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Physiological bases of genetically determined variation in growth of marine invertebrate larvae: A study of growth heterosis in the bivalve *Crassostrea gigas*

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Abstract

Many species of marine animals have larval stages whose rates of growth in the plankton are regulated by complex combinations of biological and environmental factors. In this study, we focus on the physiological bases that underlie endogenous variation in growth potential of larvae. Our approach was based on experimental crosses of gravid adults from pedigreed families of the Pacific oyster, *Crassostrea gigas*. This produced large numbers of larvae with different growth rates when reared under similar environmental conditions of food and temperature. A total of 35 larval families were reared to test hypotheses regarding the physiological bases of growth variation. Growth rate of these larval families varied over a five-fold range, from 3.4 (±0.5, S.E.) to 17.6 (±0.6) μm day^{-1}. The suite of integrated measurements applied to study growth variation included size, biochemical compositions, rates of particulate and dissolved nutrient acquisition, absorption efficiencies, respiration rates and enzyme activities. We show that a complex set of physiological processes regulated differences in genetically determined growth rates of larvae. One-half of the energy required for faster growth came from an enhanced, size-specific feeding ability. Differences in absorption rates were not significant for slow- and fast-growing larvae, nor were differences in size-specific respiration rates. Metabolic processes accounted for the additional 50% of the energy "savings" required to explain enhanced growth rates. We propose that different protein depositional efficiencies could account for this energy saving. Quantitative analyses of the endogenous physiological factors that cause variation in growth rate will allow for a more sophisticated understanding of growth, survival and recruitment potential of larvae.

Keywords: Experimental genetic crosses; Feeding; Growth; Hybrid vigor; Larvae; Metabolism; Pacific oyster *Crassostrea gigas*; Physiology; Protein depositional efficiency

1. Introduction

The majority of marine animals have a complex life history involving a larval form (Hjort, 1914; Thorson, 1950; May, 1973). A major issue in larval biology has been to understand the factors that cause variability in growth, which can affect recruitment success and

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population connectivity. For the larval forms of many species, highly variable rates of growth are routinely observed even for sibling larvae reared under identical, controlled laboratory conditions. The possible adaptive value and mechanisms of such variation have been widely discussed (Lucas, 1982; Huntley and Boyd, 1984; Crisp et al., 1985; Paulay et al., 1985; Olson and Olson, 1989; Fenaux et al., 1994; Anger, 1995; Bochenek et al., 2001; Beaumont et al., 2004; Marsh and Fielman, 2005). The rates of development and growth of such larval forms are highly complex processes that are regulated by both endogenous biological and exogenous environmental factors (i.e., "Nature and Nurture"). Considerable progress has been made in understanding how exogenous environmental factors–such as food, temperature and salinity–regulate larval growth rates (e.g., Mann and Gallager, 1985; Pechenik, 1987; Starr et al., 1990; Marsh et al., 1999; Torres et al., 2002; Anger, 2003; Moran and Manahan, 2004). For species with feeding larval forms, food availability has been studied extensively because of its obvious significance for regulating growth rates of larvae. Most larval forms are very likely to be faced with food limitations during their life span, at least transiently (Cushing, 1990; Starr et al., 1990; Platt et al., 2003 and preceding references). In addition to considerations of food quantity, many studies have focused on the effects of different food types on larval growth. For instance, it has long been known that larvae grow faster when fed combinations of different species of unicellular algae (e.g., bivalve larvae: Davis and Guillard, 1958; Loosanoff and Davis, 1963). Some species of larvae can acquire nutrients through ingestion of bacteria (Douillet and Langdon, 1993) or by direct absorption from seawater of dissolved organic material (Manahan, 1990).

It has been more difficult, however, to understand the endogenous biological mechanisms that underlie variability in larval growth rates. Scant progress has been made on these endogenous components, in large part owing to a lack of genetically based experimental 'model' organisms in marine larval biology (cf. fruit flies, nematode worms and other model organisms with advanced genetics). Yet research on such model organisms tells us that individuals of the same species differ in physiological potential as a result of genetic variability. Thus, in order to make progress in understanding the variability of growth rates in marine invertebrate larvae, it is necessary to use an organism in which growth rate can be genetically manipulated so that study of physiological mechanisms is enabled.

In this study, we focus on the endogenous mechanisms causing variation in larval growth rate. Our approach is based on crossing adults from inbred lines of the Pacific oyster, *Crassostrea gigas* (Hedgecock et al., 1995, 1996; McGoldrick and Hedgecock, 1997) to produce offspring (larvae) with different rates of growth when cultured under identical environmental conditions of food and temperature. By making a diverse series of physiological measurements on these families of larvae, we partition genetically determined differences in growth rates into the components of a modified energy balance equation (Winberg, 1956): Growth = Consumption − Respiration − Excretion.

2. Methods

2.1. General experimental design

Sets of factorial crosses using inbred lines produced over 25 million larvae in 35 different larval families. Different parental lines were used, some in multiple crosses, affording cross-generational replication of some crosses. For example, the adults from line 5 used in one cross (Fig. 1C) were reared from larvae displayed in the 5 × 5 cross (Fig. 1A). Also, gravid adults from line 3 used in Fig. 1D were grown from the same 3 × 3 larval family shown in Fig. 1A. The pedigrees of all parents were confirmed with unlinked microsatellite DNA markers (Hubert and Hedgecock, 2004).

2.2. Larval culturing conditions

Gametes from single male and female parents were used to produce numerous sibling larvae in experimental crosses. For the cross shown in Fig. 1A parents from inbred lines named "2, 3 and 5" were used to produce three families of inbred larvae (sire × dam: 2 × 2, 3 × 3 and 5 × 5) and six families of hybrid larvae (sire × dam: 2 × 3, 2 × 5, 3 × 2, 3 × 5, 5 × 2 and 5 × 3). Fertilized eggs were placed at a concentration of 5 ml^{-1} in two or three replicate 100-l culturing vessels, depending on the fecundity. All cultures were kept in a heated room at 23 °C (±2). Upon developing to the first feeding stage within 24 to 48 h, the "D-hinge" veliger larvae were removed from the cultures by concentrating on to mesh sieves, rinsed thoroughly with filtered seawater and resuspended in 100 l of preheated (titanium heat-exchanger), 1-μm (pore size) filtered seawater. Cultures were fed *Isochrysis galbana* Parke. This culturing protocol of water change and feeding was continued every other day for the duration of the experiment (e.g., 18 days for Fig. 1B). Cultures were fed at an algal concentration of 50 cells μl^{-1} that was increased, as larvae grew, to a concentration of 100 cells μl^{-1} following established hatchery manual protocols for rearing larvae of the genus *Crassostrea* (Davis and Guillard, 1958;

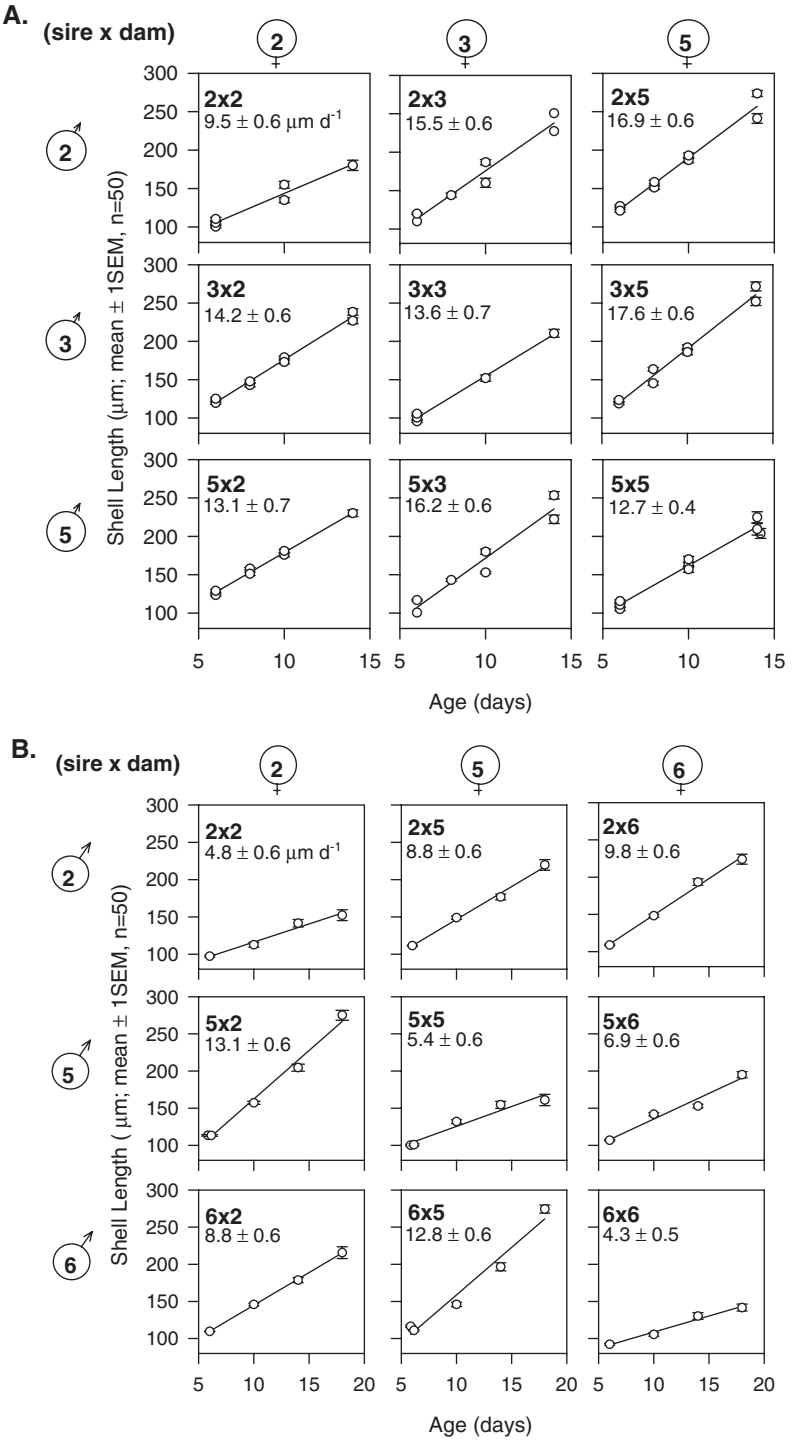

Fig. 1. Growth rates of larvae of *Crassostrea gigas*. Each nine-panel part of the figure shows the growth rate of larvae from different factorial genetic crosses of parental inbred lines (e.g., a larval family from an experimental cross of family 1 × 1, with male parent listed first—"sire × dam"). Each data point is the mean of 50 shell length measurements of different larvae. Error bars are ±1 S.E.M. Where errors bars are not shown, the error fell within the graphical representation of the data point. Linear regression analyses were used to calculate the growth rate (shown as µm day^{-1} with error) of each larval family. Each of the four panels (A–D) represents a separate set of crosses where larval families within each specific set of crosses were reared under identical environmental conditions of food and temperature (e.g., panels C and D represent all larvae cultured at 30 cells µl^{-1} of the alga *Isocyrysis galbana* that were used for direct comparison of feeding and growth).

