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COULOMETRIC MEASUREMENT OF OXYGEN CONSUMPTION DURING DEVELOPMENT OF MARINE INVERTEBRATE EMBRYOS AND LARVAE

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Summary

Determining the metabolic rate of larval invertebrates from aquatic habitats is complicated by the problems of small size and the scarcity of suitable measurement techniques. In this study, coulometric respirometry (a new technique for the study of marine embryos and larvae) was used to explore several issues associated with the rate of energy use during embryonic and larval development of marine invertebrates from three phyla. Coulometric respirometry measures rates of oxygen consumption under normoxic conditions by electrochemically replacing the oxygen consumed by organisms during an experiment. This technique is based on the assumption that all electrons consumed by the anodic reactions result in the production of oxygen. We verify this assumption using direct measurements of oxygen production and show that the technique is sensitive enough (1 nmol O_2 h^{-1}) to quantify the oxygen consumption of a single individual swimming freely in a relatively large volume (2 ml). Continuous measurements can span days, and embryos in the coulometric respiration chambers develop to the larval stage at normal rates of differentiation. Measurements of metabolic rates were made with the coulometric respirometer during the complete life-span of larvae of three species (asteroid, *Asterina miniata*; bivalve, *Crassostrea gigas*; echinoid, *Dendraster excentricus*). For these species, metabolic power equations had mass exponents near unity (0.9–1.1), showing that metabolic rate scales isometrically with mass during larval growth. Metabolic rates were independent of the concentration of larvae used in the respirometer chambers for a range of larval concentrations from 4 to 400 larvae ml^{-1} (coulometric respirometer) and from 241 to 809 larvae ml^{-1} (polarographic oxygen sensor). Metabolic rates were measured using coulometric respirometry and two other commonly used techniques, polarographic oxygen sensors and Winkler's titration. Polarographic oxygen sensors in small, sealed chambers (100 μl) consistently gave the lowest values (by as much as 80 %) for the asteroid, echinoid and molluscan larvae. By comparison, rates of oxygen consumption measured using coulometric respirometry and Winkler's titration (to measure the change in oxygen concentration over time) were similar and consistently higher. Although the polarographic oxygen sensor is the most widely used method for measuring the metabolism of small animals in sealed 100–1000 μl chambers, it appears that the metabolism of some larvae is adversely affected by the conditions within these respirometers.

Key words: metabolism, larval development, cost, coulometric respirometry, polarographic oxygen sensors, Winkler assay, *Asterina miniata*, *Crassostrea gigas*, *Dendraster excentricus*, *Strongylocentrotus purpuratus*, *Strongylocentrotus franciscanus*, *Artemia*, *Acartia tonsa*.

Introduction

The majority of marine invertebrates have a life cycle that involves a larval stage (Thorson, 1950). Measurements of energy requirements during the larval stage have mostly been carried out using rates of oxygen consumption as a measure of metabolic rate (indirect calorimetry). Zeuthen (1947) used a modified Cartesian diver, and Scholander *et al.* (1952) used the reference diver technique to make some of the first measurements of the consumption of oxygen by invertebrate embryos and larvae. These techniques, however, depend on small volumes with high numbers of larvae per volume (Sprung, 1984). For example, Scholander *et al.* (1952) used approximately one individual per microlitre in the micro-respiration chamber, which is equivalent to 1000 individuals per millilitre. More recent studies have used highly sensitive (small cathode) polarographic oxygen sensors (Gnaiger and Forstner, 1983), which have permitted more routine measurement of the metabolic rates of small organisms enclosed in fixed volumes (Gerdes, 1983; Manahan, 1983;

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McDonald, 1988; Yasumasu *et al.* 1988; Jaeckle and Manahan, 1989; Shilling and Manahan, 1990). None the less, the concentrations of larvae required to produce a measurable decline in oxygen tensions are still high (e.g. 290 individuals ml^{-1} based on 50–150 larvae per 345 μl respiration chamber, Jaeckle and Manahan, 1989). Another disadvantage of relying on decreasing oxygen tensions is the possibility that unknown stresses on the larvae result in atypical metabolic rates. This problem has been solved to some extent by flow-through respirometry systems that pass a continuous stream of fresh sea water through the experimental chamber. Very high concentrations of larvae (e.g. 30 000 in 25 ml, Wang and Widdows, 1991), however, are still required to obtain significant rates of oxygen use and heat dissipation (direct calorimetry) as the sea water flows through the respiration chamber.

Little information is available on the metabolic scaling of the developing stages of marine invertebrates (Zeuthen, 1953; Sprung, 1984; Sprung and Widdows, 1986; Manahan *et al.* 1989). This is largely because of methodological difficulties in working with small organisms and the limitations of extrapolating short-term measurements, obtained using polarographic oxygen sensors (POS) over the much longer time course of development. Questions concerning (i) the limitations of short-term oxygen consumption measurements, (ii) the possible stress effects of high numbers of animals per respiration chamber, and (iii) the effect of low oxygen tensions can be addressed using coulometric respirometry.

Coulometric respirometers measure oxygen consumption by the quantitative, electrolytic replacement of the oxygen used by an organism during an experiment (Heusner *et al.* 1982). The amount of oxygen added to the respiration chamber is continually recorded and is used to calculate the metabolic rate of the organism under normoxic conditions. The coulometric respirometer offers several advantages over previous techniques used to measure the metabolic rate of small animals. The coulometric respirometer continually replaces the oxygen used by an organism, allowing metabolic rates to be measured under stable normoxic conditions that can continue for periods of hours to days. In addition, the coulometric respirometer is, under the conditions reported here, about 100-fold more sensitive than the more widely used POS respirometer. Hence, the number of animals used in experiments can be drastically reduced (e.g. to 1–4 larvae ml^{-1}). Finally, coulometric respirometry allows for the measurement of metabolic rates over developmentally relevant periods (hours to days), thereby providing data on the total amount of energy used by a developing organism. Such long-term measurements are either difficult or impossible to obtain using previous methodologies.

Materials and methods

Culturing conditions

Adult asteroids, *Asterina miniata* (Brandt), and echinoids, *Dendraster excentricus* (Eschscholtz), *Strongylocentrotus purpuratus* (Stimpson) and *S. franciscanus* (Agassiz), were induced to spawn by intracoelomic injection of either 1 mmol l^{-1} 1-methyladenine (asteroids) or 0.5 mol l^{-1} KCl (echinoids). All culturing and experiments were carried out in sea water that had been passed through a 0.2 μm (pore-size) Nuclepore filter. Following fertilization, zygotes were transferred to 50 l culture vessels which were stirred (approximately 10 revs min^{-1}) using small rotating paddles (30 cm×30 cm paddle area). Fertilization success ranged between 95 and 100 %. Sea water in the culture vessels was changed every fourth day, and algal food (10^4 cells ml^{-1}, *Duneliella tertiolecta* Butcher) was added every second day starting at 48 h after fertilization. Cultures were maintained at 14–15 °C, except for some cultures of *D. excentricus* which were maintained at 20 °C. Encysted embryos of *Artemia* sp. (San Francisco brand) were raised to the larval stage in sea water at 20 °C. Veligers of the Pacific oyster *Crassostrea gigas* (Thunberg) were supplied by the Coast Oyster Company (Quilcene, Washington). Veligers ranged in size from newly formed D-veligers (valve length=82.0±0.68 μm, S.E.M.) to competent pediveligers (323.0±5.57 μm). Veligers were kept in autoclaved sea water for 24 h (20 °C) after being shipped from Washington, USA, and prior to use in experiments in California, USA. Nauplii and adult copepods (*Acartia tonsa*) were caught, using plankton nets, from Fish Harbor (San Pedro, California) and were kept in sea water (15 °C) for 24 h prior to any measurements.

