Elsevier Science Publishers
Journal of Experimental Marine Biology and Ecology
156 (1992), 273-284

Experimental Manipulations of the Organic Chemistry of Seawater: Implications for Studies of Energy Budgets in Marine Invertebrate Larvae

W. B. Jaeckle and Donal Manahan

Abstract: Correct measurement of changes in biomass and metabolic rates over time are two essential elements for the accurate construction of energy budgets for invertebrate larvae. Both components of larval energetics are altered by changes in the organic chemistry of the seawater. Axenic (bacteria-free) veliger larvae (88 μm shell length) of the bivalve *Crassostrea gigas* (Thunberg, 1795) had a 53% enhancement of their metabolic rate relative to control values (5.8 $\pm$ 0.6 pmol larva^{-1} h^{-1}, $\bar{x} \pm 1$ SE) when exposed to seawater to which 1 μM glucose had been added. Gastrulae, prism-stage, and pluteus-stage larvae of the sea urchin *Lytechinus pictus* (Verrill, 1867) had significantly higher metabolic rates when 1 μM amino acids (16 amino acids at 62.5 nM each) was added to seawater. Gastrulae increased their rate of respiration by 35%, from 10 $\pm$ 0.9 to 13.5 $\pm$ 1.4 pmol O_2 embryo^{-1} h^{-1}; prism-stage larvae by 33% from 40.9 $\pm$ 2.0 to 54.4 $\pm$ 2.8; and pluteus-stage larvae by 50%, from 33.4 $\pm$ 1.5 to 50.3 $\pm$ 3.1. Lecithotrophic larvae of the gastropod *Haliotis rufescens* Swainson, 1822, either had no change (Day 1, trochophore larvae) or a significant increase (Day 2, veliger larvae) in dry organic weight when reared in natural seawater that had been passed through a filter of 0.2-μm pore size (to remove particles). In contrast, sibling larvae always decreased in dry organic weight when reared in seawater which had first been passed through a sand-filter (a treatment that alters the organic chemistry of seawater), and then a 0.2-μm (pore size) filter. These data show that alterations of the organic chemistry of seawater can affect the growth and metabolism of invertebrate larvae. If such modifications are not controlled, energy budgets constructed from laboratory experiments on larvae in altered seawater may bear little resemblance to the energetics of larvae in the field.

Introduction

Examinations of the energetics of growth and development of marine invertebrates usually involve measuring the changes in rates of respiration, dry organic weight, or biochemical composition (e.g., for echinoderms: Turner & Rutherford, 1976; Lawrence et al., 1984; McClintock & Pearse, 1986; for molluscs: Millar & Scott, 1967; Holland & Spencer, 1973; Pechenik, 1980; Erickson, 1984; Mann & Gallager, 1984, 1985; Jaeckle & Manahan, 1989a). During studies that involve larval rearing, care has

generally been taken to maintain the physical aspects of the culture environment (e.g., salinity, temperature) at levels approximating those found in nature; less attention, however, has been paid to maintaining the organic chemical milieu of the experimental medium at levels similar to those in the field. This is largely a function of the difficulties in measuring and monitoring dissolved organic material (DOM) in seawater (e.g., Williams, 1975; Wangersky, 1978). Specific organic compounds in solution are known to have a distinct influence on the physiology of marine larvae, as evidenced by changes in behavior and morphology during larval settlement and metamorphosis (e.g., Hadfield, 1977; Morse et al., 1979; Burke, 1984; Coon et al., 1985; Pawlik, 1986). However, the effect of the entire pool of DOM in seawater on larval physiology, prior to the attainment of metamorphic competency, is comparatively unknown. Assessment of the influence of the mixture of organic materials in natural seawater on larvae is hampered by the fact that most marine laboratories treat the seawater that enters the facility in ways (e.g., sand filtration, settlement tanks) that are known to alter the organic composition of the seawater (Manahan & Stephens, 1983).