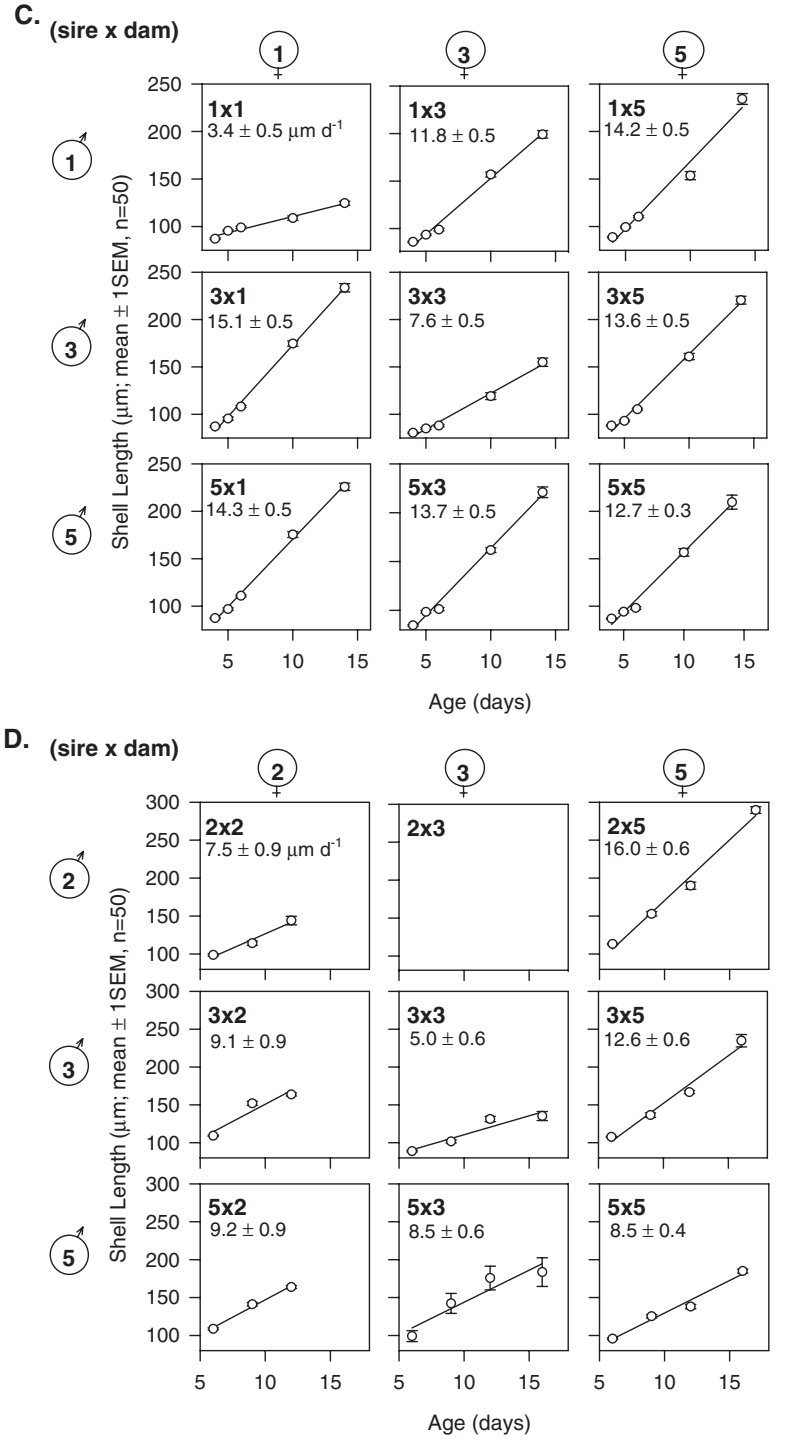

Fig. 1 (*continued*).

Breese and Malouf, 1975). For experiments in which growth and feeding were directly compared, the amount of algae was kept at the same concentration used for all feeding rate measurements (30 cells μl^{-1}) for the entire time of larval culturing. The differing algal concentrations used in different experiments were monitored daily with a Coulter Counter and restocked as needed to maintain the required concentration of algae for the different cultures.

2.3. Growth: size (shell length), with indices of mass (protein) and volume (organic osmolytes)

(a) Shell length: The average rate of larval growth in each culture vessel was determined by measuring shell length for 50 randomly selected larvae at the time intervals specified in the appropriate graphs (see Results). Length from anterior to posterior edge of each larval shell was measured under a microscope, using a calibrated ocular micrometer. Rates of shell growth (μm ± S.E. day^{-1}) were determined from the slope of shell length versus age ($y = mx + b$). Analysis of growth differences was performed statistically using a general linear model (not a regression equation), shell length = family + day + day * family. Homogeneity of growth rates was tested using the SAS statistical software package (SAS Institute Inc., Cary, NC). Standard errors of family growth rates were generated using the general linear model. If growth rates were not different (i.e., day * family *t*-test, $P > 0.05$), then a separate comparison of size-at-age was conducted to test differences in *y*-intercepts.

(b) Protein content: After establishing the differential growth rates of different larval families with shell length as a measure, the "condition index" of the same larval families was measured using total protein content. Known numbers of larvae (250–500, depending on larval size) were removed from the culture containers, rinsed and frozen in 1.7-ml test tubes for later analysis of total protein content. Homogenized samples of larvae were assayed for protein content using a modified (Jaeckle and Manahan, 1989) Bradford assay (Bradford, 1976).

(c) Organic osmolyte content: Taurine is the major constituent of the free amino acid pool of larvae of *C. gigas* (Welborn and Manahan, 1995). As larvae grow and increase in size and volume, there is a corresponding increase in taurine content (Welborn and Manahan, op. cit.). In the current study, taurine was measured in slow- and fast-growing larvae as an index of larval organic volume. This series of measurements was undertaken to measure the condition index of larvae to test for possible differences in organic volume in different larval families. Taurine content in larvae was measured using reverse-phase high-performance liquid chromatography (HPLC) (general HPLC method in Manahan et al., 1983; specifics of taurine analysis in Welborn and Manahan, 1995).

2.4. Consumption: rates of energy acquisition (particulate and dissolved nutrients)

(a) Particulate nutrients: The clearance rates from seawater of the alga *I. galbana* by veligers of *C. gigas* were measured for slow- and fast-growing larvae. Feeding assays were conducted at 23 °C and with 30 algal cells μl^{-1}. Larvae were removed from the culture vessels, rinsed with filtered seawater and resuspended at 15 larvae ml^{-1} in a series of replicate 25-ml experimental chambers (e.g., see Fig. 3B for numbers of replicates used). After an equilibration period of 30 min, 30 cells μl^{-1} were added and the feeding experiments commenced. Prior to being added to the feeding experiment, algae were centrifuged to remove excess algal nutrient medium (F/2) and resuspended in 0.2-μm (pore size) filtered seawater. Under similar conditions, additional replicate 25-ml experimental chambers were set up as controls, with algae but with no larvae present (controls for possible algal growth). A further series of replicate controls, with larvae present but no algae added, was used to correct for any possible particulate contamination that might have been introduced with the larvae themselves. Both experimental and control feeding chambers were wrapped in aluminum foil to eliminate light and prevent algal growth during the feeding experiments. During a typical 3–4 h feeding experiment, larvae were seen to be actively swimming and each feeding chamber was inverted gently by hand every 10–15 min to ensure mixing of algae and larvae. Clearance rate determinations were based on the change in the concentration of algae in each feeding chamber during the 3–4-h time course of larval feeding. Algal concentration was measured with a Coulter Counter (model ZM) using a 100-μm aperture. After the Coulter Counter's settings were calibrated according to the manufacture's specifications, counting accuracy of *I. galbana* was further checked by comparing Coulter Counter values to direct counts of cells by microscopy on a haemocytometer. Feeding rates were determined using the standard clearance rate equation:

$$\mathrm{CR} = \frac{\ln C_{\mathrm{initial}} - \ln C_{\mathrm{final}}}{N * T} * V$$

Where CR is clearance rate in units of $\mu l\ larva^{-1}\ h^{-1}$, C_{initial} is the algal concentration at the start of

the experiment, C_{final} is the concentration at the end of the experiment, N is the number of larvae in the feeding chamber, T is the duration of the feeding assay, and V is the total volume of the feeding chamber.

(b) Dissolved nutrients: Previously, a detailed kinetic analysis of the transport of dissolved amino acids from seawater showed that maximum transport capacities (J_{max}) for larvae of *C. gigas* are attained at substrate concentrations of 200 μM (Manahan, 1989; Manahan et al., 1989). In the present study, the maximum transport rates (J_{max}) by larvae of dissolved glycine from seawater were measured using ^{14}C-labeled glycine (NEN, 3.95 MBq μmol^{-1}). Transport assays were conducted at 23 °C in 7 ml of 0.2-μm (pore size) filtered seawater at a larval concentration of 300 ml^{-1}. A combination of ^{14}C-glycine and non-radioactive glycine was used and adjusted to a final concentration of 200 μM (185 kBq total radioactivity in 7 ml). To measure rates of glycine transport by larvae, a 1-ml aliquot containing 300 larvae was removed every 4 min during time series measurements over 24 min (i.e., N=6 samples). Larvae in each sample were filtered onto an 8-μm (pore size) membrane (Nucleopore) and rinsed three times with 15 ml of seawater to remove excess radioactivity. The exact number of larvae taken in each 1-ml sample and placed on the membrane filter was enumerated under a microscope (general protocol in Manahan, 1983). The amount of radioactivity (Bq) in each sample was determined using a quench-corrected liquid scintillation counter. After determining the radioactivity per larva, the moles of substrate transported were calculated by correcting for the specific activity of glycine in the seawater. Knowing the number of larvae per sample and the moles of glycine in that sample, the rate of glycine transport was calculated (pmol glycine larva^{-1} h^{-1}).

2.5. Respiration: metabolic rates (oxygen consumption), aerobic capacity (citrate synthase) and ion pump activity (Na^+,K^+-ATPase)

(a) Metabolic rates: Changes in rates of oxygen consumption as a function of size were measured for slow- and fast-growing larvae, from newly formed D-hinge veligers (80 μm) to larvae about to undergo metamorphosis at ~350 μm. Oxygen consumption, calculated as a rate per individual larva, was measured as described in Marsh and Manahan (1999). To summarize the method briefly, larvae of known size were placed in custom-made small, biological oxygen demand vials (500–700 μl) with oxygen-saturated filtered (0.2-μm pore size) seawater. Each series of measurements of larval respiration rate consisted of 7–10 replicates that encompassed a range of larval concentrations, purposely made different by design, to test for possible concentration-dependent effects on measured rates of respiration calculated for an individual larva of *C. gigas* (see Fig. 1B in Moran and Manahan, 2004). Controls for possible changes in oxygen content in the microrespiration vials not due to larvae were performed by having vials containing filtered seawater only. Each respiration chamber of known volume, containing a known number of larvae, was incubated for 2–3 h at 23 °C (exact incubation time used varied as a function of larval size and, hence, the rate of respiration). At the end of the defined incubation time, each respiration chamber was opened and a sample (500 μl) was removed with a gas-tight syringe and injected onto a Clark-type electrode to measure oxygen tension. The Clark-type electrode was mounted in a Strathkelvin (model RC-100) constant-temperature glass housing at 23 °C. A universal interface (Sable Systems) connected to a personal computer was used to convert readings of oxygen tension into moles of oxygen, using appropriate calibrations. From the number of larvae enumerated in each respiration chamber, the volume of that chamber and the amount of oxygen removed during each incubation, the rate of respiration was calculated as pmol O_2 larva^{-1} h^{-1}.

(b) Aerobic capacity (citrate synthase): The Kreb's cycle mitochondrial enzyme, citrate synthase, was measured as an index of maximum aerobic capacity in larvae of *C. gigas*. The purpose of this measurement was to compare metabolic potential (citrate synthase activity) to actual rates of energy consumption, as measured by respiration, in slow- and fast-growing larvae. The general method for the measurement of citrate synthase is given in Srere (1969), modified for measurements in larvae of *C. gigas* as in Moran and Manahan (2004). Briefly, larval tissue was homogenized in 50 mM Tris–HCl (pH 7.5) in a known volume (100–200 μl, depending on larval size). Standard assay conditions used 250 μM DTNB (Ellman's Reagent), 500 μM oxaloacetate and 400 μM acetyl-coenzyme A at 23 °C in a final volume of 800 μl. Reaction rates were monitored at 412 nm. Net

reaction rates were calculated with a least-squares linear regression analysis of absorbance against time. Citrate production was calculated from the DTNB extinction coefficient and given as pmol citrate larva^{-1} min^{-1}.

(c) Ion pump activity (Na^+,K^+-ATPase): total enzyme activity and determination of the physiologically active fraction.

Total enzyme activity—The sodium pump is a major determinant of metabolic rate in animals and, in marine invertebrate larvae, has been found to account for up to 40% of metabolic rate (sea urchin larvae: Leong and Manahan, 1997). In the current study, we examined the possibility that variations in total and physiologically active fractions of the sodium pump might regulate a component of metabolic rate in slow- and fast-growing larvae. Conventionally, Na^+,K^+-ATPase activity is measured as the ouabain-sensitive ATPase activity. In the current study, however, our initial experiments showed that the Na^+,K^+-ATPase in *C. gigas* was insensitive to ouabain even at high concentrations (>2 mM). We therefore employed a different assay to estimate the activity of Na^+,K^+-ATPase activity in *C. gigas*. K^+-pNPP phosphatase activity is a measurement of Na^+,K^+-ATPase-specific K^+-activated hydrolysis of *p*-nitrophenol phosphate (pNPP, an artificial substrate) in the absence of Na^+ (Esmann, 1988). This assay does not require the presence of ouabain for the activity to be determined and is suitable for estimating Na^+,K^+-ATPase activity in *C. gigas*. To measure the K^+-pNPP phosphatase activity, 100 μg of larval protein were added to each reaction tube with pNPP. The product of hydrolysis of pNPP is *p*-nitrophenol, an intensely yellow product that was measured spectrophotometrically at 410 nm. The activity of K^+-pNPPase was measured as the difference in activity between assays in the presence and absence of K^+ (with and without Na^+—i.e., reaction mixture = 150 mM KCl without NaCl; reaction blank = 150 mM NaCl without KCl). From the total number of larvae in each assay condition, the total amount of Na^+,K^+-ATPase was calculated as pmol ATP converted to ADP (measured as P_i) larva^{-1} h^{-1}.