Biomass measurements

Biomass is defined here as ash-free dry organic mass and was measured using a mass-calibrated electrobalance (Cahn, model 29). Eggs, embryos or larvae (200–500 individuals used per determination) were washed three times in isotonic ammonium formate solution (3.4 %, 0.2 μm filtered) and added to small aluminium vessels (pre-ashed at 500 °C for 6 h) prior to being dried at 58 °C to constant mass (which occurred within 10 days). Samples were weighed (total dry mass) and then ashed at 458 °C for 6 h, and weighed again (ash mass). Biomass was calculated as the difference between the total dry mass and the ash mass. For *C. gigas*, the relationship between valve length (l, μm) and biomass (m, μg dry organic mass) was: $\log m = 2.82 \log l - 6.594$ (r^2=0.99, N=30). Several controls were run to ensure that the aluminium vessels were completely ashed and that complete sublimation of ammonium formate had occurred. Ammonium formate solution (100 μl) was added periodically to a number of ashed aluminium vessels and the vessels treated as above. The mass differences in these control vessels was less than 2 % of typical ash-free dry organic masses seen during mass determinations (data not shown).

Coulometric respirometry

The rates of oxygen consumption of embryos and larvae at various stages of development were measured using a coulometric respirometer. All respirometry measurements (coulometric and other techniques) were carried out at the temperature at which the embryos and larvae were cultured.

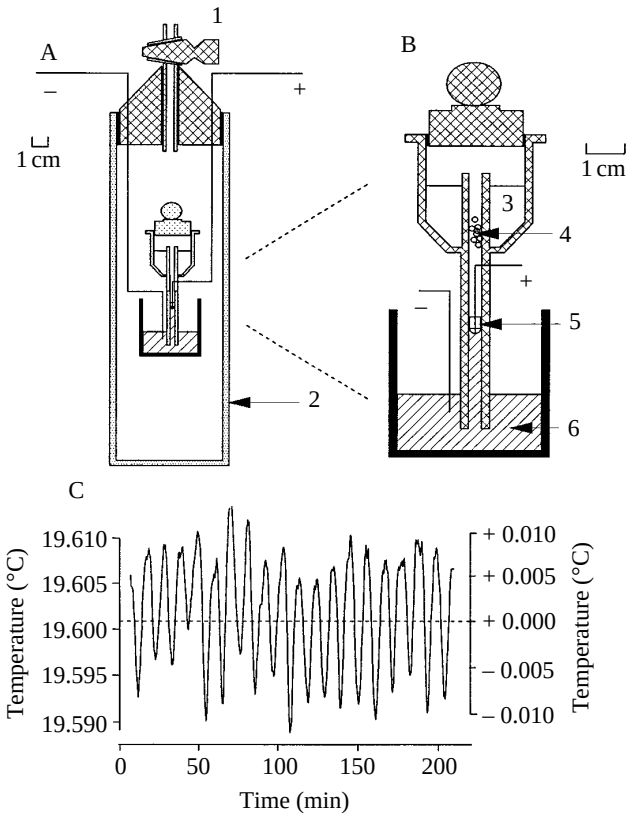

Fig. 1. (A) Design of the coulometric respirometer used to measure the metabolic rates of developing marine invertebrate larvae. The stopcock (1) sealed off the thermobarometric chamber (2) that housed the respirometry chamber. (B) Respirometry chamber for incubating marine invertebrate larvae in 2 ml of sea water (3). The CO_2 absorbent Ascarite II (4, Thomas Scientific, New Jersey) was placed in the tube to absorb CO_2 and was held in place by a small plug of cotton wool. A Teflon trigger assembly (5) encircled the anode (+) that was in discontinuous contact with a saturated solution of copper sulphate (6), which was drawn up into the glass tube by the slightly higher pressure of the thermobarometric chamber relative to the respirometry chambers. When the pressure dropped in the coulometric chamber, the $CuSO_4$ made contact with the anode, which led to the release of oxygen into the coulometric chamber. The number of capacitor discharges occurring within set time intervals (e.g. 20 min) were stored in dynamic RAM (random access memory) and later downloaded and analyzed on a personal computer. The electronics controlling the discharge of the capacitor and storing the number of capacitor discharges were made according to the specifications of Heusner *et al*. (1982). (C) Stability of temperature inside a thermobarometric chamber following equilibration. The measurements was made by inserting a calibrated platinum thermistor into the air-space within the chamber.

The design of our system was based on that described by Heusner *et al.* (1982). Our solid-state current generator supported and collected data from four independent respirometry chambers. Data from each respiration chamber were transmitted from the coulometric device *via* an RS-232 port to a personal computer, where they were loaded into standard worksheet programs. The thermobarometer (Fig. 1A) and respiration chambers (Fig. 1A,B) were constructed according to Heusner and Tracey (1984). Each respiration chamber was placed within a thermobarometer chamber (Fig. 1A) and, following temperature equilibration (2–3 h), the thermobarometer was sealed using a greased glass stopcock. The animals were placed in the 2.5 ml respirometer chamber (Fig. 1B). As the animals respired, oxygen was removed and the released CO_2 was captured by a CO_2 absorbent. The net drop in partial pressure in the respirometer chamber resulted in an increase in the height of the saturated $CuSO_4$ solution which, as a result, made contact with a sharpened platinum anode. Oxygen was released upon contact of the saturated $CuSO_4$ solution with the anode, through which current was passed from a precision capacitor (1.6704 μF in our device). The platinum anodes were sharpened electrolytically as described by Wolbarsht *et al.* (1960) to reduce the breaking tension between the anode and $CuSO_4$ solution and thus to ensure a regular rate of capacitor discharge.

The coulometric respirometers were placed vertically within a 40 l insulated acrylic holding tank. Four respiration chambers (each within a separate thermobarometric chamber and each containing 2 ml of sea water) were used simultaneously. The concentration of animals in the respiration chambers ranged from 1 to 2000 ml^{-1} depending on the species and the experiment. One chamber in each experiment contained filtered sea water as a control. The temperature of the holding tank had to be kept to within ±0.01 °C (see Fig. 1C) as coulometric respirometers are highly sensitive to changes in partial pressure caused by changes in temperature (Heusner *et al.* 1982). Temperature control was achieved by placing a circulator with proportional heating control (model N-3, Haake) in the 40 l holding tank. Antagonistic cooling was provided by passing water from an external circulating waterbath (model RDL40, Precision Scientific Group) through a cooling coil (mounted on the Haake circulator). Water in the cooling coil was 5 °C lower than the experimental temperature set-point.

Calibration of coulometric respirometer

Contact of the anode with the $CuSO_4$ solution results in the release of one oxygen molecule for every four electrons delivered to the anode (Heusner, 1970). Heusner and colleagues (Heusner, 1970; Heusner *et al*. 1982; Heusner and Tracey, 1984) calibrated coulometric respirometers by using the ideal gas law. The calculated oxygen release (in these cases) was based on the assumption that all electrons delivered to the anode are used in oxygen production. During calibration, the pressure within the respirometer chamber was decreased and the number of electrons delivered to the anode (as capacitor discharges) recorded. The volume withdrawn from the respirometry chamber (the method used to decrease pressure by Heusner *et al*. 1982) was measured by monitoring the movement of a bead of mercury moved within a calibrated capillary tube using an optical comparator. In the present study, we also used the ideal gas law in our calibration procedure, but

describe a new method that uses a solid-state pressure sensor that permits rapid and sensitive determination of the small volume changes. In addition, we have also tested the assumption that all electrons delivered to the anode produce oxygen. The number of electrons being delivered to the anode per unit time was controlled by the coulometric device and recorded as the number of capacitor discharges. The number of capacitor discharges was converted into the number of electrons from the electrostatic expression: $q=C\Delta V$; where q is charge, C is capacitance and $\Delta V=V_0-V_t$ (where V_0 is the voltage before discharge and V_t is the voltage after discharge) and Faraday's Law (to convert charge to moles of electrons). In the present study, the theoretical yield (within 0.05 %) was 4.20 pmol O_2 per capacitor discharge (C=1.6704 μF, ΔV=0.970±0.0005 V).