The work of Wilson (1951) and Wilson & Armstrong (1961) were among the first to report an influence on larval development by differences in the chemistry of seawater. They found that echinoid larvae had different growth patterns when reared in filtered seawater taken from a number of localities. The authors speculated that the absence or presence of some dissolved compound(s) in seawater was responsible for the observed variability in larval development. The ability to take up specific fractions of the total pool of DOM in seawater is widespread among larvae of soft-bodied marine invertebrates (amino acids: reviewed in Manahan, 1990; sugars: Welborn & Manahan, 1990). While the rates of transport of specific organic compounds from seawater have been extensively documented, the metabolic responses of larvae to DOM in seawater has received comparatively little attention. The purpose of the present study was to ascertain if modifications of the organic chemistry of seawater affect the respiration and growth of soft-bodied invertebrate larvae.

Materials and Methods

COLLECTION OF GAMETES AND LARVAE

Adult bivalves *Crassostrea gigas* (Thunberg) were supplied by Whiskey Creek Oyster Farms, Netarts Bay, Oregon. Axenic (bacteria-free) suspensions of both sperm and eggs of *C. gigas* were collected aseptically by direct removal from the gonads, following the procedures of Langdon (1983) as modified by Manahan (1989). Fertilized eggs of *Haliotis rufescens* Swainson were obtained from the Ab Lab, Port Hueneme, California. Adults of the sea urchin *Lytechinus pictus* (Verrill) were purchased from Marinus, Long Beach, California. Spawning of both male and female sea urchins was induced by intracoelomic injection of 0.5 M KCl. The xenic eggs were collected and washed three times in seawater. The sperm were collected dry and diluted in seawater immediately prior to fertilization.

REARING CONDITIONS

For studies of the effects of specific DOM additions on respiratory rates, larvae were cultured as follows. Embryos and larvae of *L. pictus* were held in natural seawater (17 °C) that had been passed through a filter of 0.2-μm pore size (Nuclepore). Axenic *C. gigas* larvae were maintained at 20 °C under sterile conditions in filtered (0.2-μm), then autoclaved, natural seawater. A comparison of the growth of gastropod larvae (*H. rufescens*), reared in natural seawater of different chemical compositions, was carried out with sibling larvae cultured in parallel treatments at 17 °C. In each culture vessel, the concentration of *H. rufescens* fertilized eggs was ≈5 ind ml^{-1}. The culture seawater was changed every day after the formation of the larval shell (Day 2).

EFFECT OF DOM ADDITIONS ON RESPIRATORY RATES OF SEA URCHIN AND BIVALVE LARVAE

The effect of an addition of specific organic compounds on the respiratory rate of invertebrate larvae was examined for xenic gastrulae and both prism and pluteus larvae of *L. pictus* and axenic larvae of *C. gigas*. The embryos and larvae were collected by gentle sieving onto a polyester mesh (80-μm mesh size). They were then washed and resuspended in fresh, isothermal, seawater. All seawater used for experiments had previously been filtered (0.2-μm pore size), then sterilized by autoclaving.

Embryos and larvae were removed and placed in the respiration chamber (see below) in either seawater (control), or in seawater to which specific organic compounds had been added. All added substrates were made up from individual powders (Sigma Chemical). For experiments with gastrulae, prism-stage larvae, and pluteus-stage larvae of *L. pictus*, an equimolar mixture of 16 amino acids (62.5 nM each) was added to give a final concentration of 1 μM amino acids (individual amino acids listed in Table I). For the experiments with axenic veliger larvae of *C. gigas*, the respiration measurements were made in 1-μM solution of glucose in seawater and in seawater controls (without additions).