Physiologically active fraction —An important regulatory component determining metabolic rates is the degree to which an enzyme is active kinetically (i.e., the proportion of total enzyme activity that is functioning in vivo in living cells). This physiologically active fraction of Na^+,K^+-ATPase can be calculated for living cells by measuring the rate of K^+ transport using $^{86}Rb^+$, a physiological analog of K^+ (Hilden and Hokin, 1975; Leong and Manahan, 1997, 1999). Due to the insensitivity of the sodium pump to ouabain in larvae of *C. gigas*, the following method was used to determine the in vivo rate of $^{86}Rb^+$ transport in slow- and fast-growing larvae that can be attributed to Na^+,K^+-ATPase. Our rationale is that since Na^+,K^+-ATPase activity is sensitive to change in intracellular [Na^+] (Rossier et al., 1987), complete removal of extracellular Na^+ should inhibit all in vivo Na^+,K^+-ATPase activity by eliminating all Na^+ influx into the cells. Here, we measured the difference in K^+ ($^{86}Rb^+$) transport rate for the *C. gigas* larvae in the presence and absence of Na^+ in the artificial seawater (Marine Biological Laboratory formula, Woods Hole, MA). This difference, which represents Na^+-dependent K^+ transport rate, is a measure of the in vivo Na^+,K^+-ATPase activity for larvae, after the contribution from any potential Na^+,K^+,2Cl transporter activity is accounted for.

For each transport assay, a known number of larvae (ca. 4000, depending on size) was removed from a culture, rinsed in filtered artificial seawater and resuspended in 7 ml in a series of 20-ml glass vials. Artificial seawater was prepared in the presence or absence of Na^+. Sodium-free artificial seawater was prepared by replacing NaCl with the isosmotically correct amount of choline chloride. Prior to each ion transport assay 1.85 MBq of $^{86}Rb^+$ was added to each vial (New England Nuclear, ^{86}RbCl volume activity of different batches of isotopes purchased from NEN ranged from 444 to 1665 MBq ml^{-1}). During time-course experiments of 15 min, a series of 1-ml samples with a known number of larvae were removed every 2–3 min (N=5–7, depending on larval size). Larvae in each sample were placed onto a membrane filter (Nucleopore: 25 mm, 8-μm pore size), washed three times with artificial seawater and the amount of $^{86}Rb^+$ radioactivity in larvae determined as described in Leong and Manahan (1997). Each data point given in Fig. 8B for the in vivo rate of transport is the rate calculated from each different time-course experiment (with N=5–7) described above.

The difference in $^{86}Rb^+$ transport rates by larvae in the presence and absence of Na^+ was used to measure the total in vivo Na^+-dependent K^+ activity. The portion of this in vivo activity that

was attributable specifically to Na^+,K^+-ATPase was calculated as the difference in K^+ transport rates in the presence (ASW) and absence (Na^+-free ASW) of Na^+ by correcting for any possible contribution from Na^+,K^+,2Cl cotransport activity. The latter was measured using bumetanide, a specific inhibitor for the Na^+,K^+,2Cl cotransporter (Haas, 1994). For these experiments, 100 μM of bumetanide was used, as this concentration is within the range (10^{-5} to 10^{-4} M) known to completely inhibit Na^+, K^+,2Cl cotransport activity in marine organisms (Altamirano et al., 1988: squid; Hannafin et al., 1983: dogfish; O'Grady et al., 1986: flounder). The measurement of the in vivo Na^+,K^+-ATPase-facilitated K^+ transport rate is the rate of K^+ transport that was Na^+-dependent and insensitive to the presence of the Na^+,K^+,2Cl inhibitor, bumetanide.

2.6. Excretion (retention efficiency of ^{14}C-labeled algae)

The possibility was tested that differential rates of absorption efficiency might account for the growth differences between slow- and fast-growing larvae. The efficiency with which larvae of *C. gigas* retained ingested carbon from their algal diet was analyzed using radiolabeled *I. galbana*. Our algal labeling and feeding experiments using radioactive carbon were conducted following established procedures (Pechenik and Fisher, 1979; Gallager et al., 1994; Reinfelder and Fisher, 1994). Aliquots of *I. galbana* ($\sim 20 \times 10^6$ cells) were incubated for 3 days in 20 ml of seawater with 74 kBq of ^{14}C-bicarbonate (NEN). Following the period of labeling, algae were centrifuged (4000 rpm, clinical centrifuge) and resuspended in filtered seawater. This washing step was repeated several times until the excess isotope that was not incorporated into the algal cells was removed from seawater. When ^{14}C-labeled algae were provided to larvae of different sizes (180 and 250 μm shell length), in all cases tested during initial experiments, larvae rapidly filled their guts and by ~30 min the amount of radioactivity ingested from ^{14}C-labeled algae reached a maximum. Aliquots of radiolabeled algae, each with a known amount of ^{14}C per algal cell, were used to simultaneously measure both the clearance rate and the carbon retention efficiency of larvae. The clearance rate of radiolabeled algae was measured as described above using a Coulter Counter. The retention efficiency was calculated as the percent of the ^{14}C present in ingested algae that was subsequently absorbed following feeding. This is a conservative estimate of absorption efficiency as the possible fraction respired as $^{14}CO_2$ was not measured. At the start of each series of feeding and retention efficiency experiments, a known amount of radioactive algae was added to 50 ml of seawater containing 10 larvae ml^{-1}. Following a 3-h incubation with radioactive algae, larvae were removed, washed three times with filtered seawater and placed in seawater containing non-radioactive algae at an identical concentration to that used during the feeding experiment with radiolabeled algae (i.e., 30 cells μl^{-1}). Microscopic examination revealed that larvae used in our experiments cleared their gut in less than 1 h. The procedure of incubating larvae in non-radioactive algae following a feeding period with radiolabeled algae was used to remove any unassimilated ^{14}C-labeled algal material from the larval gut. The amount of ^{14}C label still present in larvae following gut clearance was considered to be algal carbon absorbed by larval tissues. The amount of ^{14}C so absorbed was measured by filtering larvae onto an 8-μm pore size (Nucleopore) membrane, enumerating the exact number of larvae in each sample (~500 per sample) and determining the amount of ^{14}C in larvae by quench-corrected liquid scintillation counting. Data given in Fig. 9 are expressed as the percent of the total amount of ^{14}C that was initially ingested, but remained in larval tissue after gut clearance (i.e., herein defined as the absorbed fraction).

3. Results

The growth rates of 35 larval families, representing 12 inbred and 23 hybrid families, are shown in Fig. 1A–D. These families of larvae were then used to test hypotheses regarding the physiological mechanism(s) that might cause genetically determined differences in growth for larvae reared under identical environmental conditions of food and temperature. These tests of physiological performance were based on specific measurements following a modified balanced energy equation of Winberg (1956):

$$\text{Growth} = \text{Consumption} - \text{Respiration} - \text{Excretion}$$

3.1. Growth: size (shell length), mass (protein) and volume (organic osmolytes)

(a) Shell length: For the nine larval families shown in Fig. 1A, growth rates ranged from 9.5±0.6 (S.E.) μm day^{-1} for the '2×2' family to 17.6±0.6 (S.E.) μm day^{-1} for the '3×5' family. The variations in growth rates within a family were negligible compared to the difference among families (Fig. 1A,

see replicate culture vessels shown as different data points on the same day of sampling). Growth rates for hybrid larvae exceeded those of the faster inbred parent in nearly all cases. The maximum difference in growth rate observed was in larvae from the cross of inbred lines 1 and 3 (Fig. 1C). For these larvae, which showed heterosis for growth, hybrid larvae (1×3 and 3×1) grew at 11.8±0.5 and 15.1±0.5 μm day^{-1}, respectively, while inbred larvae (1×1 and 3×3) grew at lower rates of 3.4±0.5 and 7.6±0.5 μm day^{-1}, respectively. This represents a dramatic, maximum difference in larval growth rates of over four-fold (i.e., 15.1, cf. 3.4 μm day^{-1}). These results demonstrate the power of the experimental design used to produce substantial and reproducible differences in growth rates of larvae.

(b) Protein content: The relationship of the amount of protein per larva as a function of size (shell length) was determined for the 18 larval families shown in Fig. 1A,B. For all 18 larval families measured, no differences were evident in larval "condition" for slow- and fast-growing larvae (Fig. 2A). ANOVA of the combined regressions for inbred and hybrid larvae showed there to be no significant differences in slopes ($P=0.23$, $df=1, 45$) or intercepts ($P=0.55$, $df=1, 45$). The regression shown in Fig. 2A is the pooled data for all larvae measured for size and protein content. The equation describing the pooled data is: log(protein content)=2.57 log(shell length)−3.53 ($r^2=0.77$).

(c) Organic osmolyte content: Taurine, known to be the major organic osmolyte of the free amino acid pools of larvae of *C. gigas* (Welborn and Manahan, 1995), was measured in six larval families for which the shell growth rates are shown in Fig. 1A (families: 2×2, 3×3, 5×5, 2×3, 3×2 and 3×5). ANOVA of the combined regressions for inbred and hybrid larvae ($df=1, 16$) showed no significant differences in slopes ($P=0.53$) or intercepts ($P=0.053$). The regression shown in Fig. 2B is the pooled data for all larvae measured for size and taurine content: log(taurine content)=2.91 log (shell length)−4.48 ($r^2=0.93$).

3.2. Consumption: rates of energy acquisition (particulate and dissolved nutrients)

(a) Particulate nutrients: Measurements of algal clearance rates were performed to test the degree to which differences in feeding rates caused differential growth rates of fast- and slow-growing larvae. Fig. 3A illustrates that larvae removed algae from seawater at predictable rates over time that justified end-point analyses based on five independent replicates (Fig. 3B: "larvae and algae"). Control experiments showed that algal concentrations remained constant when no larvae were present (Fig. 3A,B: "algae only"). As a

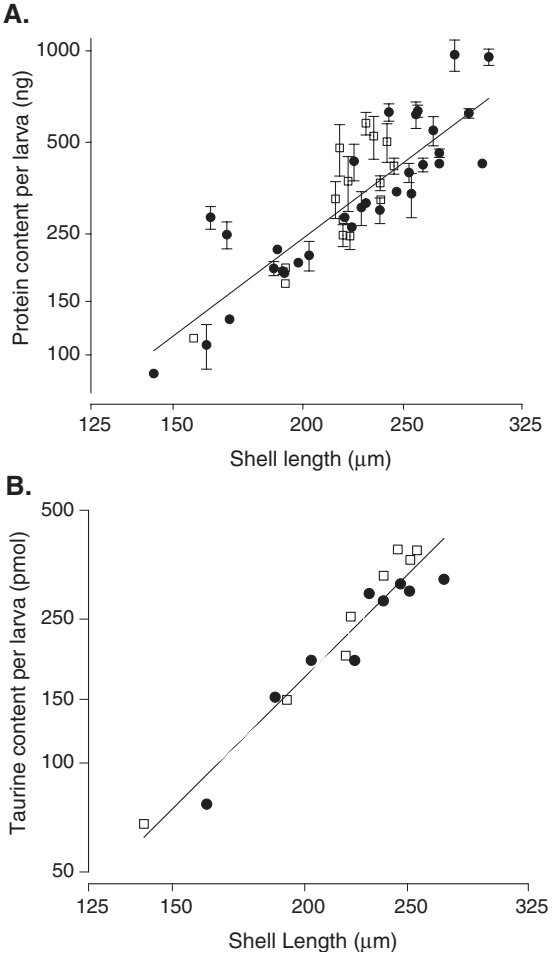

Fig. 2. Relationship of biochemical content as a function of size for larvae of *Crassostrea gigas*. Open squares=slow-growing inbred larvae; solid circles=fast-growing hybrid larvae. (A) Increase in protein content as a function of shell length ($N=3$ for each data point ± 1 S.E.M.). (B) Increase in free amino acid pool content (taurine) as a function of shell length (each data point is a single measurement by high-performance liquid chromatography). Separate regressions of protein and taurine content in slow- and fast-growing larvae were not significantly different (statistical data given in Results). The regression line shown is for the pooled data for all measurements for protein (A) and taurine (B) content. Regression equation for protein content (A): log(protein content)=2.57 log(shell length)−3.53, $r^2=0.77$. Regression equation for taurine content (B): log(taurine content)=2.91 log (shell length)−4.48, $r^2=0.93$.