The calibration device in the present study (Fig. 2A) consisted of a solid-state pressure sensor (NPH82.5D, NovaSensor, California) mounted between two sealed chambers. In this device, the upper chamber served as a thermobarometric reference, whereas the lower chamber enclosed a small air space (less than 500 μl) above a saturated solution of $CuSO_4$. Both the anode and cathode were in contact with this solution, with the anode passing through the solution within a glass jacket and just coming into electrical contact with the surface. The cathode was in contact with the solution at the base of the lower chamber. The signal from the pressure sensor was amplified by circuitry employing an instrumentation amplifier chip (Fig. 2B; LM363, National Semi-conductor Corporation, California) and the resulting voltage recorded using a 16-bit A-to-D convertor and data acquisition software (Datacan IV, Sable Systems, California). The calibration chamber and part of the electronic circuitry (Fig. 2B, dashed box) were placed within a water-tight container and immersed in the constant-temperature holding tank described above. A calibrated microsyringe (1.0 μl total volume with 1.0 nl intervals, Unimetrics, Illinois) was connected to the lower

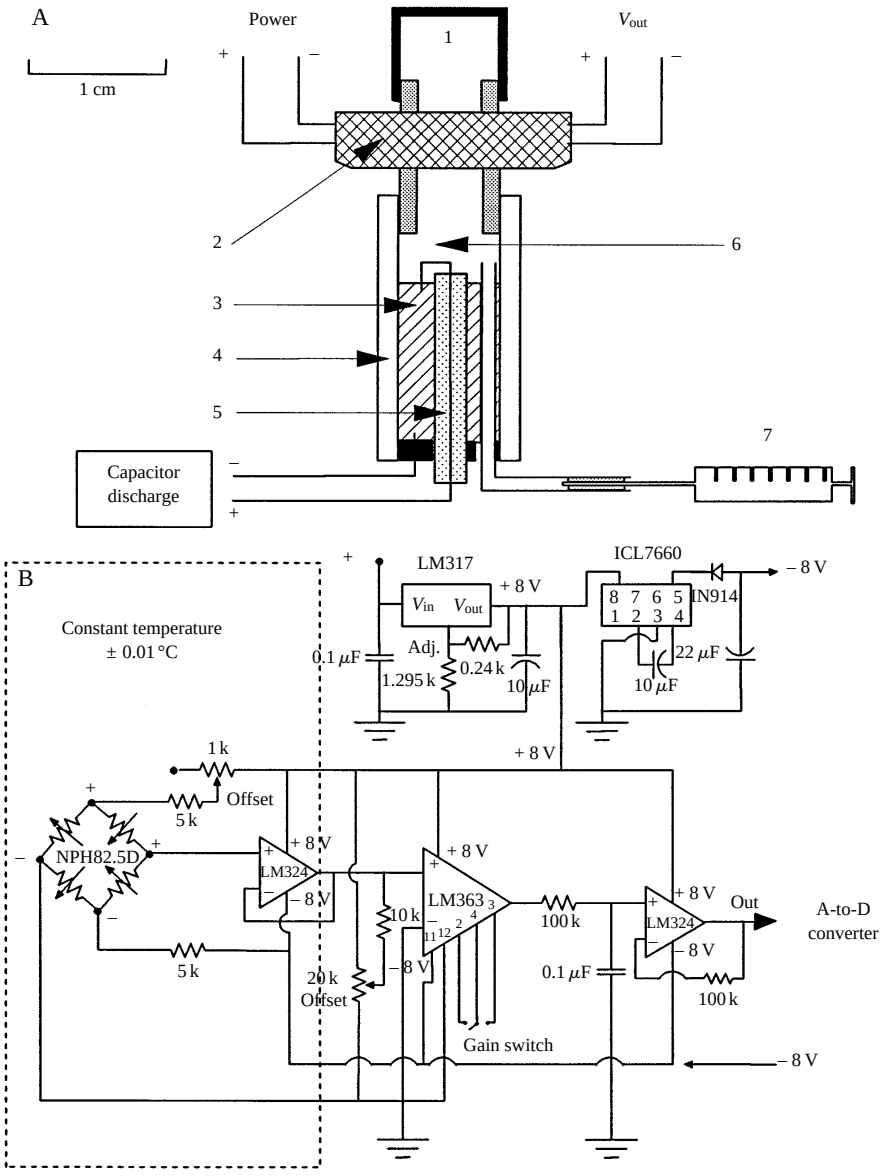

Fig. 2. (A) Design of the calibration device. A solid-state pressure sensor (2) separated the thermobarometric reference chamber (1) from the main oxygen-generating chamber (4). In the oxygen-generating chamber, the electrolytically sharpened platinum anode (+) sat in continuous contact with the surface of a saturated solution of $CuSO_4$ (3), after having been passed through the solution within an insulated glass coating (5). The platinum cathode (−) contacts the bottom of the $CuSO_4$ solution. A calibrated microsyringe (7) was connected to an air-space above the $CuSO_4$ solution (6) and was used to calibrate the pressure transducer output to volume. Electronics that control the discharge of the precise capacitance used in the coulometric respirometer (see Fig. 1) were connected to the anode (+) and cathode (−). (B) Circuit diagram showing the electronics used to power the pressure transducer and to amplify its signal. Part of the circuit, including the hardware shown in the dashed box, was housed in a thermobarometric chamber immersed in a waterbath kept to within ±0.01 ˚C of the set-point. The signal from this circuit was fed into a 16-bit A-to-D convertor controlled by a data acquisition software system.

chamber and used to change the volume, thereby allowing the conversion of voltage output from the pressure sensor to volume changes (Fig. 3A,B). Following calibration of the pressure sensor, current from the coulometric device (as number of capacitor discharges) was delivered to the anode and the resulting change in volume was recorded (Fig. 3C). From these data, the amount of oxygen liberated (assuming all electrons are used at the anode in the production of oxygen) was calculated using the molar volume of gas at the measurement temperature (Fig. 3D). The amount of oxygen (predicted by the pressure sensor technique assuming all the gas produced is oxygen) released by the anode per discharge was 4.19 pmol (see regression equation, Fig. 3D legend). This value was similar to the theoretical yield of 4.20 pmol O_2 per discharge (see calculations above of theoretical yield).

To test the assumption that all electrons delivered to the anode are used in the production of oxygen, the gas produced at the anode was collected in a stream of oxygen-free nitrogen. This was done using a respiration chamber (Fig. 1B) with the following modifications. The anode and the cathode were kept in electrical contact using a wick (Whatman filter paper) soaked in saturated $CuSO_4$. No CO_2 absorbent was necessary. Nitrogen gas was supplied at a constant rate (using a mass flow meter, model FMA 2DPV, Omega Engineering Inc., Connecticut, USA) at the top end of the manometric glass tube, and the gas stream drawn from the bottom end of the tube at a constant flow rate over the anode (Flow Controller, model R-2, Applied Electrochemistry Inc., California, USA). The oxygen produced by the anode (after a known number of capacitor discharges) was measured using an oxygen sensor and analyzer (model N-37M, Applied Electrochemistry Inc., California, and model S-3A, Ametek Thermox, Pennsylvania, USA). The output signal was recorded using the Datacan data acquisition system. Fig. 3E shows an actual tracing of the pulse of oxygen produced during 7139 capacitor discharges. Similar measurements were made over a range of discharge events from 0 to 60 000 capacitor discharges per pulse (Fig. 3F). The measured amount of oxygen produced per individual capacitor discharge was calculated from the slope of the regression (given in the legend to Fig. 3F) and found to be 4.18 pmol O_2 per discharge (cf. pressure sensor=4.19 pmol O_2 per discharge; theoretical yield=4.20 pmol O_2 per discharge). These data indicated that the original assumption underlying the use of coulometric respirometry in metabolic studies was true (i.e. all electrons used at the anode produce oxygen).