All respiration measurements were made using a Clark-type polarographic oxygen sensor. This consisted of a Strathkelvin Instruments oxygen meter (Model 781), an oxygen sensor (No. 1302), and a microrespiration chamber (RC-200). The respiration chamber was calibrated to 100 μl total volume and maintained at culture temperature (±0.02 °C) through external circulation by a water bath (Model RDL 20, Precision Instruments). Embryos and larvae were added to the chamber and the rate of decrease in the partial pressure of oxygen was determined for at least 20 min. The analog output from the oxygen meter was converted to a digital signal and transferred to an IBM-XT computer using an analog/digital converter and the Datacan computer software package (Sable Systems, Los Angeles, California). At the end of each trial, the animals were removed from the sample chamber, and counted. The respiratory rates of the embryos and larvae were converted from the change in the partial pressure of O_2 over time to the amount (mol) of O_2 ind^{-1} h^{-1}. This was achieved by calibrating the sensor relative

to the molar amount of O_2 present in the seawater, the latter being determined by the Winkler titration method (Parsons et al., 1984). For each batch of embryos or larvae, trials with and without the additions of organic compounds were alternated in series. This allowed for the detection of any changes in respiration rates due solely to developmental changes which might have occurred during the course of each experiment. After each trial involving the addition of organic compounds, the sample chamber was washed four times with seawater to remove any residual amino acids or glucose. Prior to any experiment, the oxygen consumption rate of the sensor itself was determined in both natural seawater and in seawater to which organic substrates had been added. The self-consumption rate of the sensor ($< 10\%$ of experiment trials) was then subtracted from the measured respiratory rates of the embryos and larvae for each treatment.

GLUCOSE TRANSPORT BY AXENIC BIVALVE LARVAE

The rate of glucose transport by axenic larvae of *C. gigas* was measured using larvae with a mean shell length of 88 μm. Larvae were exposed to a 1.1 μM solution of glucose in 10 ml of filtered seawater. After the addition of the isotope and cold carrier (0.433 μM ^{14}C-glucose (353 μCi μmol^{-1}) and 0.657 μM ^{12}C-glucose, both from Sigma Chemical), the suspension of larvae was thoroughly mixed, and samples removed every 1–2 min for a total time of 20 min. All samples of larvae were processed and analyzed following the procedures described by Manahan (1989).

EFFECT OF SAND FILTRATION ON AMINO ACID CONCENTRATION IN SEAWATER

The concentration and composition of dissolved free amino acids in samples of seawater were measured to assay for any differences in the organic chemistry of seawater that was caused by passing the seawater through a sand-filter. Seawater samples were taken upstream and downstream of a sand-filter using chemically clean pipettes. A 500-μl sample was gently filtered through a 0.2-μm (pore size) polycarbonate filter housed in a 13-mm filter holder (Nuclepore) and immediately frozen on dry ice. The samples were stored in a $-20\,^{\circ}$C freezer until analyzed. Differences in the concentrations of individual amino acids were determined using high-performance liquid chromatography (HPLC) following Manahan's (1989) modifications of the procedures described in Lindroth & Mopper (1979) and Jones et al. (1981).

EFFECTS OF SEAWATER TREATMENTS ON LARVAL GROWTH

To assess the influence of different seawater treatments on larval growth, the change in organic weight was measured for sibling larvae reared in differently treated seawaters. Growth of *H. rufescens* larvae was measured as differences in the dry organic weight ind^{-1} (see Methods in Jaeckle & Manahan, 1989a). For each experiment, a batch of sibling fertilized eggs was divided into two groups. One group of fertilized eggs was placed in seawater which had only been passed through a 0.2-μm (pore size) filter to

remove particles. The other group of fertilized eggs was placed in seawater which had been first passed through a sand-filter, and then through a 0.2-μm (pore size) filter. Sand-filtered seawater was used because when seawater is processed in this way the organic chemical composition is altered, as demonstrated by changes in the concentrations of free amino acids (Manahan & Stephens, 1983; see Results).

