *Crassostrea virginica* and *Mercenaria mercenaria* collected over a 2-year period are presented relative to their ORO lipid index in Table 3. Cultures assessed with an average ORO lipid index of 0 or 1 never exceeded a total survival of 0.02%; total survival of cultures with an index of 2 or 3 ranged from 0.1 to 17%. High survival was accompanied by high ORO lipid content; however, high ORO lipid content did not guarantee good survival.

Lipid class composition of healthy and starved larvae of *Crassostrea virginica* and *Mercenaria mercenaria* reared at 25°C is presented in Table 6. Triacylglycerol (TRI) and phospholipids (PHOSPHO) comprised the bulk of the lipid in all stages of both species and were in roughly equal proportion throughout development of healthy larvae. Steryl esters (SE) ranged from about 4 to 8.8% and 2 to 15.8% of the total lipid in *M. mercenaria* and *C. virginica*, respectively.

TABLE 6

Lipid class composition of two species of healthy and starved bivalve larvae

Age (days)	Days fed	Days starved	Total lipid		TRI		PHOSPHO		SE		1-2 Di		STER		FFA	
			%	ng	%	ng	%	ng	%	ng	%	ng	%	ng	%	ng
Mercenaria mercenaria: 22°C																
0	eggs	0	8.3	5.2	39.3	2.0	31.2	1.6	5.4	0.3	1.7	0.1	1.0	0.1	–	–
1	1	0	2.6	2.7	13.2	0.7	60.4	2.4	4.0	0.2	–	–	3.4	1.9	6.5	0.4
5	5	0	6.2	55.7	23.3	13.0	44.8	25.0	6.1	3.4	–	–	0.3	0.2	14.5	8.0
8	8	0	7.7	135.5	30.6	41.5	32.7	44.3	8.8	11.9	–	–	0.9	1.2	1.5	2.0
8	5	3	2.9	21.9	7.3	1.6	82.1	18.0	3.5	0.8	–	–	2.8	0.6	6.6	1.4
11	8	3	6.2	93.8	12.6	11.8	53.0	49.7	7.8	7.3	–	–	2.8	2.6	3.2	3.0
Percentage loss from starvation																
0	0	1				92.8%		+		7.1%		–		+		+
8	5	3				41.3%		25.3%		9.5%		–		–		24.1%
11	8	3				86.7%		–		13.2%		–		–		+
Crassostrea virginica: 25°C																
0	eggs	0	23.0	9.2	39.1	3.6	34.7	3.2	3.3	0.3	2.2	0.2	–	–	1.0	0.1
1	1	0	6.6	4.1	19.5	0.8	46.3	1.9	7.3	0.3	–	–	–	–	4.8	0.2
3	3	0	4.7	8.2	31.7	2.6	34.1	2.8	15.8	1.3	–	–	–	–	2.4	0.2
6	6	0	3.3	17.0	28.2	4.8	31.7	5.4	13.5	2.3	–	–	7.6	1.3	3.5	0.6
9	9	0	2.0	26.0	27.6	7.2	36.1	9.4	5.0	1.3	–	–	5.0	1.3	2.7	0.7
15	15	0	1.9	39.0	35.9	14.0	37.9	14.8	2.3	0.9	–	–	1.5	0.6	1.5	0.6
9	6	3	2.1	14.8	11.5	1.7	35.1	5.2	14.9	2.2	–	–	–	–	4.7	0.7
18	15	3	0.8	33.0	25.7	8.5	43.0	14.2	1.8	0.6	–	–	1.8	0.6	4.5	1.5
Percentage loss from starvation																
0	0	1				68.3%		31.7%								+
9	6	3				91.2%		5.8%		2.9%		–		–		–
18	15	3				84.6%		9.2%		6.2%		–		–		+

(+) indicates an increase in a lipid class upon starvation; (%) percentage of dry weight; ng, lipid class content per larva; TRI, triacylglycerol; PHOSPHO, phospholipid; SE, steryl esters; 1-2 Di, diacylglycerol; STER, free sterol; FFA, free fatty acid; –, below level of detection.

The free fatty acid (FFA) fraction only exceeded 6.6% in one instance (*M. mercenaria* at 5 days), suggesting a possible anomaly due to oxidation of lipid components in that sample (Sasaki and Capuzzo, 1984).

It is clear that TRI were predominantly catabolized during starvation, exceeding 80% of the total lipid utilized in most cases, while the contribution from PHOSPHO and SE ranged from 0 to 31.7%. Of particular interest was the high percentage utilization of TRI during embryonic development (i.e., 92.8%) reminiscent of the rapid loss of stainable material also seen at this stage. Increase in a particular lipid class during starvation probably did not represent true synthesis, since, where this occurred, the values were within the coefficient of variation for the lipid class separation and quantification technique (C.V. = 6.4–10.5%).