Comparison of coulometric respirometry with other methods

The rates at which oxygen was consumed by larvae of

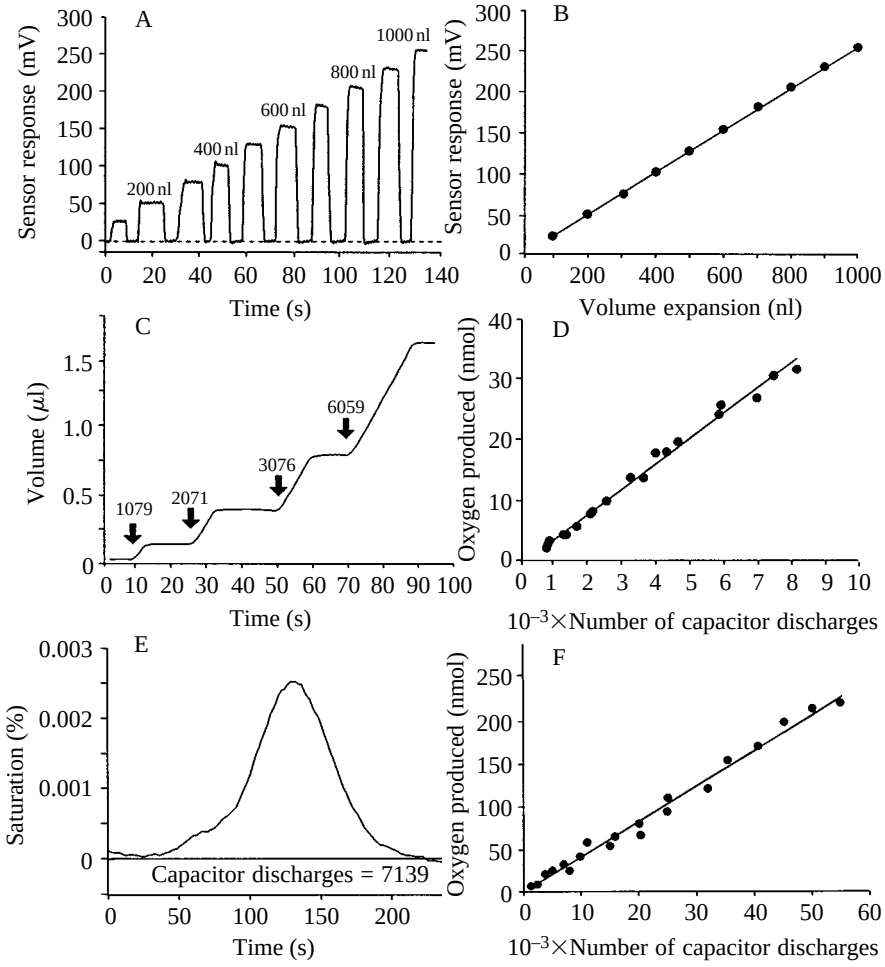

Fig. 3. (A) Voltage output from the pressure transducer during its calibration with volume changes. Each plateau is a separate volume expansion event, with events started at 100 nl and ending with 1000 nl of expansion. (B) Relationship between sensor response (mV) and expansion volume (nl). Linear regression for data shown: y=0.256x+0.887 (r^2=0.9999, N=10). (C) Trace showing the effect of supplying current (as capacitor discharges) to the oxygen generator circuit (see Fig. 2B). Each arrow indicates a separate burst of capacitor discharges, with the number of discharges indicated as a number above each arrow. (D) Relationship between predicted oxygen production (nmol; assuming 100 % efficiency) and the number of capacitor discharges (total current delivered). Linear regression: y=4.19x+0.097 (r^2=0.990, N=19). (E) Output of an oxygen analyzer (model S-3A, Ametek Thermox, Pennsylvania) during a pulse of oxygen resulting from 7139 capacitor discharges. (F) Relationship between oxygen produced (nmol) and the number of capacitor discharges (total current delivered). Linear regression: y=4.18x−1.388 (r^2=0.984, N=20). Values on the x-axis for D and F were divided by 1000 (the actual value is 1000 times greater). Note in F that the units are nmol O_2 per 1000 discharges; hence, the calculation in the text of pmol O_2 per discharge.

Artemia sp. (2-day-old nauplii), *Dendraster excentricus* (six-arm plutei) and *Asterina miniata* (bipinnaria) were measured independently using coulometric respirometry and two other methods. Larvae used with each of the three methods were at the same stage of development and taken from the same culture. Metabolic rates were measured using (i) coulometric respirometry, (ii) Winkler's titration of dissolved oxygen, and (iii) polarographic oxygen sensors (POS). The latter system consisted of three oxygen meters (model OM 780, Strathkelvin Instruments, Glasgow, UK), each connected to an oxygen sensor (model SI 1302) located either at the base of a 100 μl (model RC 200) or at the top of a 300–1000 μl (model RC 300) micro-respirometer chamber. The outputs from all three meters were recorded simultaneously using a data acquisition system (Datacan). Oxygen consumption rates were calculated from the rate of decline in oxygen concentrations over a 30 min period following a 30–60 min period that allowed the animals to acclimate to the respirometer chamber. Calibration of the POS to an oxygen-free solution was carried out at the beginning of each experiment in sea water flushed with nitrogen. POS were calibrated with saturated sea water, the oxygen content of which was measured using the Winkler's assay (Parsons *et al.* 1984). For all cases, the measured amount of oxygen was within 5% of the value calculated using corrections for temperature, salinity and atmospheric pressure. The concentration of larvae in the chambers ranged from 30 to 400 per 100 μl for most experiments, and from 214 to 809 per 1000 μl for measurements testing the effect of the concentration of larvae on metabolic rate (see Results). Background oxygen consumption rates (filtered sea water only) were interspersed between readings taken in the presence of animals and were less than 0.5% of a typical reading. Leakage of oxygen into the chambers when they were sealed was also negligible (tested by measuring the rate of change in oxygen-depleted sea water). The temperature of each chamber was controlled to within ±0.02 °C using an external circulation water bath (model RDL20, Precision Scientific Group). For the Winkler's method (Parsons *et al.* 1984), seven containers (300 ml, biological oxygen demand bottles) each containing larvae of a given species (per ml: *Artemia* sp., 21 larvae; *D. excentricus*, 20 larvae; *A. miniata*, 16 larvae) were incubated for up to 36 h. At intervals of 4–5 h (the first bottle at time zero), the oxygen content of an individual bottle was measured. The rate at which oxygen was used was calculated per larva per unit time by subtracting the oxygen content of each bottle from the oxygen concentration at time zero.