RESULTS

CHANGES IN RATES OF LARVAL RESPIRATION IN PRESENCE OF GLUCOSE AND AMINO ACIDS

Axenic larvae of *C. gigas* increased their respiratory rate, relative to controls, when exposed to 1 μM glucose in seawater (Fig. 1). Larval respiration increased by an average

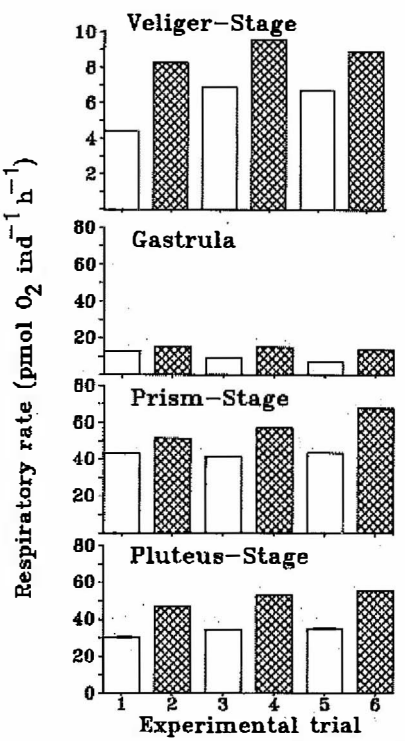

Fig. 1. Influence of addition of specific organic compounds on respiratory rates of embryos and larvae of *C. gigas* and *L. pictus*. (upper graph) A comparison of respiratory rates of axenic veliger larvae of bivalve *C. gigas* (88 μm shell length) in presence of seawater with and without addition of 1 μM glucose. (lower graphs) Respiratory rates of gastrulae, prism-stage, and pluteus-stage larvae of sea urchin *L. pictus* with and without addition of 1 μM amino acids to seawater (16 individual amino acids at 62.5 nM per compound, listed in Table I). Each bar represents a single experiment trial; r^2 for all respiration rates was >0.95 ($n \geq 86$). Open bars represent trials with larvae in natural seawater (control); cross-hatched bars depict trials with larvae in presence of either 1 μM glucose or 1 μM amino acids added to natural seawater.

of 53% above the control value of 5.8 pmol O_2 larva^{-1} h^{-1} (ANOVA, $p \leqslant 0.05$, $n = 3$ trials treatment^{-1}; Table I).

TABLE I

Respiration rates (pmol O_2 ind^{-1} h^{-1}) of veliger larvae of *C. gigas* and developmental stages of *L. pictus* in presence and absence of specific additions of organic compounds. All data are presented at $\bar{x} \pm 1$ SE. A single asterisk denotes a statistical significance at $p \leq 0.05$, a double asterisk indicates a statistically significant difference at $p \leq 0.01$.

Veliger larvae of *C. gigas*			
Shell length (μm)	No addition (control)	plus 1 μM glucose	VR
88	5.8 ± 0.6	8.9 ± 0.4	11.2*

Gastrulae and larvae of *L. pictus*				
Age (day)	Stage	No addition (control)	plus 1 μM amino acids[a]	VR
1	Gastrula	10.0 ± 0.09	13.5 ± 1.4	8.8*
2	Prism-stage	40.9 ± 2.0	54.4 ± 2.8	11.1*
3	Pluteus-stage	33.4 ± 1.5	50.3 ± 3.1	38.8**

[a] 1 μM amino acid mixture was composed of 16 individual compounds each at a substrate concentration of 62.5 nM. Amino acids used were: alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine.