DISCUSSION

The assemblage of color photographs presented in this study, depicting normal ORO lipid accumulation in healthy bivalve larvae, and depletion associated with stressful conditions, was designed as a reference guide for comparison with user-obtained results of the lipid-specific staining technique. Analytically derived total lipid and lipid class composition data have been provided as a semi-quantitative calibration of the visual technique and suggest that ORO-stainable lipid is largely comprised of neutral lipids (direct quantification of stained lipid is the subject of another report; Gallager and Mann, 1986b). We suggest that both laboratory researchers and bivalve hatchery operators could derive benefit from this technique by its use as a predictive index of potential larval success in routine culture work.

Lipid has been shown by numerous investigators to be an important energy source during stress in larvae of many planktotrophic invertebrates (see Holland, 1978, for review of early literature; Waldock and Holland, 1978; Waldock and Nascimento, 1979; Gallager and Mann, 1981; Mann and Gallager, 1984, 1985); however, only the studies of Holland and Spencer (1973) and Mann and Gallager (1984, 1985) have considered, in detail, catabolism of energy substrates other than lipid (e.g., protein and carbohydrate) in their analyses. These authors agree that while catabolism of carbohydrate is negligible in most cases, protein may provide 40 to 70% of the calories expended during short starvation periods. Although protein as a source of stored energy should not be underemphasized, the value of lipid as an indicator of physiological condition becomes apparent from its inherent physical properties; coalesced lipid droplets are localized in discrete organs of bivalve larvae (Bayne, 1976; Elston, 1980) and monitoring their accumulation and depletion with histological techniques thus becomes a more tractable prospect. Buoyancy control has recently been shown to be necessary in bivalve larvae that experience short-term nutritional stress, and lipid is fundamental to survival in this respect (Gallager, 1985; Gallager,

in review). The combination of rapid response to environmental change and the integrative nature of lipid energy reserves suggests that they are an ideal mechanism for evaluating physiological condition in bivalve larvae populations.

The present study demonstrates that endogenous lipid supply provided to eggs by the parent during vitellogenesis is rapidly metabolized during embryogenesis prior to the development of a functional mechanism for particulate feeding. This observation is supported by a previous study by Lucas and Rangel (1983) who used fluorescence microscopy to show that prior to shell formation (~24 h at 25°C), developing embryos of *Crassostrea virginica* and *C. gigas* did not ingest autofluorescing phytoplankton cells.

At least 68% of the lipid catabolized during embryogenesis was TRI in both oviparous species studied here, while concomitant observations of stained larvae visually substantiated the loss of lipid. Helm et al. (1973) suggested that the viability of newly released *Ostrea edulis* larvae was correlated with the neutral lipid content. It is possible, therefore, that the degree of individual variability in lipid content between newly formed straight hinge larvae in the present study may have contributed to the variable culture success. Lannan et al. (1980) have demonstrated that broodstock management strongly influences subsequent larval viability. The influential mechanism was not known by the authors but may be related to the initial triacylglycerol quota supplied to each egg by the parent during the conditioning period (Gallager and Mann, 1986a).

Soon after ingestion had begun in straight hinge larvae, lipid (probably triacylglycerol) was accumulated in the digestive gland and associated digestive organs. Waldock and Nascimento (1979) showed that this also occurs in larvae of *Crassostrea gigas* irrespective of the quantity or species of algae food source; however, algal species that promoted fastest growth of *C. gigas* larvae also promoted accumulation of the greatest triacylglycerol reserve. These results are important by virtue of the coupling between good growth and accumulation of neutral lipid; the implication being that the level of neutral lipid is indicative of potential growth.

Evidence of the nutritional value of a food species is indicated by the low level of ORO lipid accumulated by *Crassostrea virginica* larvae fed *Phaeodactylum tricornutum*. This diatom species is of notoriously poor food value (review by Epifanio, 1976); however, its comparison with *Isochrysis galbana* in the present study illustrates how the lipid-staining technique could be used to screen local phytoplankton isolates for their potential as optimal sources of nutrition.