Results

Rates of oxygen consumption measured with the

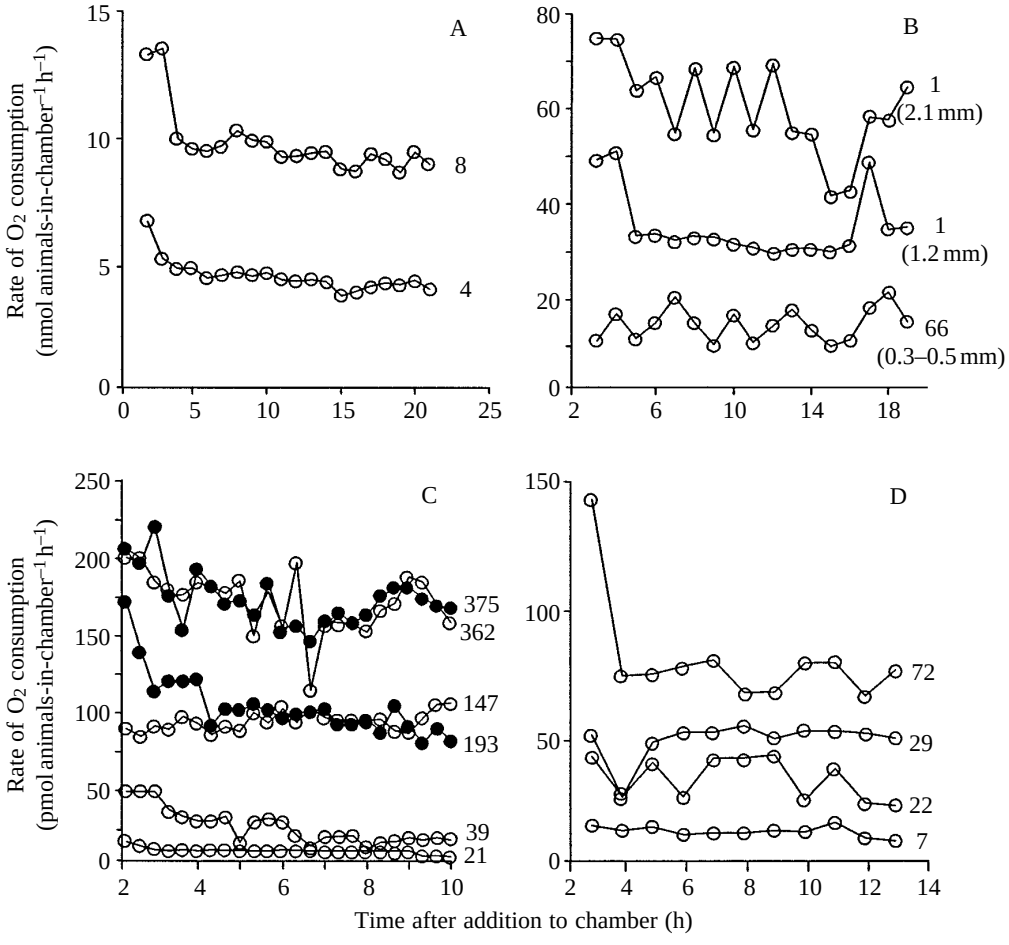

Fig. 4. Oxygen consumed over time within chambers of coulometric respirometers that contained different numbers of animals. (A) *Artemia* sp. nauplii (3 days old; at 20 °C). (B) *Acartia tonsa* adults (top two traces) and 66 nauplii (bottom trace); sizes (body lengths) shown in parentheses (at 15 °C). (C) Veligers of *Crassostrea gigas* (276.5 μm valve length; measurements made every 20 min; at 20 °C). (D) Competent plutei of *Dendraster excentricus* (at 20 °C). The number of individuals per chamber (2 ml) is indicated at the end of each trace.

coulometric respirometer were stable for at least 20 h, after an initial period of 3–4 h to allow for temperature equilibration (Fig. 4A). The sensitivity of the coulometric respirometer is illustrated by the measurement of the metabolic rate of a single copepod swimming freely in 2 ml of sea water (Fig. 4B). The response of the respirometer was proportional to the number of animals per respiration chamber (Fig. 4C,D). The conditions within the respiration chamber allowed adults (copepods) and larvae to survive for several days. Development within the chambers also proceeded normally for days, from newly fertilized eggs to the formation of feeding larvae (e.g. pluteus, Fig. 5A,B,C). Larvae in the respirometry chambers (inspected at the end of each experiment) reached developmental stages identical to those of siblings reared under normal culture conditions, indicating that conditions in the respiration chamber did not affect the rate of development. Development rates and metabolic rates were consistent among chambers when measurements were made simultaneously with three coulometric respirometers (Fig. 5B,C). Immediately after fertilization, metabolic rates increased steadily. At hatching, the rate of oxygen consumption began to increase very rapidly to a peak. Approximately 5 h after the beginning of hatching, metabolic rates decreased and returned to rates similar to those just prior to hatching (Fig. 5A,B,C).

The influence of the concentration of animals per coulometric chamber was investigated for larvae of *Crassostrea gigas* and *Dendraster excentricus*. In both cases, there was no significant change (least-squares linear regression, Fig. 6A,B) in the rate of oxygen consumption as a function of increasing concentrations of larvae (e.g. for *C. gigas* from 4 to 400 individuals ml^{-1}). Increasing the concentration of animals had no effect on the metabolic rate of *C. gigas* (241–809 larvae ml^{-1}) measured using the POS respirometer (Fig. 6C).

Metabolic rates measured with three different techniques were compared (Fig. 7A; given as a percentage of the Winkler titration value; see legend for absolute rates used in statistical analyses). The measured metabolic rate of bipinnaria larvae of *Asterina miniata* did not differ (analysis of variance, ANOVA) when assessed with the coulometric respirometer or the Winkler's method [variance ratio, VR=0.52, $F_{0.05(1,10)}$=4.96]. The metabolic rate of the same larvae measured using the POS respirometer was, however, significantly lower [VR=14.62, $F_{0.001(2,15)}$=11.3] than that measured using the two other methods. The same pattern of comparison held for pluteus larvae of *Dendraster excentricus*. Again, rates measured with the coulometric respirometer did not differ from those yielded by the Winkler's method [VR=0.51, $F_{0.05(1,7)}$=5.59], whereas rates measured with the POS respirometers differed from the other two [VR=14.62, $F_{0.001(2,15)}$=11.3]. There were no significant differences in the metabolic rates of *Artemia* sp. measured with the three techniques [VR=2.77, $F_{0.05(2,12)}$=3.89].

In the case of *C. gigas*, where metabolic rates were measured with coulometric and POS respirometers, rates measured with the latter technique were lower throughout the entire lifespan of the larvae (Fig. 7B). Both the mass exponents [VR=27.47, $F_{0.001(1,111)}$=12.0] and mass coefficients [VR=259.67, $F_{0.001(1,111)}$=12.0] were different for the metabolic rates measured with the two techniques.

The metabolic rate of *Artemia* sp. decreased rapidly as the concentration of oxygen in the 100 μl chambers decreased as the larvae consumed oxygen in the POS respirometer (Fig. 8A). In contrast, there was relatively little influence of

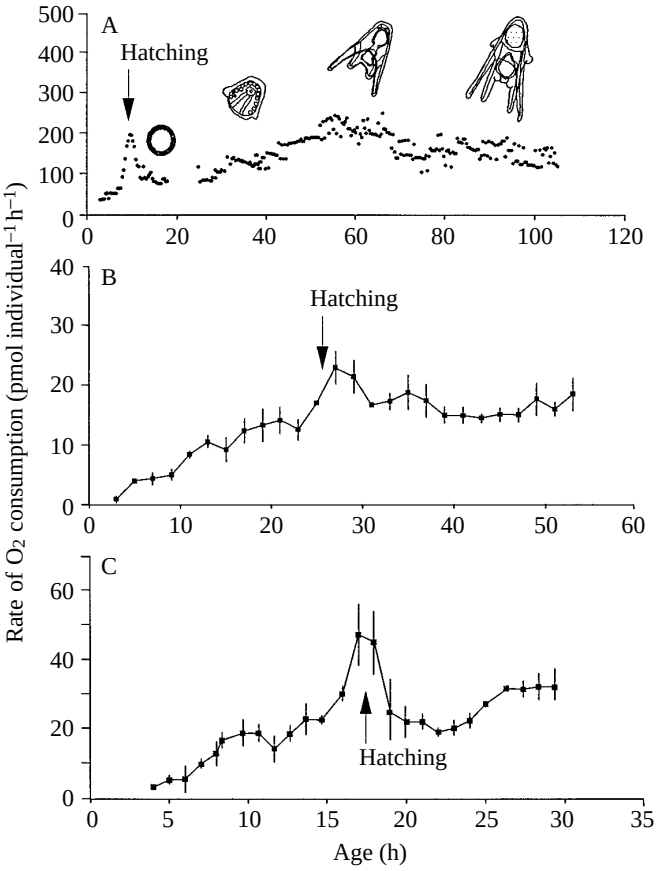

Fig. 5. The metabolic rates of embryos and larvae during early development. (A) Single respiration chamber containing 340 individuals of the sand dollar *Dendraster excentricus* (at 20 °C). Each data point represents the capacitor discharges accumulated over a measurement interval of 20 min. At the end of 20 min, the total number of capacitor discharges was recorded and the next measurement interval started. The sequence of measurements shown was interrupted at 18.5 h, the larvae were removed and a new set of larvae from the same culture added to the respiration chamber. Larvae were inspected at the end of each run and were found to have reached the same developmental stage as siblings raised under normal culture conditions. Drawings above the curve show the developmental stage of larvae from these sibling cultures. (B) *Strongylocentrotus purpuratus* (average concentration of embryos in the chamber was 564 individuals ml^{-1}; at 15 °C). (C) *S. franciscanus* (average concentration was 490 individuals ml^{-1}; at 15 °C). Capacitor discharges were recorded over 2 h. Three chambers were run simultaneously. Each point represents the mean and standard deviation for three chambers.