The respiratory rates of gastrulae, prism-stage larvae, and pluteus-stage larvae of the sea urchin *L. pictus* were higher in the presence of a mixture of 1 μM amino acids (Fig. 1) than in control trials without additions. For gastrulae (1-day-old), the rate of respiration increased 35% above the average control value of 10.0 pmol O_2 embryo^{-1} h^{-1} (ANOVA, $p \leqslant 0.05$; Table I). Following development of the embryos to the prism-stage (2-day-old), the control respiratory rates averaged 40.9 pmol O_2 larva^{-1} h^{-1}. Again in the presence of 1 μM amino acids, the respiratory rate of these larvae increased by 33% (ANOVA, $p \leqslant 0.05$; Table I). These larvae were reared in the absence of any particulate foods (feeding begins in the prism-stage) and, not surprisingly, the respiratory rate of 3-day-old larvae (now pluteus-stage) was lower than that of prism-stage larvae, averaging 33.4 pmol O_2 larva^{-1} h^{-1}. However, the plutei still showed an average increase of 50% in their rates of O_2 consumption in the presence of 1 μM amino acids (ANOVA, $p \leqslant 0.01$; Table I).

RATE OF GLUCOSE TRANSPORT BY AXENIC BIVALVE LARVAE

Axenic larvae of *C. gigas* (88 μm) transported glucose (1.1 μM) from seawater at a rate of 12 fmol glucose larva^{-1} h^{-1} ($r^2 = 0.92$, $n = 6$).

EFFECT OF SAND-FILTRATION ON CONCENTRATION OF DISSOLVED FREE AMINO ACIDS IN SEAWATER

When natural seawater was passed through a sand-filter, there was a large decrease in the concentrations of all detectable amino acids (Fig. 2A, compare upper and lower chromatograms). Following filtration, the concentration of aspartic acid decreased from 167 to 16 nM (90%). Similarly, there were decreases in glutamic acid (61%, 51 to 20 nM), serine (87%, from 533 to 69 nM), alanine (86%, 216 to 31 nM), and tyrosine (82%, 57 to 10 nM) after this treatment.

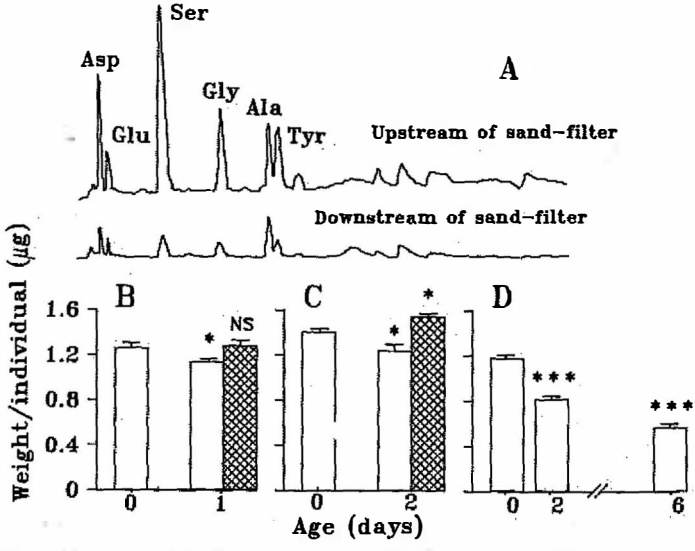

Fig. 2. Change in concentration of dissolved free amino acids in seawater before and after passage through a sand-filter and effects of this seawater treatment on growth of larvae of *H. rufescens*. Upper portion (A) shows HPLC chromatograms depicting change in total amino acid concentrations in seawater which was sampled upstream and downstream of a sand-filter. For each amino acid, peak areas are proportional to substrate concentrations. Bar graphs (B–D) show changes in dry organic weight of larvae when reared in differently treated seawater; each graph represents larvae obtained from an independent spawning. Data are presented as $\bar{x}$ + SE. Open bars represent dry organic weight of larvae reared in seawater which had been passed through a sand-filter, and then a 0.2-µm (pore size) filter. Cross-hatched bars depict dry organic weight of larvae reared in filtered seawater (0.2-µm pore size) which was not passed through a sand-filter. Day 0, egg; Day 1, trochophore-stage larva; Days 2–6, veliger stage larva. *Abbreviations:* Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Gly, glycine; Ala, alanine; and Tyr, tyrosine. A single asterisk denotes a statistical significance at $p \leqslant 0.05$, a triple asterisk indicates a statistically significant difference at $p \leqslant 0.001$.