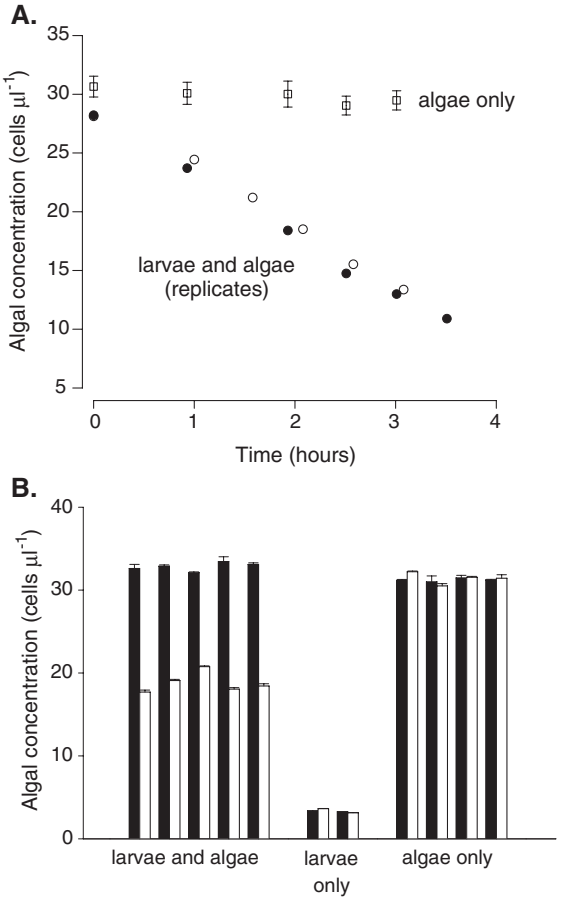

Fig. 3. Feeding (clearance) rates of the alga *Isocyrysis galbana* by larvae of *Crassostrea gigas*. (A) The relationship of the removal with time of algae from seawater by the feeding activity of larvae. Open squares: control experimental containers containing algae, but with no larvae present. Error bars represent the S.E.M. ($N=3$). Open and solid circles: two replicate containers with algae and larvae to illustrate the removal of algae with time by feeding larvae. (B) Experimental and control containers used to measure clearance rates by larvae. Histograms labeled as "larvae and algae" ($N=5$) show the experimental containers with larvae and algae at the start (solid) and end (open) of a 3-h incubation. Those labeled as "larvae only" ($N=2$) show containers with larvae, but no algae present. Histograms labeled "algae only" show control containers with algae only ($N=4$). Error bars represent replicate determinations ($N=4$) of algae in each experimental container.

further control, larvae were placed in seawater with no algae added (Fig. 3B: "larvae only"). These measurements were used to correct for any possible particulate contamination that might have been introduced with the larvae themselves.

The algal clearance rates were measured for larvae with known different growth rates. From the larval families shown in Fig. 1B, for example, clearance rates were measured on larvae from crosses 2×2, 5×5 and 5×2, where the 5×2 larvae (Fig. 4A) had an average of a 154% faster growth rate (Fig. 4A: inset—average inbred growth rate of 2×2 and 5×5 cf. 5×2 hybrid). The fast-growing larvae had consistently higher clearance rates that were maintained during growth (Fig. 4A). When the equations for clearance rates for the different larval families were solved for a standard-sized larvae of 220 µm, the faster-growing larvae (5×2) had a higher clearance rate of 13.8 µl larva^{-1} h^{-1}, compared to 7.3 µl larva^{-1} h^{-1} for the slower-growing inbred larvae (Fig. 4A: pooled regression of 5×5, 2×2). This equates to an 89% difference in clearance rate (Table 1). From the differences in growth rate of 154% and in clearance rate of 89%, we conclude that growth rate difference cannot be fully accounted for by the difference in clearance rate for this particular set of larval families.

A more extensive series of measurements, involving 24 different sets of clearance rates was performed on larval families resulting from the crosses shown in Fig. 1C. As would be anticipated based on growth differences, there was variation in clearance rates among larvae of different families (Fig. 4B). As shown in Table 1, the faster growing hybrid larvae in this case had a size-specific clearance rate that was 158% higher than comparable inbred larvae. This finding of higher size-specific clearance rates for faster-growing larvae was further tested with additional sets of larval families. For the different growth rates given in Fig. 1D, the corresponding clearance rates are given in Fig. 4C. Here again, hybrid larvae maintained a clearance rate during growth that was 68% faster than comparably sized inbred larvae (Table 1). Finally, for the different growth rates given in Fig. 1A, the hybrid larvae had a 22% faster size-specific clearance rate than inbred larvae (Fig. 4D).

Based on the 332 different clearance rate measurements (all replicates included, Fig. 4A–D), we conclude that a common physiological scaling principle applies, where clearance rates increase as a function of size at similar rates for slow- and fast-growing larvae. As illustrated in Fig. 4 and Table 1, the slopes of the relationship of size and feeding rate were not significantly different. However, size-specific feeding rate ("intensity") was always higher in the hybrid larvae and that difference was set by the differences in y-axis intercepts (i.e., these were higher in hybrid larvae: Table 1). When all data for inbred and hybrid larvae were combined for a pooled analysis of size and

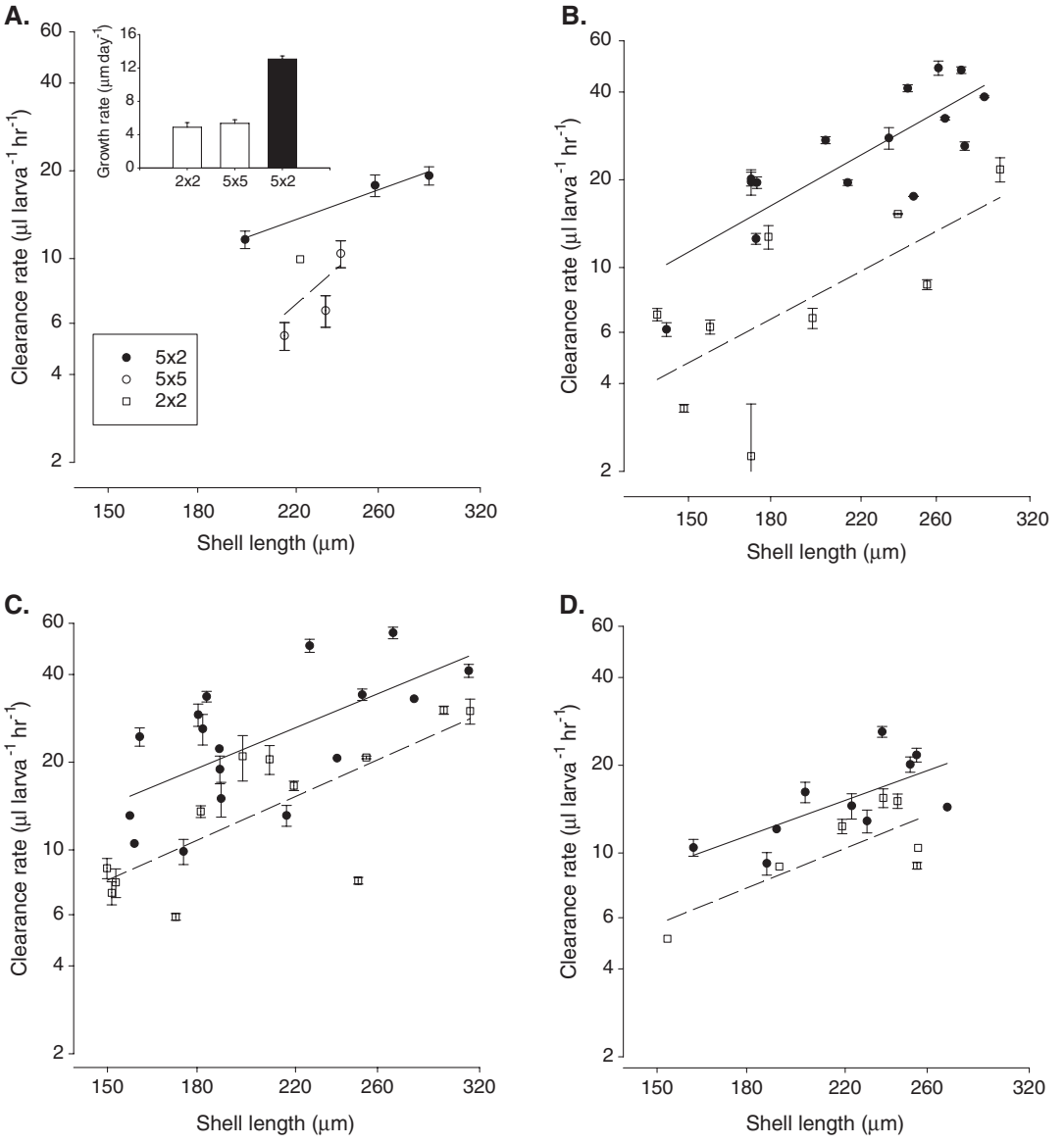

Fig. 4. Relationship of clearance rates of the alga *Isocyrysis galbana* as a function of larval size of *Crassostrea gigas*. Solid circles: fast-growing hybrid larvae. Open circles and squares: slow-growing inbred larvae. All data points shown are means (N=4–5±1 S.E.M.). (A) The relationship of size and algal clearance rates for larvae of three different families (5×2, 5×5, 2×2). Inset graph shows the corresponding growth rates of the larvae that were used in measurements of clearance rates (from Fig. 1B). (B) Relationship of size and clearance rates for larvae with growth rates shown in Fig. 1C. (C) Relationship of size and clearance rates for larvae with growth rates shown in Fig. 1D. (D) Relationship of size and clearance rates for larvae with growth rates shown in Fig. 1A. See Table 1 for regressions equations.

clearance rates, slower-growing inbred larvae of 220 µm shell length had a clearance rate of 11.4 µl larva^{-1} h^{-1} compared to a much higher rate of 21.7 µl larva^{-1} h^{-1} for faster-growing hybrid larvae. An ANOVA of combined regressions for inbred and hybrid larvae showed that the slopes of the change in clearance rates as a function of size were not significantly different (P=0.68). As above, differences in physiological rates were again set by the highly significant differences of the y-axis intercepts (P<0.00001).

(b) Dissolved nutrients: Transport of dissolved organic material (DOM) directly from seawater is another possible component of the "consumption" term of the balanced energy equation. The maximum transport capacity (J_{max}) of larvae to

Table 1
Feeding on the alga *Isochrysis galbana* by larvae of *Crassostrea gigas*

Parental inbred lines crossed	Clearance rate data	Regression results (log clearance rate = slope × log shell length + *y*-intercept)			Clearance rate for 220 μm larva (μl h^{-1})	Percent difference (%)
			Slope	*y*-intercept		
1, 3, 5 (Fig. 1C)	Fig. 4B (N=24)	Hybrid	2.01^{ns}	−3.31***	Hybrid: 25.0	
		Inbred	1.89	−3.44	Inbred: 9.7	158
2, 5 (Fig. 1B)	Fig. 4A (N=7)	Hybrid	1.40^{ns}	−2.14*	Hybrid: 13.8	
		Inbred	3.40	−7.10	Inbred: 7.3	89
2, 3, 5 (Fig. 1D)	Fig. 4C (N=29)	Hybrid	1.60^{ns}	−2.34***	Hybrid: 25.6	
		Inbred	1.73	−2.87	Inbred: 15.2	68
2, 3 (Fig. 1A)	Fig. 4D (N=17)	Hybrid	1.40^{ns}	−2.14**	Hybrid: 13.8	
		Inbred	1.56	−2.60	Inbred: 11.3	22

Differences in clearance rates for slow- and fast-growing inbred and hybrid larvae. The results of statistical analysis of four groupings of hybrid and inbred larvae for differences in slope and *y*-intercept values are presented as 'ns' = no statistical difference (P>0.05); *P<0.05; **P<0.01; ***P<0.001. Clearance rates in μl $larva^{-1}$ h^{-1} are calculated from each regression and standardized to a larval size of 220 μm shell length.

transport dissolved amino acids from seawater was determined using glycine as a substrate. The larvae with different growth rates used to test for possible difference in transport capacities of dissolved amino acids were from the larval families shown in Fig. 1A,B. Transport rates increased with increasing size (shell length) for both inbred and hybrid larvae (Fig. 5). There were, however, no differences in amino acid transport capacities between inbred and hybrid larvae (ANOVA of combined regressions, df=1, 36, slope: P=0.41, *y*-intercepts: P=0.99). The linear equation describing transport rate for the pooled data set is: log(transport rate)=1.96 log (shell length)−3.01 (r^2=0.57).

3.3. Respiration: metabolic rates (oxygen consumption), aerobic capacity (citrate synthase) and ion pump activity (Na^+,K^+-ATPase)

(a) Metabolic rates: Rates of oxygen consumption for larvae (Fig. 6) with different growth rates (Fig. 1C,D) were measured as a function of size during the entire larval growth phase, from newly formed D-hinge veligers (80 μm) to larvae about to undergo metamorphosis at ~350 μm. The increase in respiration as a function of size was not significantly different between slow- and fast-growing larvae (Fig. 6). ANOVA of the combined regressions for inbred and hybrid larvae (df=1, 36) showed there to be no significant differences in slopes (ANOVA, P=0.09) or intercepts (ANOVA, P=0.16). The regression shown in Fig. 6 is the pooled data for all larvae measured for size and respiration rate. The linear regression equation for the pooled data relating respiration rate to shell length is: log(respiration rate)=3.05 log (shell length)−5.02 (r^2=0.88).