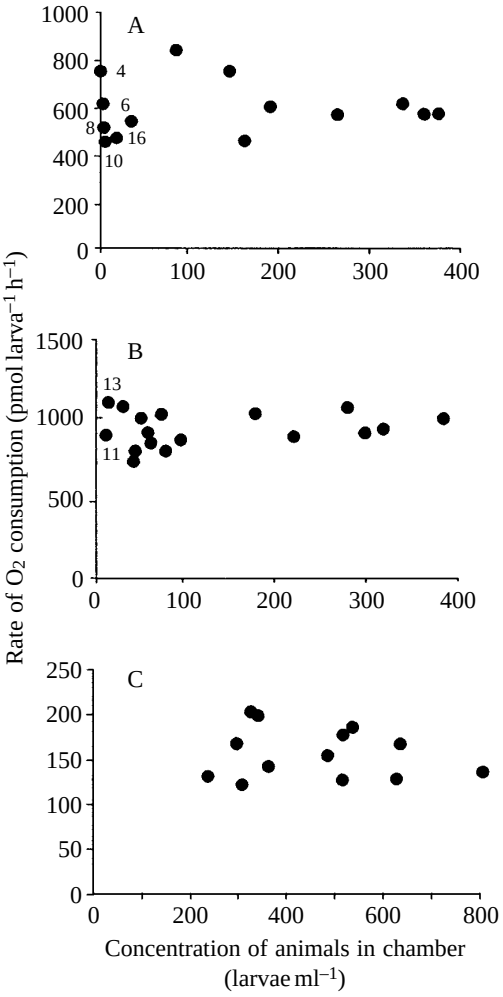

Fig. 6. The metabolic rate as a function of the concentration of larvae in the respirometry chambers (coulometric respirometer with 2 ml of sea water; polarographic oxygen sensor respirometer with 1 ml of sea water; all measurements at 20 °C). (A) Veligers of *Crassostrea gigas* (276.5 μm valve length; linear regression: $y = 0.450x + 509.781$, r^2=0.27, slope not significantly different from zero, P>0.05) in the coulometric respirometer. (B) Competent plutei of *Dendraster excentricus* (average ash-free dry organic mass=1004 ng larva^{-1}; linear regression: $y = 0.094x + 932.102$, r^2=0.01, slope not significantly different from zero, P>0.05) in the coulometric respirometer. (C) Veligers of *Crassostrea gigas* (271 μm valve length; slope not significantly different from zero, P>0.05) in the polarographic respirometer.

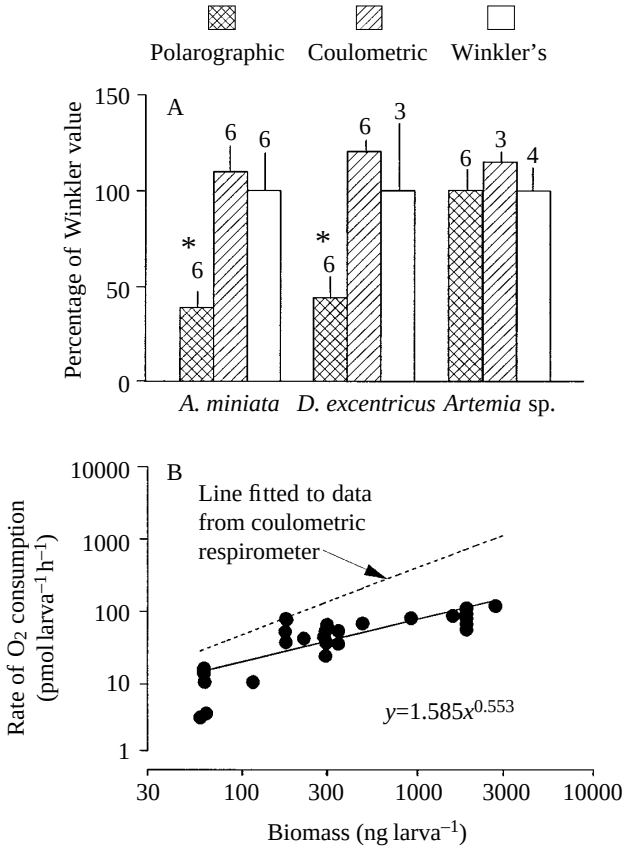

Fig. 7. (A) Comparison of metabolic rates measured using polarographic oxygen sensors (P), coulometric respirometry (C) or Winkler's assay (W) for determining oxygen concentrations in sea water. The single asterisks (for graphical simplicity) indicate values that are significantly different from the other two treatments (P≤0.001), numbers refer to the number of replicate measurements and error bars represent 1 S.E.M. The bipinnaria larvae of *Asterina miniata* (15 °C) each weighed 823.0±24.89 ng. Metabolic rates (as pmol O_2 larva^{-1} h^{-1} ± 1 S.E.M.) were 58.5±8.8 (P), 171.8±15.3 (C) and 150.3±20.2 (W). The six-arm plutei of *Dendraster excentricus* (15 °C) each weighed 780.1±12.16 ng and had metabolic rates (in pmol O_2 larva^{-1} h^{-1}) in the three systems that were 56.2±10.4 (P), 172.5±7.5 (C) and 149.4±36.2 (W). The nauplii of *Artemia* sp. (20 °C) were 3 days post-hatching and had metabolic rates (in pmol O_2 larva^{-1} h^{-1}) of 1079.5±116.6 (P), 1420.6±74.9 (C) and 1121.6±140.5 (W). (B) Metabolic rates of veligers of *Crassostrea gigas* (20 °C) as a function of ash-free dry organic mass, determined using polarographic oxygen sensors. Regression line for data from the polarographic oxygen sensor is shown as a solid line: log$y$ = 0.553log$x$ + 0.200 or $y = 1.585x^{0.553}$, r^2=0.57 (N=39). The dashed line shows the data for *C. gigas* measured using the coulometric respirometer (data from Fig. 9C). Veliger valve length was measured for each batch and converted to a measure of mass using the relationship given in the Materials and methods section determined using six different sized batches of veligers (range 81.5–314.6 μm; r^2=0.99, N=30).

decreasing oxygen concentration on the metabolic rate (down to 20 % of full saturation) of the larvae of *Dendraster excentricus* (Fig. 8B) and *Asterina miniata* (data not shown). It is also notable that metabolic rates of *Artemia* sp. were only similar in coulometric and POS respirometers at oxygen saturation (Fig. 8A; the open circle represents coulometric data from Fig. 7A). All measurements of metabolic rates with the coulometric respirometer were carried out at oxygen concentrations near saturation. The production of oxygen by the anode in the coulometric respirometer maintains the partial pressure of oxygen in the air space above the sea water in the respirometer chamber. Under these conditions, the coulometric respirometer is capable of maintaining normoxic conditions. In the data shown in Fig. 8C, a polarographic oxygen sensor was