INFLUENCE OF SAND-FILTERED SEAWATER ON GROWTH OF MOLLUSCAN LARVAE

When the change in dry organic weight larva^{-1} was compared for sibling larvae that were reared in either treated (passed through a sand-filter) or untreated seawater, there was a significant difference between the treatments. Larvae reared in untreated seawater

(Fig. 2A, upper chromatogram) either exhibited no change (ANOVA, $p > 0.05$, 0–1 days, Fig. 2B) or increase (0–2 days, ANOVA, $p \leqslant 0.05$; Fig. 2C) in dry organic weight. In contrast, sibling larvae from both cultures that were reared in treated seawater (Fig. 2A, lower chromatogram) decreased in organic weight (ANOVA, $p \leqslant 0.05$ each; Fig. 2B,C). A third culture of larvae was reared in sand-filtered (treated) seawater until the larvae were competent to settle. For this group of larvae, there was both a net decrease in organic weight during both the first 2 days of development and throughout the entire 6-day larval lifespan (ANOVA for both comparisons between eggs and 2- and 6-day-old larvae, $p \leqslant 0.001$; Fig. 2D).

Discussion

Certain specific organic compounds in seawater are recognized as modulators of larval physiology after metamorphic competency has been attained (Hadfield, 1977; Morse et al., 1979; Burke, 1984; Coon et al., 1985; Pawlik, 1986). However, the influence of organic compounds dissolved in seawater on the physiology of precompetent larvae has received less attention. The purpose of the present study was to empirically ascertain if alterations in the chemistry of seawater affect the physiology of larvae. It is necessary to understand the influence of DOM (both defined and undefined fractions) on larval metabolism because most energy budgets of larvae are based on rates of O_2 consumption and changes in biomass (expressed as either energy or C). This is particularly important if the results of such laboratory studies are to be used to estimate the metabolism of larvae in the field.

Axenic veliger larvae of the bivalve *C. gigas* increased their respiratory rates by 53% when exposed to 1 μM glucose. The fact that an increase in O_2 consumption occurred in these axenic larvae indicates that bacteria were not responsible for the enhanced rate of O_2 consumption after the addition of glucose. The increase in the average metabolic rate of these larvae exceeded the energy gained solely by the transport of the glucose itself, given the stoichiometric relationship that 1 mol of glucose requires 6 mol of O_2 for complete combustion. The rate of glucose transport by these larvae was measured to be 12 fmol glucose larva^{-1} h^{-1}. If all of this glucose was completely oxidized to CO_2, then the expected increase in the rate of respiration would be 72 (12 × 6) fmol O_2 larva^{-1} h^{-1}. The measured increase in the respiratory rate of axenic larvae in the presence of 1 μM glucose was 8.9 pmol O_2 larva^{-1} h^{-1}, or 3100 fmol above the mean control value of 5.8 pmol O_2 larva^{-1} h^{-1} (Table I). Therefore, only 2.3% (72/3100) of the increase in the respiratory rate of these larvae could be explained by the complete oxidation of the transported substrate. The increase in metabolic rates is neither restricted to the presence of monosaccharides in seawater, nor solely to bivalve larvae. When exposed to 1 μM mixture of amino acids, gastrulae, prism-stage, and pluteus-stage larvae of *L. pictus* also increased their respiratory rates by 33–50% above controls.

Similar metabolic responses to specific dissolved organic compounds have been described for adult mussels (*Mytilus edulis;* Thompson & Bayne, 1972) and for scyphistomae of a scyphozoan (*Aurelia aurita;* Shick, 1975). Thompson & Bayne (1972) reported that the respiratory rate of adult mussels increased by as much as 50% when the animals were exposed to 10 mM glucose. A greater increase in respiration (to 150% of controls) was observed when a mixture of organic compounds (a filtrate of ruptured cells of the flagellate *Tetraselmis suecica*) was added to the experimental chamber. Shick (1975) reported that the rate of oxygen consumption was significantly greater in starved scyphistomae of *A. aurita* exposed to dissolved glycine (800 nM), relative to starved controls with no addition of glycine.