It is noteworthy that larvae with different growth rates had similar size-specific respiration rates. This is illustrated in Fig. 6 by comparing the inset graphs showing larval growth rate with the corresponding larval families labeled on the regression of respiration and shell length. The different growth rates for larvae, as shown in Fig. 1C, are given in Fig. 6 as the inset showing the growth rates for larvae of the 3×3, 5×3 and 3×5 crosses. Both larval families 5×3 and the reciprocal 3×5 had an 80% faster growth rate than larvae from the 3×3 cross. ANOVA of the combined regressions of growth rates for 3×3 and 3×5 (df=1, 498) were significantly different (P<0.0001). Yet the larvae from these families had similar respiration rates for larvae of similar size (250 μm shell length) of ~210 pmol O_2 $larva^{-1}$ h^{-1}. For another set of larval families from lines 2 and 5, the faster growing larvae (2×5) had a growth rate that was at least 88% higher than two families of slower growing larvae (2×2 and 5×5). ANOVA of combined regressions between 5×5 (the faster-growing inbred) and 2×5 (df=1, 394) showed growth rates to be highly significantly different between the two families (P<0.0001). Again, these larvae with very different growth rates all had similar respiration rates for a given size of larva. These data strongly suggest that differences in growth rates for faster-growing larvae were not explained by differences in size-specific respiration rates, but were more likely due to different metabolic allocation of energy given a constant metabolic rate (see Discussion).

(b) Aerobic capacity (citrate synthase): The amount of the mitochondrial enzyme, citrate synthase,

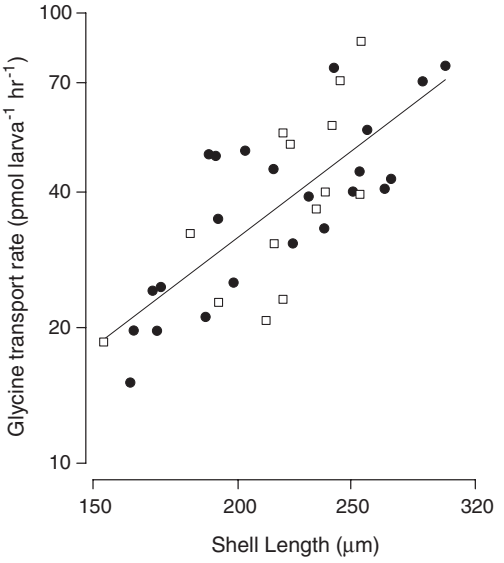

Fig. 5. Relationship of maximum transport capacity (J_{max}) by larvae of dissolved glycine from seawater as a function of larval size of *Crassostrea gigas*. Open squares: slow-growing inbred larvae; solid circles: fast-growing hybrid larvae. Each data point represents the transport rate of glycine based on a time-course experiment of 6 data points. For all 37 transport assays shown, the mean lack-of-fit error of the linear regressions was 5% (r^2=0.95). Separate regressions of glycine transport by slow- and fast-growing were not significantly different (statistical data given in Results). The regression line shown is for the pooled data for all measurements: log(transport rate)=1.96 log(shell length)−3.01, r^2=0.57.

was measured in slow- and fast-growing larvae (Fig. 7A). Larvae for these measurements were from larval families shown in Fig. 1A,B. The relationship of the increase of total citrate synthase activity with size in larvae of *C. gigas* was similar between inbred and hybrid larvae (ANOVA of combined regressions, *df*=1, 56, slope: *P*=0.64), so both data sets were pooled as shown in Fig. 7A. The linear equation describing the pooled data set is: log(citrate synthase activity)=3.23 log(shell length)−6.05 (r^2=0.66). The relationship of citrate synthase and respiration rate (Fig. 7B) showed that citrate synthase is a good predictor of respiration rate for both slow- and fast-growing larvae (Fig. 7B). No differences were measured between inbred and hybrid larvae (ANOVA of combined regressions, *df*=1, 20, slope: *P*=0.66; *y*-intercept: *P*=0.60). The equation for the pooled data shown in Fig. 7B is: respiration rate=2.1(CS activity)+40.0 (r^2=0.58). The faster growth rates of hybrid larvae were not attributable to a greater metabolic capacity in terms of either mitochondrial densities or mitochondrial metabolic output (as indicated by citrate synthase activity and oxygen consumption, respectively).

(c) Ion pump activity (Na^+,K^+-ATPase): Total Na^+, K^+-ATPase activity, measured as K^+-pNPPase activity, was significantly higher in slow-growing larvae compared to fast-growing larvae throughout development (Fig. 8A). For total enzyme activity, the rate of increase was similar for both groups of larvae (slope); the difference was set by the higher size-specific enzyme activity of the inbred larvae (intercept) (ANOVA of combined regressions: *df*=1, 21, *P* value of slope=0.77, *P* value for intercept=0.001).

For the physiologically active fraction of total enzyme activity (Fig. 8B), the in vivo Na^+,K^+-ATPase-facilitated K^+ transport activity was higher in hybrid than in inbred larvae. Again, the rate of increase was similar for both groups of larvae (slope); however, in this case, the difference was set by the higher size-

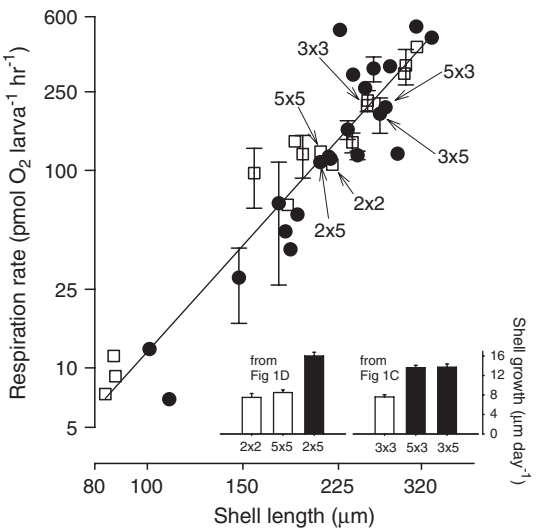

Fig. 6. Relationship of respiration as a function of size for larvae of *Crassostrea gigas*. Open squares: slow-growing inbred larvae; solid circles: fast-growing hybrid larvae. Inset graph shows the corresponding growth rates (e.g. larvae from the 5×5 family, from Fig. 1D) of the larval families labeled on the graph showing the regression of respiration and shell length. Each data point shown for respiration represents the rate of oxygen consumption based on a linear regression analysis using different numbers of larvae in a series of 7–10 respiration vials (details in Methods). Error bars for each data point are ±1 S.E. of the slope. Where errors bars are not shown, the error fell within the graphical representation of the data point. Separate regressions of oxygen consumption by slow- and fast-growing larvae were not significantly different (statistical data given in Results). The regression line shown is for the pooled data for all measurements: log (respiration rate)=3.05 log(shell length)−5.02, r^2=0.88.

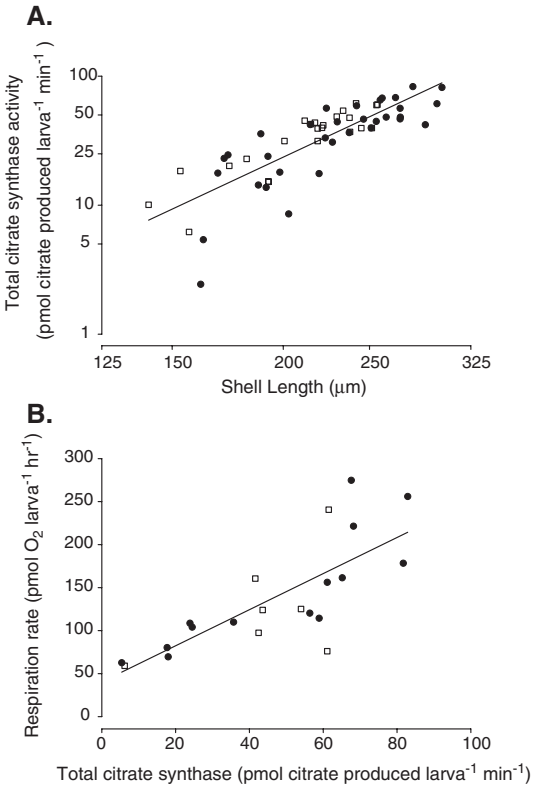

Fig. 7. Citrate synthase (CS) activity in larvae of *Crassostrea gigas*. Open squares: slow-growing inbred larvae; solid circles: fast-growing hybrid larvae. (A) Relationship of total CS activity as a function of larval size. Separate regressions of CS activity in slow- and fast-growing larvae were not significantly different (statistical data given in Results). The regression line shown is for the pooled data for all measurements: log(CS)=3.23 log (shell length)−6.05, r^2=0.66. (B) Oxygen consumption rate as a function of total CS activity. The regression line shown is for the pooled data for all measurements: respiration rate=2.1(CS)+40.0, r^2=0.58.

specific ion transport rate of the hybrid larvae (intercept) (ANOVA of combined regressions: *df*=1, 20, *P* value of slope=0.77, *P* value for intercept=0.042). The proportionality of the regulation of these differences during larval growth in total enzyme activity and the corresponding changes in physiologically active fractions are illustrated in Fig. 8C. This graph shows that while fast-growing larvae have less total enzyme (from Fig. 8A), a larger fraction of that total activity is physiologically active (Fig. 8C). Solving these regression equations (given in legend for Fig. 8C) for 50 units of total sodium pump activity (units=pmol P_i $larva^{-1}$ h^{-1}), fast-growing larvae had 48% higher in vivo activity than slow-growing larvae. This difference can be interpreted to mean that the sodium pump in fast-growing larvae was kinetically closer to the maximum velocity (V_{max}) value of Na^+, K^+-ATPase enzyme than it was in the slow-growing larvae, given the same total amount of enzyme in both groups of larvae.

3.4. Excretion (retention efficiency of ^{14}C-labeled algae)

Another component of the balanced energy equation is the relative efficiency with which slow- and fast-growing larvae retain ingested, particulate foods. There was no difference in the retention efficiency of slow- and fast-growing inbred and hybrid larvae (Fig. 9). By analysis of variance (ANOVA, *df*=1, 52) of the combined regressions, there were no significant differences in slopes (*P*=0.99) and *y*-intercepts (*P*=0.21) for the retention efficiencies of inbred and hybrid larvae. When all the data for retention efficiencies were pooled, there was a decrease in retention efficiency with increasing size of larvae (ANOVA of pooled regression showed that the decrease in slope was statistically significant, *P*=0.003). For a doubling of larval size (e.g., from 150 to 300 μm), the retention efficiency decreased from 46% to 25%. We conclude that, while there was a difference in clearance rates as a

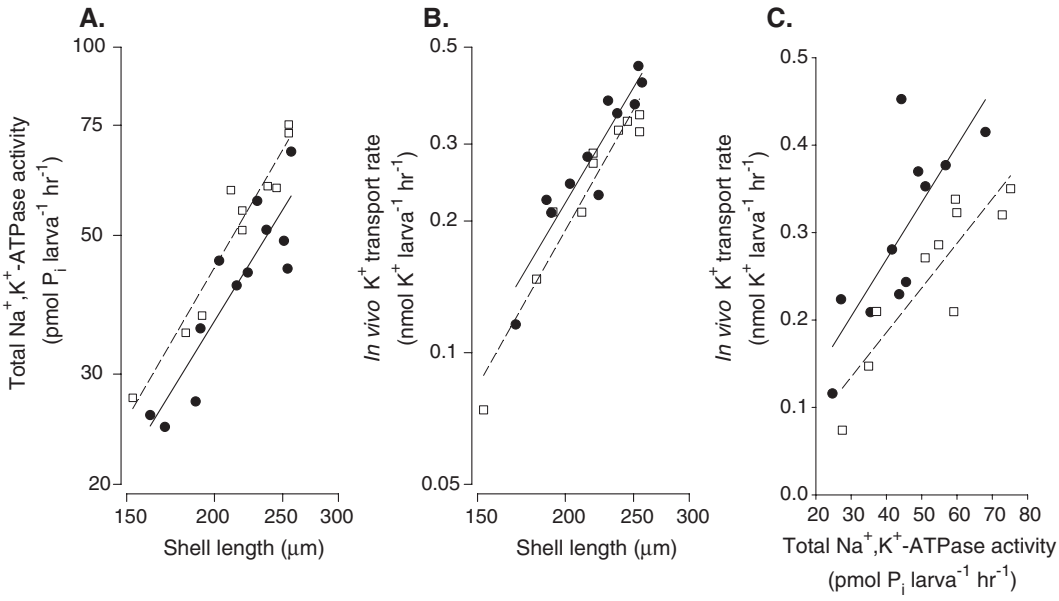

Fig. 8. Na^+,K^+-ATPase activity in larvae of *Crassostrea gigas*. Open squares: slow-growing inbred larvae; solid circles: fast-growing hybrid larvae. (A) Total activity of Na^+,K^+-ATPase measured in vitro for larvae of different sizes ($N=3$ for each data point). Regression equations, inbred larvae: log (in vitro activity) = 1.94 log(shell length) − 2.82, $r^2=0.93$; hybrid larvae: log(in vitro activity) = 1.84 log(shell length) − 2.66, $r^2=0.80$. (B) Physiologically active Na^+,K^+-ATPase measured in vivo for larvae of different sizes. Each data point represents the transport rate of $^{86}Rb^+$ based on a time-course experiment of 5–7 data points. For all 21 transport assays shown, the r^2 values were >0.90. Regression equations, inbred larvae: log(in vivo activity) = 2.85 log(shell length) − 7.28, $r^2=0.93$; hybrid larvae: log(in vivo activity) = 2.72 log(shell length) − 6.92, $r^2=0.88$. (C) Relationship of the physiologically active Na^+,K^+-ATPase to the total enzyme activity. Regression equations, inbred larvae: log(in vivo activity) = 1.32 log(in vitro activity) − 2.89, $r^2=0.81$; hybrid larvae: log(in vivo activity) = 1.11 log(in vitro activity) − 2.36, $r^2=0.69$. Results of ANOVA of combined regressions for A, B and C given in Results section.