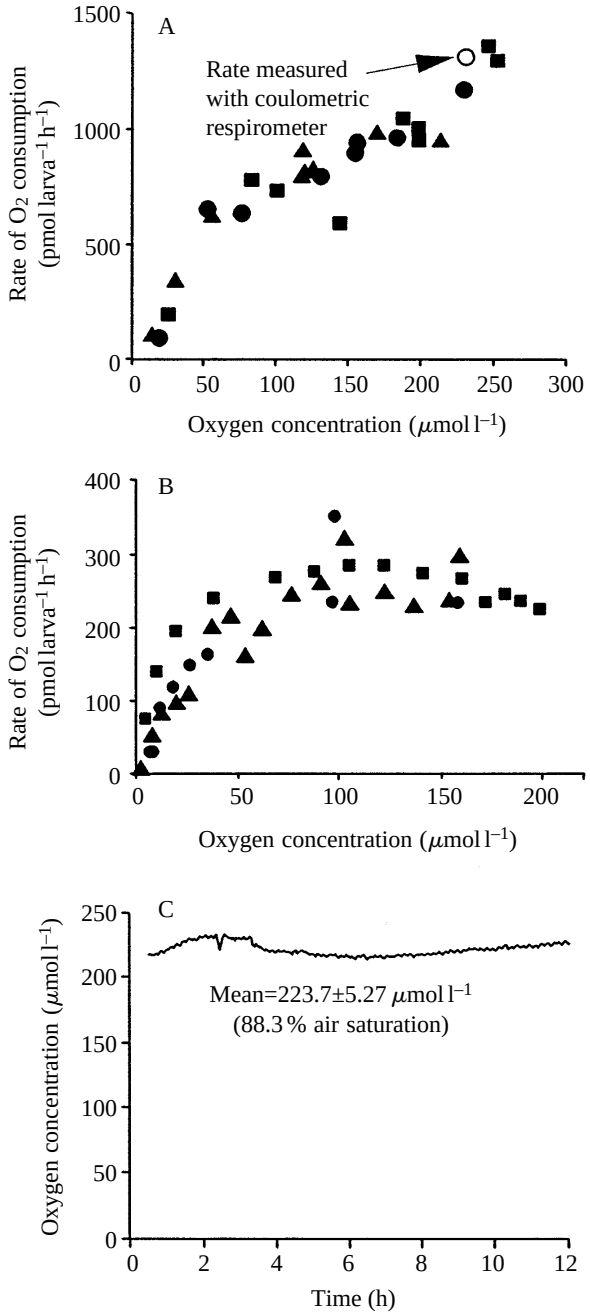

Fig. 8. Metabolic rate of larvae as a function of average oxygen concentration (at 20 ˚C), which declined as larvae respired. Different symbols represent different chambers. (A) 3-day-old *Artemia* sp. (20 ˚C) nauplii measured using polarographic oxygen sensors in 100 μl respirator chambers (N=3). The arrow indicates to the metabolic rate of *Artemia* sp. determined for the same batch of larvae using the coulometric respirometer. (B) Competent eight-arm plutei of *Dendraster excentricus* (20 ˚C, N=3). (C) Oxygen concentration of a coulometric respirometer chamber as a function of time. The chamber contained 862 veligers of *Crassostrea gigas* (20 ˚C) in 2 ml of sea water.

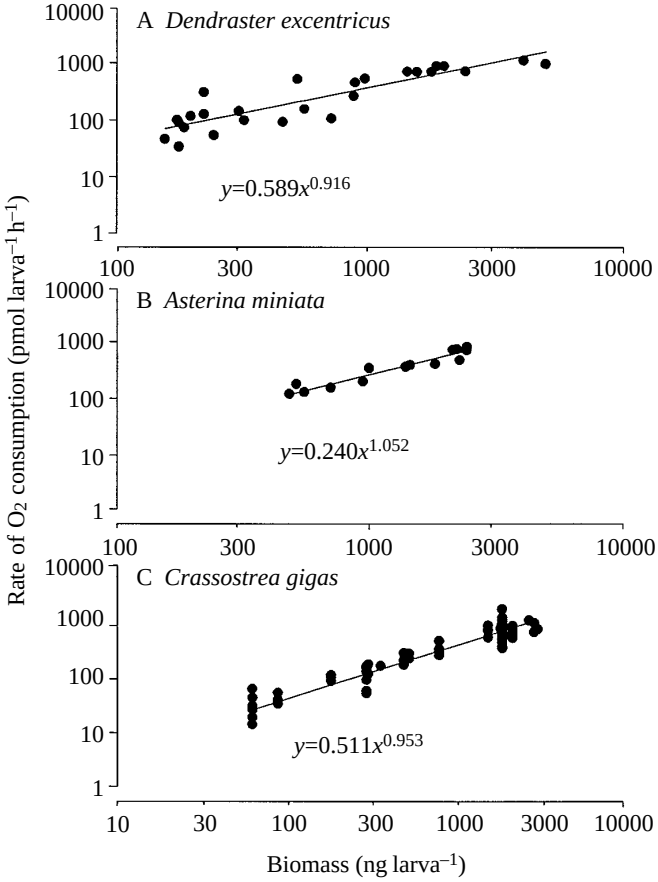

Fig. 9. Metabolic rate as a function of ash-free dry organic mass for growing and differentiating larvae. (A) *Dendraster excentricus* (15 ˚C, regression line: log$y$ = 0.916log$x$ − 0.230 or y = 0.589$x^{0.916}$, r^2=0.79, N=26); (B) *Asterina miniata* (15 ˚C, regression line: log$y$ = 1.052log$x$ − 0.619 or y = 0.240$x^{1.052}$, r^2=0.91, N=14), (C) *Crassostrea gigas* (20 ˚C, regression line: log$y$ = 0.953log$x$ − 0.292 or y = 0.511$x^{0.953}$, r^2=0.90, N=74).

inserted into the bottom of a modified respiration chamber which contained 862 larvae in 2 ml of sea water (*C. gigas*). Throughout a 12 h experiment, the oxygen concentration of the sea water in the respiration chamber was maintained at 88.3 % of air saturation despite the large oxygen demand imposed by the high concentration of larvae.

The relationship between the increase in biomass (ash-free dry organic mass) during growth and the corresponding increase in metabolic rate (measured with coulometric respirometry) is given in Fig. 9 for larvae of three species. The mass exponents of the scaling equations describing this relationship were high and close to unity (0.9–1.1). Therefore, metabolic rate increases linearly (slope=1) with increasing biomass for growing larvae of *Dendraster excentricus* (Fig. 9A), *Asterina miniata* (Fig. 9B) and *Crassostrea gigas* (Fig. 9C).

Discussion

Accurate measurement of the metabolic rate of embryos and larvae is fundamental to studies of the energy requirements of

developing marine invertebrates. In this study, coulometric respirometry was used to measure the metabolic rate of embryos and larvae that were growing and differentiating. Coulometric respirometry allows measurements of metabolic rates over long periods (from fertilized egg to feeding larva) and has no effect on the course and timing of development. Assessing the effect of the measurement system on embryos and larvae is an important but often neglected feature of most other techniques used to measure respiration during development. Fertilized eggs placed in the respirometer developed to the larval stage at rates that were similar to those of sibling larvae reared in culture, showing that measurements made with this technique are on normal and actively developing animals.

The need to use high concentrations of larvae to measure metabolic rates has long been a 'sore point' (quote from Sprung, 1984; see also Walne, 1966; Millar and Scott, 1967; Crisp, 1976; McDonald, 1988). Reducing the concentration of larvae in these other systems is, however, a problem. Crisp (1976), for example, pointed out the unreliable nature of respiration data obtained for large dilute cultures of larvae. In his re-evaluation of the data of Millar and Scott (1967), Crisp showed that the larvae were not the largest sink for oxygen at low concentrations of larvae and, hence, that the higher oxygen consumption per larva in the most dilute cultures was an artefact. McDonald (1988) included controls to correct for oxygen consumption not attributable to larvae and yet still reported a 35 % reduction in oxygen consumption of bivalve larvae as the concentration of individuals increased. His data, however, show that the relationship between decreasing larval concentrations and increasing metabolic rates is a power function with an asymptote approaching infinity (at zero larvae). The conclusion from his data is that the respiration of a single larva in dilute culture is many times greater than that of larvae at high concentrations.

We investigated the effect of increasing the concentration of larvae on metabolic rates of echinoid and bivalve larvae (*Dendraster excentricus* and *Crassostrea gigas*) using both the coulometric and POS respirometers (the latter for *C. gigas* only). After correcting for the very low background rates of oxygen consumption in the control chamber, metabolic rates (in the coulometric respirometer) remained constant for both species over a 100-fold increase in larval concentration (Fig. 6A,B). A similar conclusion was obtained for metabolic rates measured using the POS respirometer and concentrations of bivalve larvae ranging from 200 to 800 larvae ml^{-1} (Fig. 6C). The conclusions of our study differ (for bivalve larvae) from those of McDonald (1988) and Millar and Scott (1967), but are similar (for *Artemia* sp.) to those of Varo *et al.* (1993*a*), and suggest that the effect of larval concentration may depend on conditions associated with the technique used.