In contrast to the more rapid shell growth at 30°C, elevated temperature had a profound negative effect on ORO lipid accumulation in *Crassostrea virginica* larvae. The dependence of growth and metabolic rate on temperature in bivalve larvae has been reviewed by Bayne (1976). More recently, Sprung (1984) reported Q_{10} values for temperature-dependent respiration by larvae of *Mytilus edulis* (L.) to range from 10.8 (egg) to 1.6 (pediveliger) and 1.4 (egg) to 2.5 (pediveliger) for the temperature ranges 6–12° and 12–18°C, respectively. This

suggests a relatively greater influence on metabolism at the lower temperatures. Q_{10} values for the loss of total lipid during starvation of *C. virginica* in the present study were very high, 8 to 24, indicative of a tight coupling between lipid metabolism and temperature. Moreover, the absence of accumulated ORO lipid in the digestive glands of larvae reared at the elevated temperature implied an enhanced metabolic rate that exceeded the rate at which lipid could be stored. Intestinal ORO lipid probably represented a secondary less labile repository, but this too was depleted upon prolonged enforced starvation. Absorptive cells containing large electron-lucent, osmophilic vacuoles (most likely lipid droplets) occur in both the digestive gland and intestine of *Crassostrea virginica* larvae (Elston, 1980). Elston also noted that vacuolation was highly variable in the intestine but concentrated near the stomach–intestine commissure when present.

It is clear from the results of cultures reared at the elevated temperature that shell growth alone can be a deceiving indicator of culture condition. Indeed, the inability of competent larvae to complete metamorphosis successfully at 30°C may have been attributed to the reduced lipid level stored during planktonic life. Water-born toxic substances and microbial metabolites that stimulate larval metabolism could elicit a similar response in intensive culture systems.

The morphogenic events related to mechanisms of feeding during transition from metamorphosing planktonic bivalve larvae to benthic adult are complex, but important in a discussion of energy storage. The induction of metamorphosis in competent pediveliger larvae is followed by rapid disintegration of the velum, thereby limiting locomotion and feeding. Two or three primordial gill lamellae may be present that aid respiration but appear not to be functional with respect to retention of food particles (*Ostrea edulis* — Cole, 1938; Hickman and Gruffydd, 1971; Waller, 1981; *Mytilus edulis* — Bayne, 1965; *Crassostrea virginica* — Elston, 1980). Upon settlement, the velum is completely resorbed and poorly defined feeding currents of the gill become organized over a period of 2 to 8 days. Holland (1978) reviewed the appropriate literature and suggested that, during this transition, energy requirements are met by lipid reserves stored prior to metamorphosis.

An analogous situation exists in the non-feeding cyprid stage of the barnacle, *Balanus balanoides*. Cyprids contain numerous large oil droplets which accumulate during planktonic life and are subsequently catabolized and disappear while attempts are made to locate a suitable substrate on which to metamorphose (Holland and Walker, 1975; Lucas et al., 1979). The latter study showed that the cyprid cannot complete metamorphosis to the juvenile stage if a substrate is not located by the time about 50% of the endogenous lipid is utilized. Qualitative observations of many stained pediveliger larvae combined with analytical total lipid and lipid class data suggest that a minimum of 21 ng of triacylglycerol, 47% of the initial level, is required to fuel the transition period

between velar resorption and gill activation in *C. virginica* larvae at 25 °C. By comparison, 70 ng of triacylglycerol, 48% of the initial level, is required by larvae of *Mercenaria mercenaria* to complete the transition period at the same temperature. It is apparent that a minimum threshold lipid level must be present in larvae initiating metamorphosis for them to complete that process successfully (Gallager and Mann, in prep.).

Survival of larvae throughout development was correlated with the qualitative ORO lipid index. Cultures with an overall average index of 0 or 1 never exceeded a total survival of 0.02% while survival of cultures with an index of 2 or 3 ranged from 0.1 to 17%. The implication is clear: high survival must be accompanied by high lipid content but high lipid content does not necessarily guarantee good survival. Other biochemical, genetic or environmental factors undoubtedly affected larval survival, but irrespective of these, the potential for good growth and survival was always concomitant with a high ORO lipid index.

High lipid levels may not always be desirable in developing larvae. Elston et al. (1981) consistently found abnormally large lipid vacuoles deposited in the stomach, style sac, digestive gland and intestinal tissues early in the progression of the larval disease vibriosis. They suggested the condition was a useful diagnostic feature of the disease because of the early appearance of this metabolic dysfunction. The possibility exists that routine visual monitoring of the ORO lipid distribution in bivalve larvae may provide an early and distinctive indication of the presence of this disease.

The present study attempts to illustrate the usefulness of visualizing lipid content in bivalve larvae as an indicator of physiological condition and potential for successful metamorphosis. The photographs depict general conditions and suggest how the principle of lipid staining may be employed. Comparing results of stained larvae from cultures in question with the reference photographs presented here should provide a valuable addition to the culturist's repertoire of available techniques used to assess the condition of larvae in culture.

The possibility of applying the staining technique to field populations should not be overlooked. Recruitment of bivalve larvae and spat is affected by the physiological condition of natural larval assemblages. Employing a similar method to that described here would facilitate the use of ORO lipid index as a predictive tool in the natural marine environment.

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