For adult mussels, there appears to be a behavioral component to the increase in the respiratory rate (Thompson & Bayne, 1972). Concomitant with the change in the oxygen consumption, there was an increase in the rate of filtration (volume of water passed through the mantle cavity per unit time). Further, when the added organic molecules were flushed from the experimental system, the respiratory and filtration rates of the mussels returned to control values. For larvae we cannot be certain whether the mechanism(s) responsible for the increase in respiratory rates in the presence of either glucose or amino acids is due to a behavioral change (e.g., increased swimming speeds) or a result of an increase in metabolism, independent of any alteration in behavior. Regardless of the mechanism, it is clear that additions of organic material can increase the metabolic rates of larvae by as much as 50%. Because of this metabolic change, it appears likely that energy budgets calculated for larvae that were reared in chemically altered seawater may not be an accurate predictor of the metabolic cost of development of larvae in the field.

In most marine biology laboratories and commercial aquaculture enterprises, invertebrate larvae are raised in seawater that is usually passed through a sand-filter in order to reduce the amount of particulate material (e.g., Loosanoff & Davis, 1963; Ebert & Hook, 1984). Although these treatments successfully reduce the particulate load in seawater, it is known that the organic chemical composition of the seawater is also modified (Manahan & Stephens, 1983; present study). Larvae of *H. rufescens* cannot capture particulate foods and their only potential exogenous food source is DOM (Jaeckle & Manahan, 1989b). As a consequence, any alteration in the total amount of DOM in seawater may be reflected by changes in the biomass of larvae. In the present study, when larvae of *H. rufescens* were reared in seawater with an organic chemistry that approximated the composition and concentrations of natural seawater, their dry organic weight either increased or remained constant during the first 1–2 days of development. Yet, when sibling larvae were reared in treated (sand-filtered) seawater, a loss of dry organic weight occurred. However, even though larvae lose weight throughout their entire larval development when reared in treated seawater, they are still capable of successful metamorphosis (J. McMullen, Ab Lab, pers. comm.). As the eggs of *H. rufescens* are supplied with more than enough energy to satisfy the metabolic demands of complete larval development (Jaeckle & Manahan, 1989a), the ability to

assimilate DOM from seawater may serve as a means to supplement endogenous reserves. The experimental design employed in the present study, however, does not exclude the possibility that some unknown chemical was released into the seawater during the passage through the sand-filter, and that it was this chemical that inhibited larval growth. Nonetheless, the observed increases in dry organic weight of embryos (Shilling & Manahan, 1990) and larvae that lack a functional digestive system (Jaeckle & Manahan, 1989a, present study) suggest that these stages of development can use DOM as a food source.

The chemistry of seawater can have a profound influence on the physiology of marine invertebrate larvae. The inorganic components in solution in seawater are vital for the production of structural components in growing larvae (e.g., Gallager et al., 1988). Less attention has been paid to the importance of the organic components in seawater for successful larval development. If laboratory-based studies are to be of value for predicting the growth and metabolism of larvae in nature, the physiological responses of larvae to dissolved organic compounds in seawater should be considered.

Acknowledgments

We thank J. McMullen (Ab Lab) for providing fertilized eggs of *H. rufescens* and L. Hanson (Whiskey Creek Oyster Farms) for supplying adult *C. gigas*. This manuscript was significantly improved by the comments of F. Shilling, C.J. Hurlbut, S. Colwell, S. Nourizadeh, and J. Welborn. This research was supported by grants from NOAA Office of Sea Grant (USC Grant R/RD-27, 35) and from the National Science Foundation (OCE-86–0889) to D. Manahan.

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