The data obtained for the POS respirometer (Fig. 6C) suggest that the lower estimates of metabolic rate obtained using this system were not due to reduced oxygen tension in the respirometer chambers. These data were collected using a 1 ml respiration chamber for the POS respirometer. The metabolic rates of larval *C. gigas* at the minimum and maximum concentrations did not differ. For example, at 241 larvae ml^{-1}, the metabolic rate was 131.4 pmol $larva^{-1}$ h^{-1}, similar to the value of 137.3 pmol $larva^{-1}$ h^{-1} measured for a concentration of 809 larvae ml^{-1}. These data for *C. gigas* also allow calculation of the time required for different concentrations of larvae to deplete fully the oxygen in the POS chambers. Given a starting oxygen concentration of 235 μmol l^{-1}, and bivalve larvae with metabolic rates calculated from the average of the two rates above, it would take between 2.1 and 7.2 h for larvae present at the highest and the lowest concentrations used in this study to use completely the oxygen in a 1 ml chamber. In all these measurements, the larvae were in a sealed 1 ml chamber for 30–90 min (the time required for making a typical measurement). Therefore, at most (highest concentration of larvae, 809 ml^{-1}), approximately 70 % of the oxygen was depleted during the course of a 90 min measurement. That this extent of depletion had no effect on the metabolic rate of the larvae is evident in the comparison of these rates with rates measured at the lowest concentrations of larvae, where only 21 % of O_2 had been depleted. As has been previously reported (e.g. Widdows *et al.* 1989), hypoxia does not affect the metabolic rates of bivalve larvae until low oxygen partial pressures are reached. This was also the case for the echinoderm larvae we studied (e.g. Fig. 8B). Larvae of *Artemia* sp. have been reported to be respiratory regulators (Varo *et al.* 1993*b*; De Wachter *et al.* 1994, and references therein), although embryos do down-regulate metabolism under anoxia (Hand, 1990; Hontoria *et al.* 1993). Varo *et al.* (1993*b*) reported that the ability of *Artemia* larvae to regulate respiration was reduced with changes in salinity. In our experiments in full-salinity sea water, the larvae of *Artemia* sp. were not respiratory regulators (Fig. 8A). In sealed POS respiration chambers, the metabolic rate of *Artemia* decreased with the decrease in oxygen concentration in the chamber caused by the animals' respiration. We used a different strain of *Artemia* sp. to those used in the study by Varo *et al.* (1993*b*). Perhaps the different abilities of *Artemia* larvae to regulate respiration in declining oxygen concentrations is strain-dependent.

The metabolic rate of larvae increased with mass during growth, as has been described for a number of species (Zeuthen, 1953). Mass exponents for echinoderm (*Dendraster excentricus*, *Asterina miniata*) and mollusc (*Crassostrea gigas*) larvae were close to 1.0 and agree with earlier studies (reviewed by Zeuthen, 1953), which concluded that the metabolic rates of embryos and larvae of marine animals scale with a mass exponent 0.95. However, when metabolic rates of *C. gigas* were measured with polarographic oxygen sensors (POS) in small (100 μl) chambers, the mass exponent was only 0.55 (Fig. 7B) and statistically different from the mass exponent of 0.953 obtained with the coulometric respirometer (Fig. 9C). These data demonstrate that metabolic rates measured with the POS respirometer were proportionally lower for older stages of *C. gigas* and support the suggestion that conditions associated with the confinement of larvae

within the POS chambers (which are presumably exacerbated for larger larvae) may explain the deviation of POS measurements from those using the coulometric technique. These comparative data highlight the importance of the measurement technique in establishing the mass exponent and may help to explain the diversity of mass exponents reported in the literature for larvae and early juvenile stages of marine animals (bivalve, 1.07–1.18, Mann and Gallager, 1985; bivalve, 0.59–0.90, Sprung, 1984; copepods, 0.62–1.14, reviewed by Conover, 1968; fish, 0.65–1.69, reviewed by Giguere *et al*. 1988).

In his review of the metabolic rates of marine invertebrate larvae, Crisp (1976) concluded that the majority of larvae have metabolic rates in the range 3–10 $ml\,O_2\,h^{-1}\,g^{-1}$ dry mass. Crisp also obtained a similar range for metabolic rates calculated indirectly from the rate of loss of energy reserves in starving larvae. Assuming that 50 % of total dry mass is ash, Crisp's range of values (corrected to the units used in our study) is 268–893 $pmol\,O_2\,h^{-1}\,\mu g^{-1}$ dry organic mass. Most of the studies since Crisp's review have supported this range. For example, Widdows *et al*. (1989) report that the metabolic rate of bivalve larvae (*Crassostrea virginica*) is 300 $pmol\,O_2\,h^{-1}\,\mu g^{-1}$ dry organic mass using direct and indirect calorimetry. Erickson (1984), using a gradient diver, reported metabolic rates of 701 $pmol\,O_2\,h^{-1}\,\mu g^{-1}$ dry organic mass for gastropod larvae. However, there are data that fall outside the lower boundary of this range; e.g. 57 and 196 $pmol\,O_2\,h^{-1}\,\mu g^{-1}$ dry organic mass (Wang and Widdows, 1991; Mann and Gallager, 1985, respectively). Our values, despite differences in temperature and species, are all consistent with the range reported by Crisp (1976). In our study, calculations using data obtained with coulometric respirometry predict that a 1 μg larva of *Dendraster excentricus*, *Asterina miniata* (both at 15 °C) or *Crassostrea gigas* (at 20 °C) would consume 330, 344 or 369 $pmol\,O_2\,\mu g^{-1}\,h^{-1}$, respectively (values calculated from scaling equations given in Fig. 9A,B,C). As with other organisms (Heusner, 1991), the metabolic explanations for the values of the mass coefficients (metabolic intensities) are unknown.

It is important to note that the values used by Crisp for his comparisons of metabolic rates were obtained using techniques other than polarographic oxygen sensors. If the rate of oxygen consumption in our study is calculated for a 1 μg larva of *C. gigas* (at 20 °C), using data from the POS respirometer (Fig. 7B), the metabolic rate would be 72 $pmol\,O_2\,h^{-1}$, or 20 % of the coulometric value. Rates measured using the POS respirometer were 34 % of the coulometric rate for the two species of echinoderm studied (*A. miniata*, *D. excentricus*). Varo *et al*. (1993*a*) also found that measurements made with the same POS system as used in our study were lower (by 10 %) than measurements made with other techniques, in their case a Gilson differential respirometer.

What might be the basis of the lower estimates of larval respiration obtained with the POS respirometer? Errors in our use of the POS are unlikely. We followed all of the manufacturer's specifications, and the standard procedures used for calibration of POS, as in previous studies of larval respiration in this laboratory (e.g. Jaeckle and Manahan, 1989, 1992; Shilling and Manahan, 1990) and others (e.g. Varo *et al*. 1993*a*). We found no significant difference in our comparison of methods used to measure respiration on the same group of *Artemia* larvae (Fig. 7A), which suggests that POS measurements can be accurate for certain animals. We have eliminated the possibilities that declining oxygen or larval concentrations might be causes of the low measurements by POS of larval respiration. We have also tested the possibility that the POS respiration chamber itself (e.g. O-ring, presence of KCl reservoir) might inhibit development and found that it did not, although embryonic development was arrested when current was applied to the POS (data not shown). We can offer no explanation as to why the POS gives low rates of respiration for all species and stages tested other than *Artemia*. Further studies that use POS for measurements of metabolic rate in larvae of benthic marine invertebrates should proceed with caution on a case-by-case basis, as no single calibration factor can be used to correct between the different methods (e.g. POS and coulometric respirometers: Fig. 7B). We conclude that coulometric respirometry offers greater accuracy and versatility for measuring the metabolic rate of developing larval forms of benthic marine invertebrates.

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