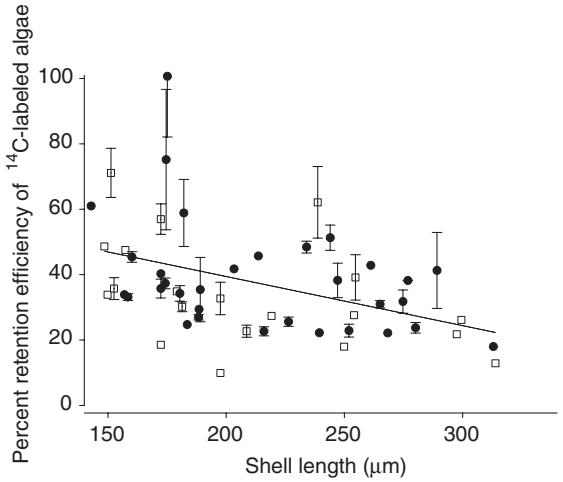

Fig. 9. Relationship of retention efficiency of ^{14}C-labeled algae by larvae of *Crassostrea gigas*. Open squares: slow-growing inbred larvae; solid circles: fast-growing hybrid larvae ($N=5$, with S.E.M. for each data point). Where errors bars are not shown, the error fell within the graphical representation of the data point. Separate linear regressions of data for slow- and fast-growing larvae were not significantly different (statistical data given in Results). The regression line shown is for the pooled data for all measurements: retention efficiency = −1.39(shell length) + 66.6, $r^2=0.17$.

function of slow and fast growth rates (Fig. 4), larvae with these physiological differences in the ability to acquire algae had, nonetheless, similar absorption efficiencies.

4. Discussion

The central goal of this study was to use genetically induced differences to investigate the physiological mechanisms of variation in larval growth rate. Our approach was to measure in slow- and fast-growing larvae the key components of an energy balance equation (growth = consumption − respiration − excretion). The suite of integrated measurements applied to this question included size, biochemical compositions, rates of nutrient acquisition (both particulate and dissolved), absorption efficiencies, metabolic rates and enzyme activities.

Inbred lines of the bivalve *C. gigas* were crossed in a series of experiments to produce 35 larval families with different growth and physiological phenotypes. Depending on the specific set of crosses, differences in growth rates of over four-fold were obtained under

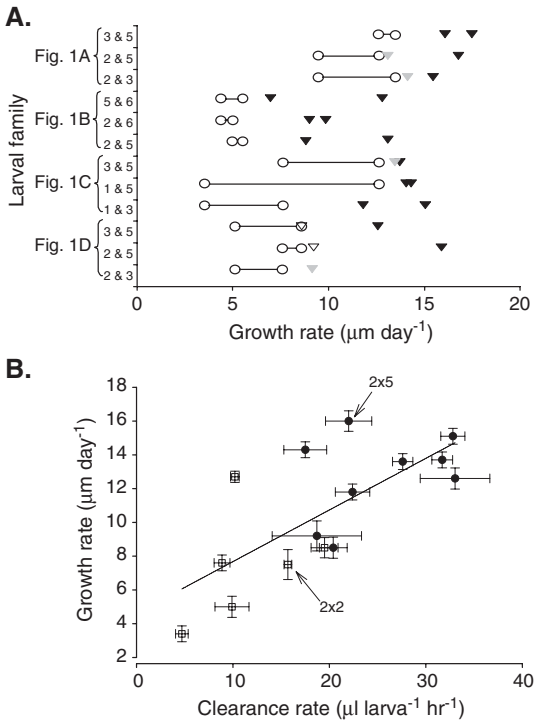

Fig. 10. Growth heterosis and the variance in growth accounted for by differential feeding rates in larvae of *Crassostrea gigas*. (A) Growth heterosis in larval families. Details of ANCOVA analysis are given in Methods. Growth rates from shell lengths (Fig. 1A–D) of families of hybrid larvae are given as triangles, with corresponding growth rates of families of larvae from parental inbred lines given as circles. Heterosis is evidenced by growth rates of hybrid and reciprocal hybrid larvae that are significantly greater than those of the fastest growing inbred larvae (ANCOVA, $P<0.05$; black triangles, 17 of 23 comparisons). Heterosis is also evidenced by hybrid families of larvae that grew at similar rates as the best inbred larvae, but were still larger in size-at-age (gray triangles, 4 of 23 comparisons; differences in size determined by testing differences in shell length at specific sampling days). Simple dominance is shown by hybrid larvae that did not grow faster nor have a larger size-at-age than the best inbred larvae (open triangles, 2 of 23 comparisons). The growth rate of no hybrid lies at the midpoint of the line connecting the points for its inbred parental lines, which would be the case if growth rate were determined by additive genetic variation. (B) Relationship of growth and feeding rate for families of hybrid (solid circles) and inbred larvae (open squares). Growth rates taken from Fig. 1C,D where larvae were reared at 30 algal cells μl^{-1}. A direct comparison of growth and clearance rate measurements was made for 15 larval families, based on a standardized larvae shell length of 220 μm. Feeding rates measured in experiments at 30 algal cells μl^{-1} were calculated from the equations for clearance rates for the same larval families (Table 1), standardized to a shell length of 220 μm. Growth rates for each larval family shown as μm day^{-1} with error. Error bars for clearance rates are S.E. of *y*-estimate for *x*-value of 220 μm shell length. Equation of regression line shown: $y=0.31x+4.62$, $r^2=0.51$, $P=0.003$ (ANOVA, $n=15$).

identical, environmentally controlled rearing conditions of food and temperature (e.g., Fig. 1C). Our measurements of growth rates using shell length were good measures of actual tissue growth as the relationships between size (shell length) and tissue (protein and taurine content) were similar between slow- and fast-growing larvae (Fig. 2A,B). These data show that the differences in shell growth reflected differences in the amount of tissue mass and volume.

The experimental crosses employed in this study produced F_1 progeny that showed hybrid vigor or growth heterosis (Shull, 1948; Crow, 1998), as well as differences in growth rate between reciprocal hybrid families. Hybrid larvae grew faster than the larvae from the best inbred parental line in 17 out of 23 comparisons (Fig. 10A). In four additional cases, hybrid larvae grew no faster than the larvae from the best inbred parental line but *y*-intercepts were larger so that hybrid larvae were always significantly larger. In only two cases were there no differences in growth rate or size (*y*-intercept) between hybrid larvae and larvae of the best inbred parental line. It is noteworthy that the growth rates of hybrid larvae always equaled or exceeded the growth rates of their respective inbred parental families (Fig. 10A). Additive genetic variation in larval growth would be evident if growth rates of hybrid larvae fell between the offspring of the respective parental inbred lines. Clearly, growth of hybrid larvae was genetically non-additive as it generally exceeded growth rates of

offspring of the best parent. Such statistically significant and consistent differences in growth rates of different families of larvae were the foundation for study of the physiological bases of differential growth under constant environmental conditions.

Before presenting our explanations of the energetic bases of differential growth rates in larvae, we should point out that the range of genetically determined physiological rates we report here are consistent with previous studies on larvae of *C. gigas*. Maximum growth rates of 13 to 19 μm shell length day^{-1} for laboratory-reared larvae of *C. gigas* have been reported by Collet et al. (1999). His and Maurer (1988) reported an average growth rate of 10 μm shell length day^{-1} for field-collected larvae (calculated from their Table 1). These field-collected larvae can be considered to be "wild-type" and correspond to growth rates for some of the hybrid larval families in our study. The average growth rates for all of the hybrid larval families shown in Fig. 1 were 15.7 μm day^{-1} (Fig. 1A), 10.0 μm day^{-1} (Fig. 1B), 13.8 μm day^{-1} (Fig. 1C) and 12.1 μm day^{-1} (Fig. 1D). Of course, lower growth rates are highly likely under field conditions if food is limiting. For feeding rates, Gerdes (1983a) reported a clearance rate at 25 °C of 7.7 μl larva^{-1} h^{-1} for "wild type" larvae of *C. gigas* of 114 μm shell length fed algae at a concentration of 25 cells μl^{-1} of *I. galbana*. Though larvae used in our feeding rate measurements were larger than those used by Gerdes, comparable clearance rates for a hybrid larva of 114 μm shell length larva can be calculated from equations in Table 1. Clearance rates of hybrid larvae ranged from 5.5 to 8.9 μl larva^{-1} h^{-1}, in close agreement with Gerdes' (1983a) value. The retention efficiencies we measured were similar to those measured for larvae of the flat oyster, *Ostrea edulis* (Gabbott and Holland, 1973), which ranged from 50% to 77% with declining efficiencies as growth proceeded. The gut clearance times measured in our study also agree with the results of Reinfelder and Fisher (1994), in which the excretion of radioactive feces by larvae of the American oyster, *Crassostrea virginica*, was found to be complete within 1 h of removal from radioactive algae in seawater. Our range of values for respiration rates given in Fig. 6 fall within the range published for larvae of *C. gigas* (Gerdes, 1983b; Hoegh-Guldberg and Manahan, 1995; Goulletquer et al., 2004). The general agreement of our measured rates with those from other studies is of further importance because it was not experimentally practical in our study to make all physiological measurements simultaneously on all 35 different larval families. The agreement of our data with those previously cited supports our approach of using a specific set of measurements, when physiologically appropriate, as being representative for *C. gigas* (for example, respiration rate: Fig. 6).

It is the differential rates of these physiological processes that are likely to be the bases for understanding the mechanisms underlying slow and fast growth in larvae under identical environmental conditions. We next consider how the integration of the changes in these physiological processes can explain differential growth in larvae. An important conclusion from our experiments is that fast-growing larvae had significantly higher size-specific clearance rates (Fig. 4). The lack of a difference in retention efficiencies between slow- and fast-growing larvae (Fig. 9) indicates that, although feeding faster, the hybrid larvae were still retaining a proportionally similar amount of nutrients from algae. Clearly, feeding and growth are related and the extent to which differential growth under our experimental conditions could reasonably be explained by differential rates of feeding is illustrated in Fig. 10B. This summary is based upon all growth data for which there were corresponding clearance rate measurements at the same algal concentration used to rear larvae (i.e., a total of 15 larval families grown at 30 cells μl^{-1} for larval families shown in Fig. 1C,D). There is a significant and positive relationship between growth rate and feeding rate (ANOVA: $n=15$, $P=0.003$). Those larvae with higher feeding rates had higher growth rates. Importantly, however, differential feeding rates did not fully account for higher growth rates, as feeding rate only accounted for about half of the variance in the rate of change of growth rate ($r^2=0.51$ for ANOVA of regression). While some smaller fraction of the variance in growth rate can be attributed to replicate errors, the level of replicate error for clearance rate as shown in Fig. 10B cannot fully account for the observed differences in growth rates. Larval family 2 × 5 had the highest growth rate of all the larval families studied in this comparison of feeding rate and growth rate. These larvae had a 2.1-fold faster growth rate than the slower-growing, half-sibling larvae from the 2 × 2 family. This growth difference cannot be explained by the 1.4-fold difference in clearance rate for these larval families (Fig. 10B). Given that, on average, 50% of the growth difference can be accounted for by enhanced feeding rates, which metabolic processes might account for the remaining 50% of the energy required to explain the faster growth rates? Obvious biological processes such as differential rates of absorption efficiency, or changes in metabolic rate, or alterations in aerobic enzyme activity cannot account for the required energy, as these processes were similar for both slow- and fast-growing larvae (Figs. 9, 6, 7, respectively). Protein accounts for the majority of the biochemical composition of larvae of *C. gigas* (Moran and Manahan, 2004). Larvae of molluscs, including *C. gigas*, are known to have high rates of protein turnover (Vavra and Manahan, 1999; Donald et al., 2001). Using our measurements of

respiration, protein accumulation and biosynthetic costs of protein synthesis, we calculate below that differential allocation of internal energy use could account for the 50% of the energy required for faster larval growth that was not provided by enhanced feeding. We base this series of calculations on the fastest growing larval family (2×5) from Fig. 10B. Our rationale for this choice is that, if the largest growth differential can reasonably be explained by differential energy allocation, then this explanation is sufficiently robust to account for the other, lesser, growth differences observed.

Quantifying the metabolic energy required for a given growth increment reveals large differences between slow- and fast-growing larvae in the amount of metabolic energy consumed. To grow from 200 μm to 240 μm shell length requires 5.3 days for the 2×2 family, but only 2.5 days for the 2×5 family (growth rates were 7.5 for 2×2 and 16.0 μm day^{-1} for 2×5, Fig. 1D). Converting respiration rate to energetic units, larvae from the 2×2 family required 8.4 mJ to grow from 200 μm to 240 μm (i.e., a total of 17.3 nmol O_2 consumed by a larva in 5.3 days, converted to energy equivalents based on 484 kJ $[mol\ O_2]^{-1}$) (Gnaiger, 1983). A similar calculation shows that larvae from the 2×5 family required only 3.9 mJ to complete the same shell growth increment due to their higher growth rates. This is a difference in energy requirements for growth from 200 μm to 240 μm of 4.5 mJ (8.4–3.9). Enhanced feeding rate could account for half of this required energy (from Fig. 10B), leaving 2.3 mJ to be accounted for by other growth efficiency processes. Fig. 11 presents a metabolic model to illustrate how changes in protein depositional efficiency could account for the remaining 50% of the energy (i.e., 2.3 mJ) required to explain the observed faster growth of larvae.

The relationship between larval size and protein content for slow- and fast-growing larvae is shown in Fig. 2A. From these data, the amount of protein growth can be calculated for a 200 μm to a 240 μm larvae. That amount is 152 ng $larva^{-1}$. This protein depositional rate of 152 ng for this increment of larval growth is positively affected by the rate of protein synthesis and negatively affected by the rate of protein degradation in larvae. Costs of protein synthesis in animals typically fall within the range of 8–10 J (mg protein synthesized)$^{-1}$ (reviewed in Houlihan, 1991). Our recent study of growing sea urchin larvae gave a cost of protein synthesis of 8.4 J (mg protein synthesized)$^{-1}$ (Pace and Manahan, 2006). This cost of protein synthesis can be used to estimate the energy cost of protein growth in larvae of *C. gigas*. Fig. 11 illustrates the relationship between protein depositional efficiency and the energy required to deposit a known amount of protein, in this case the 152 ng of protein deposited by larvae of *C. gigas* when growing from 200 to 240 μm. For a 25% protein depositional efficiency, 608 ng of protein would have to be synthesized to result in a net growth of protein of 152 ng (608×0.25=152). The metabolic cost of synthesizing

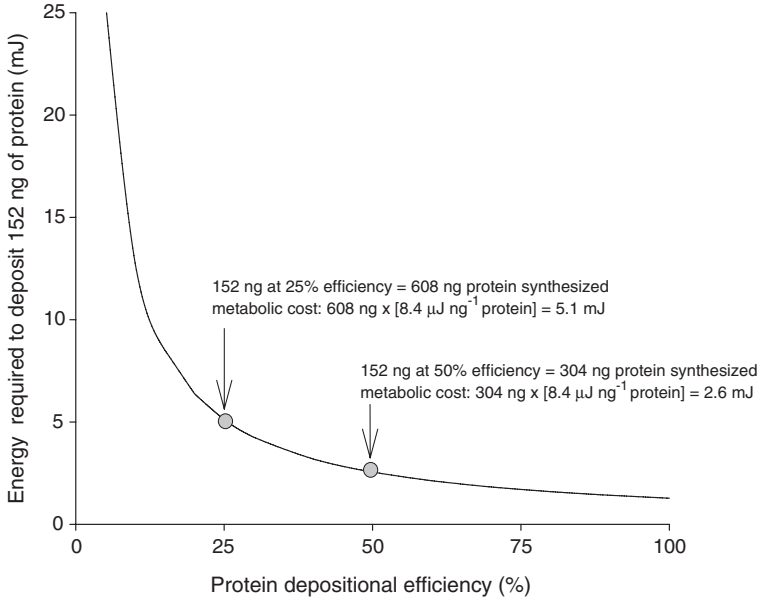

Fig. 11. Energetics of protein growth efficiency in larvae of *Crassostrea gigas*. For an increase in shell length from 200 to 240 μm, larvae increased protein content by 152 ng during this growth increment (from Fig. 2A). The *y*-axis gives energy cost of depositing 152 ng of protein due to differential depositional efficiencies (*x*-axis). Arrows indicate protein depositional efficiencies of 25% and 50%, with the corresponding amounts of protein of 608 ng and 304 ng that would have to be synthesized to deposit 152 ng of protein based on depositional efficiency of 25% and 50%, respectively.

608 ng of protein is 5.1 mJ (based on 8.4 J/mg protein synthesized). If the efficiency of protein deposition increased to 50%, then a corresponding reduction in the amount of protein synthesized would be required, at 304 ng ($304 \times 0.5 = 152$ ng protein; equivalent to 2.6 mJ). Similar ranges of depositional efficiencies have been reported for another species of marine invertebrate larva (Pace and Manahan, 2006). For adult marine bivalves, a 50% reduction in protein depositional efficiency has been reported for slow- and fast-growing mussels (Hawkins et al., 1986). For larvae of *C. gigas*, the difference in energy costs for a protein depositional efficiency of 25% and that of 50% would result in a net reduction of 2.5 mJ (5.1–2.6 mJ) in the cost of synthesizing 152 ng of protein. Thus, this reduction in cost of protein growth of 2.5 mJ could account for the calculated differential cost of growth of 2.3 mJ between slow- and fast-growing larvae that were attributable to metabolic processes.

The possible mechanisms that might explain the differential ability of fast-growing larvae to feed faster than slow-growing larvae need further investigation. The rates of increased feeding as a function of larval size are similar for both slow- and fast-growing larvae (i.e., the slopes of the increase in clearance rates are not statistically different in Fig. 4). Differences in size-specific clearance rates were due to differences in *y*-axis intercepts, suggesting a "setting" of clearance rates early in development that continued throughout subsequent larval growth. These data on the physiological scaling of body size and feeding rate are similar to scaling principles commonly found in animals (Schmidt-Nielsen, 1997, p. 192). It is likely that the differences in feeding rates we have measured in larvae of *C. gigas* are set genetically. The velum of bivalve larvae is responsible for food collection and is likely involved in differential rates of feeding. Differences in velar size relative to shell length have been observed in mollusc larvae (*C. gigas*—Strathmann et al., 1993; *Crepidula fornicata*—Klinzing and Pechenik, 2000). Preliminary measurements in our laboratory revealed no significant differences in velar length or width as a function of genotype for inbred and hybrid larvae of *C. gigas* (Appelmans, 2000). Additionally, indirect evidence from Fig. 5 suggests that there might be similar velar surface areas as no significant differences were measurable in rates of amino transport from seawater by inbred and hybrid larvae of *C. gigas* (transport of dissolved amino acids occurs across the velar surface: Manahan and Crisp, 1983). Other more subtle aspects of velar functional morphology that could result in differential feeding rates might include cirral length, beat frequency and metachronal wavelength (Strathmann and Leise, 1979; Gallager, 1988). Differences in the scaling of several processes relative to velar size could potentially have significant impacts on food particle encounter and capture. The use of larvae with genetically determined different feeding rates, coupled with sophisticated visualization technologies such as high-speed cinematography (Strickler, 1982; Emlet, 1990; Hadfield and Koehl, 2004), should greatly expand the experimental possibilities for understanding the interactions of organism, genotype and environment in the context of feeding.

We now consider how results from our laboratory crosses might reflect conditions in nature. We took advantage of the reproducible phenomenon of hybrid vigor or growth heterosis to generate slow- and fast-growing larval families for physiological analysis (Fig. 10A). This approach maximizes genetically determined variation in larval growth rate, allowing for a more precise dissection of the physiological mechanisms of growth than is afforded by traditional analysis of wild-caught animals. Because oysters are broadcast spawners, however, one might expect less growth rate variation among groups of naturally produced oyster larvae than is observed in laboratory comparisons of inbred and hybrid larval families (although there is little direct evidence concerning the natural variation of larval growth rates). We believe that our laboratory results are representative of natural conditions for three reasons.

First, the inbred lines of adult *C. gigas* used in this study were all derived from the same population of Pacific oysters and thus partitioned among them genetic components of growth rate variation that were present in that natural population. Genetic analyses of similarly derived inbred and F_2 hybrid populations reveal deleterious recessive mutations in natural oyster populations, consistent with evidence for inbreeding depression (Bierne et al., 1998; Launey and Hedgecock, 2001; Evans et al., 2003). Deleterious recessive mutations in highly fecund marine animals, such as the oyster, are likely an important source of endogenous variation in the relative fitness of individuals.

Second, despite the depression of inbred relative to hybrid larval growth rates (Fig. 10A), we note that several major physiological processes were indistinguishable between slow-growing inbred and fast-growing hybrid larvae. These included size-specific respiration rates (Fig. 6), aerobic enzyme capacities (Fig. 7), transport rates of dissolved amino acids (Fig. 5) and absorption efficiencies (Fig. 9). Even for those physiological traits that were different, such as feeding rate and the total amount of sodium pump enzyme activity, the physiological scaling with size was "normal" between inbred and hybrid larvae (similar slopes, Figs. 4 and 8A). Combined, the suite of integrative measurements that were undertaken on slow- and fast-growing larvae strongly supports the conclusion that differences in growth between inbred and hybrid

larval families were not the result of grossly "abnormal" physiology.

Third, the notion that large bivalve populations cannot possibly be affected by inbreeding depression is likely mistaken. Highly fecund marine organisms, such as oysters, have the potential for "sweepstakes reproductive success", in which a relatively small proportion of adults can account for most reproduction and recruitment, owing to chance matching of reproductive activity with temporally and spatially varying conditions conducive to fertilization, larval development and settlement (Beckenbach, 1994; Hedgecock, 1994; Li and Hedgecock, 1998). In the context of understanding the genetic components of variation in larval performance, sweepstakes reproductive success creates the potential for co-settlement and subsequent mating of full- or half-sibs in nature. Consanguineous mating, even at levels of a few percent of all matings, increases variance in individual inbreeding levels (David et al., 1997a), reducing the mean and increasing the variance in larval growth and survival. Indeed, the combination of sweepstakes reproductive success and mutational load provides a consistent explanation for the widespread correlation of fitness related traits and marker heterozygosity observed in populations of bivalves (Fujio, 1982; Gaffney et al., 1990; Beaumont, 1991; Zouros and Pogson, 1994; Britten, 1996; David et al., 1997b).

A general conclusion from the physiological research on adult stages of bivalves is that higher feeding rates combined with lower metabolic rates allow more energy to be directed to growth processes (Koehn and Shumway, 1982; Hawkins et al., 1986; Bayne et al., 1999; Bayne, 2000). The findings presented here for larvae of *C. gigas* extend these observations to the larval stages and show that a complex set of functional relationships underlies the physiological processes that regulate variation in larval growth. About 50% of the energy required to account for genetically determined growth variation in larvae comes from variation in size-specific feeding capacity. Differences in absorption rates appear not to be important. We conclude that metabolic processes must account for the additional 50% of the energy "savings" required to explain the genetically determined variation in growth rates. Further experiments using genetically determined variation in biological processes should provide new insights into the key physiological mechanisms that regulate growth of marine invertebrate larvae. Understanding the endogenous factors responsible for biological variation in larvae, combined with study of environmental variables, such as food and temperature, will allow for the development of more sophisticated ecological models of larval growth, survival and recruitment potential.

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References

Altamirano, A.A., Breitwieser, G.E., Russell, J.M., 1988. Vanadate and fluoride effects Na^+ K^+–Cl^- cotransport in squid giant axon. Am. J. Physiol. 254 (4), C582–C586.

Anger, K., 1995. Starvation resistance in larvae of a semiterrestrial crab, *Sesarma curacaoense* (Decapoda: Grapsidae). J. Exp. Mar. Biol. Ecol. 187, 161–174.

Anger, K., 2003. Salinity as a key parameter in the larval biology of decapod crustaceans. Invertebr. Reprod. Dev. 43 (1), 29–45.

Appelmans, N., 2000. Physiological energetics during the larval and metamorphic stages of marine invertebrates. PhD Dissertation, University of Southern California. 127 pp.

Bayne, B.L., 2000. Relations between variable rates of growth, metabolic costs and growth efficiencies in individual Sydney rock oysters (*Saccostrea commercialis*). J. Exp. Mar. Biol. Ecol. 251, 185–203.

Bayne, B.L., Hedgecock, D., McGoldrick, D., Rowena, R., 1999. Feeding behavior and metabolic efficiency contribute to growth heterosis in Pacific oysters (*Crassostrea gigas* Thunberg). J. Exp. Mar. Biol. Ecol. 233, 115–130.

Beaumont, A.R., 1991. Genetic studies of laboratory reared mussels, *Mytilus edulis*: heterzygote deficiencies, heterozygosity and growth. Biol. J. Linn. Soc. Lond. 44, 273–285.

Beaumont, A.R., Turner, G., Wood, A.R., Skibinski, D.O.F., 2004. Hybridisations between *Mytilus edulis* and *Mytilus galloprovincialis* and performance of pure species and hybrid veliger larvae at different temperatures. J. Exp. Mar. Biol. Ecol. 302 (2), 177–188.

Beckenbach, A.T., 1994. Mitochondrial haplotype frequencies in oyster: neutral alternatives to selection models. In: Golding, B. (Ed.), Non-Neutral Evolution Theories and Molecular Data. Chapman and Hall, N.Y., pp. 188–198.

Bierne, N., Launey, S., Naciri-Graven, Y., Bonhomme, F., 1998. Early effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. Genetics 148, 1893–1906.

Bochenek, E.A., Klinck, J.M., Powell, E.N., Hofmann, E.E., 2001. A biochemically based model of growth and development of *Crassostrea gigas* larvae. J. Shellfish Res. 20 (1), 243–265.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Breese, W.P., Malouf, R.E., 1975. Hatchery manual for the Pacific oyster *Crassostrea gigas*. Oregon Agricultural Experiment Station Special Report No. 443, and Oregon State University Sea Grant College Program Publ. No. ORESU-H-75-002. 22 pp.

Britten, H., 1996. Meta-analysis of the association between multilocus heterozygosity and fitness. Evolution 50, 2158–2164.

Collet, B., Boudry, P., Thebault, A., Heurtebise, S., Morand, B., Gerard, A., 1999. Relationship between pre- and post-metamorphic growth in the Pacific oyster *Crassostrea gigas* (Thunberg). Aquaculture 175, 215–226.

Crisp, D.J., Yule, A.B., White, K.N., 1985. Feeding by oyster larvae: the functional response, energy budget and a comparison with mussel larvae. J. Mar. Biol. Assoc. U.K. 65, 759–783.

Crow, J., 1998. 90 years ago: the beginning of hybrid maize. Genetics 148, 923–928.

Cushing, D.H., 1990. Plankton production and year-class strength in fish populations—an update of the match–mismatch hypothesis. Adv. Mar. Biol. 26, 249–293.

David, P., Perdieu, M.A., Pernod, A.F., Jarne, P., 1997a. Fined grained spatial and temporal population genetic structure in the marine bivalve *Spisula ovalis*. Evolution 51, 1318–1322.

David, P., Perdieu, Berthou, P., Noel, P., Jarne, P., 1997b. Patchy recruitment patterns in marine invertebrates: a spatial test of the density-dependent hypothesis in the bivalve *Spisula ovalis*. Oecologia (Berlin) 111, 331–340.

Davis, H.C., Guillard, R.R., 1958. Relative value of ten genera of micro-organisms as food for oyster and clam larvae. Fish. Bull. Fish Wildl. Serv. U.S. 58, 293–304.

Donald, K.M., Hawkins, A.J.S., Smerdon, G.R., 2001. Transcript analysis of the genes encoding aminopeptidase N and alanine aminotransferase, two enzymes involved in protein turnover, in the Pacific oyster, *Crassostrea gigas*. Comp. Biochem. Physiol., B 128 (3), 459–467.

Douillet, P.A., Langdon, C.J., 1993. Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae. Biol. Bull. 184, 36–51.

Emlet, R.B., 1990. Flow fields around ciliated larvae: effects of natural and artificial tethers. Mar. Ecol. Prog. Ser. 63, 211–225.

Esmann, M., 1988. ATPase and phosphatase activity of Na^+,K^+-ATPase: molar and specific activity, protein determination. Methods Enzymol. 156, 105–115.

Evans, F., Matson, S., Brake, J., Langdon, C., 2003. The effects of inbreeding on performance traits of adult Pacific oysters (*Crassostrea gigas*). Aquaculture 230, 89–98.

Fenaux, L., Strathmann, M.F., Strathmann, R.R., 1994. Five tests of food-limited growth of larvae in coastal waters by comparisons of rates of development and form of echinoplutei. Limnol. Oceanogr. 39, 84–89.

Fujio, Y., 1982. A correlation of heterozygosity with growth rate in the Pacific oyster, *Crassostrea gigas*. Tohoku J. Agric. Res. 33, 66–75.

Gabbott, P.A., Holland, D.L., 1973. Growth and metabolism of *Ostrea edulis* larvae. Nature 241, 475–476.

Gaffney, P.M., Scott, T.M., Koehn, R.K., Diehl, W.J., 1990. Interrelationships of heterzygosity, growth rate and heterozygote deficiencies in the coot clam, *Mulina lateralis*. Genetics 124, 687–699.

Gallager, S.M., 1988. Visual observations of particle manipulation during feeding in larvae of a bivalve mollusk. Bull. Mar. Sci. 43 (3), 344–365.

Gallager, S.M., Waterbury, J.B., Stoecker, D.K., 1994. Efficient grazing and utilization of the marine cynaobacterium *Synechococcus* sp. by larvae of the bivalve *Mercenaria mercenaria*. Mar. Biol. 119 (2), 251–259.

Gerdes, D., 1983a. The Pacific oyster *Crassostrea gigas*: Part I. Feeding behavior of larvae and adults. Aquaculture 31, 195–219.

Gerdes, D., 1983b. The Pacific oyster *Crassostrea gigas*: Part II. Oxygen consumption of larvae and adults. Aquaculture 31, 221–231.

Gnaiger, E., 1983. Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. In: Gnaiger, E., Forstner, H. (Eds.), Polarographic Oxygen Sensors. Springer-Verlag, Berlin, pp. 337–345.

Goulletquer, P., Wolowicz, M., Latala, A., Brown, C., Cragg, S., 2004. Application of a micro-respirometric volumetric method to respiratory measurements of larvae of the Pacific oyster *Crassostrea gigas*. Aquat. Living Resour. 17 (2), 195–200.

Haas, M., 1994. The Na–K–Cl cotransporters. Am. J. Phys., Cell Physiol. 267 (4), C869–C885.

Hadfield, M.G., Koehl, M.A.R., 2004. Rapid behavioral responses of an invertebrate larva to dissolved settlement cue. Biol. Bull. 207, 28–43.

Hannafin, J., Kinnesffran, E., Friedman, Kinne, R., 1983. Presence of a sodium–potassium chloride co-transport system in the rectal gland of *Squalus-acanthias*. J. Membr. Biol. 75 (1), 73–83.

Hawkins, A.J.S., Bayne, B.L., Day, A.J., 1986. Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis*: the basis of variable age-specific growth. Proc. R. Soc. Lond., B 229, 161–176.

Hedgecock, D., 1994. Does variance in reproductive success limit effective population size of marine organisms? In: Beaumont, A. (Ed.), Genetics and Evolution of Aquatic Organisms. Chapman and Hall, London, pp. 122–134.

Hedgecock, D., McGoldrick, D.J., Bayne, B.L., 1995. Hybrid vigor in Pacific oysters: an experimental approach using crosses among inbred lines. Aquaculture 137, 285–298.

Hedgecock, D., McGoldrick, D.J., Manahan, D.T., Vavra, J., Appelmans, N., Bayne, B.L., 1996. Quantitative and molecular genetic analysis of heterosis in bivalve mollusks. J. Exp. Mar. Biol. Ecol. 203, 49–59.

Hilden, S., Hokin, L.E., 1975. Active potassium transport coupled to active sodium transport in vesicles reconstituted from purified sodium and potassium ion-activated adenosine triphosphatase from the rectal gland of *Squalus acanthias*. J. Biol. Chem. 250, 6296–6303.

His, E., Maurer, D., 1988. Shell growth and gross biochemical composition of oyster larvae (*Crassostrea gigas*) in the field. Aquaculture 69, 185–194.

Hjort, J., 1914. Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. Rapp. P.-V. Reun. -Cons. Int. Explor. Mer. 1, 5–38.

Hoegh-Guldberg, O., Manahan, D.T., 1995. Coulometric measurement of oxygen consumption during development of marine invertebrate embryos and larvae. J. Exp. Biol. 198, 19–30.

Houlihan, D.F., 1991. Protein turnover in ectotherms and its relationship to energetics. In: Gilles, R. (Ed.), Advances in Comparative and Environmental Physiology. Springer-Verlag, New York, pp. 1–43.

Hubert, S., Hedgecock, D., 2004. Linkage maps of microsatellite DNA markers for the Pacific oyster *Crassostrea gigas*. Genetics 168 (1), 351–362.

Huntley, M., Boyd, C., 1984. Food-limited growth of marine zooplankton. Am. Nat. 124 (4), 455–478.

Jaeckle, W.B., Manahan, D.T., 1989. Growth and energy imbalance during the development of a lecithotrophic molluscan larva (*Haliotis rufescens*). Biol. Bull. 177, 237–246.

Klinzing, M.S.E., Pechenik, J.A., 2000. Evaluating whether velar lobe size indicates food limitation among larvae of the marine gastropod *Crepidula fornicate*. J. Exp. Mar. Biol. Ecol. 252, 255–279.

Koehn, R.K., Shumway, S.E., 1982. A genetic/physiological explanation for differential growth rate among individuals of the American oyster, *Crassostrea virginica* (Gmelin). Mar. Biol. Lett. 3, 35–42.

Launey, S., Hedgecock, D., 2001. High genetic load in the Pacific oyster *Crassostrea gigas*. Genetics 159, 255–265.

Leong, P.K., Manahan, D.T., 1997. Metabolic importance of Na^+/K^+-ATPase activity during sea urchin development. J. Exp. Biol. 200, 2881–2892.

Leong, P.K.K., Manahan, D.T., 1999. Na^+/K^+-ATPase activity during early development and growth of an Antarctic sea urchin. J. Exp. Biol. 202, 2051–2058.

Li, G., Hedgecock, D., 1998. Genetic heterogeneity detected by PCR-SSCP, among samples of larval Pacific oysters (*Crassostrea gigas*

Thunberg), supports the hypothesis of large variance in reproductive success. Can. J. Fish. Aquat. Sci. 55, 1025–1033.
Loosanoff, V.L., Davis, H.C., 1963. Rearing of bivalve mollusks. In: Russel, F.S. (Ed.), Advances in Marine Biology, vol. 1. Academic Press, London, pp. 1–136.
Lucas, J.S., 1982. Quantitative studies of feeding and nutrition during larval development of the coral reef asteroid *Acanthaster planci* (L.). J. Exp. Mar. Biol. Ecol. 65, 173–194.
Manahan, D.T., 1983. The uptake and metabolism of dissolved amino acids by bivalve larvae. Biol. Bull. 164 (2), 236–250.
Manahan, D.T., 1989. Amino acid fluxes to and from seawater in axenic veliger larvae of a bivalve (*Crassostrea gigas*). Mar. Ecol. Prog. Ser. 53, 247–255.
Manahan, D.T., 1990. Adaptations by invertebrate larvae for nutrient acquisition from seawater. Am. Zool. 30, 147–160.
Manahan, D.T., Crisp, D.J., 1983. Autoradiographic studies on the uptake of dissolved amino acids from sea water by bivalve larvae. J. Mar. Biol. Assoc. UK. 63, 673–682.
Manahan, D.T., Wright, S.H., Stephens, G.C., 1983. Simultaneous determination of net uptake of 16 amino acids by a marine bivalve. Am. J. Physiol. 244, R832–R838.
Manahan, D.T., Jaeckle, W.B., Nourizadeh, S.D., 1989. Ontogenic changes in the rates of amino acid transport from seawater by marine invertebrate larvae (Echinodermata, Echiura, Mollusca). Biol. Bull. 176, 161–168.
Mann, R., Gallager, S.M., 1985. Physiological and biochemical energetics of larvae of *Teredo-navalis* L and *Bankia-gouldi* (Bartsch) (Bivalvia, Teredinidae). J. Exp. Mar. Biol. Ecol. 85 (3), 211–228.
Marsh, A.G., Fielman, K.T., 2005. Transcriptome profiling of individual larvae of two different developmental modes in the poecilogonous polychaete *Streblospio benedicti* (Spionidae). J. Exp. Zool.-Mol. Dev. Evol. 304B, 238–249.
Marsh, A.G., Manahan, D.T., 1999. A method for accurate measurements of respiration rates of marine invertebrate embryos and larvae. Mar. Ecol. Prog. Ser. 184, 1–10.
Marsh, A.G., Leong, P.K.K., Manahan, D.T., 1999. Energy metabolism during embryonic development and larval growth of an Antarctic sea urchin. J. Exp. Biol. 202, 2041–2050.
May, R.C., 1973. Larval mortality in marine fishes and the critical period concept. Proc. Int. Symp. Scot. Mar. Biol. 3–19.
McGoldrick, D.J., Hedgecock, D., 1997. Fixation, segregation and linkage of allozyme loci in inbred families of the Pacific oyster, *Crassostrea gigas* (Thunberg): implications for the causes of inbreeding depression. Genetics 146, 321–334.
Moran, A.L., Manahan, D.T., 2004. Physiological recovery from prolonged 'starvation' in larvae of the Pacific oyster *Crassostrea gigas*. J. Exp. Mar. Biol. Ecol. 306 (1), 17–36.
O'Grady, S.M., Musch, M.W., Field, M., 1986. Stoichiometry and ion affinities of the Na–K–Cl cotransport system in the intestine of the winter flounder (*Pseudopleuronectes americanus*). J. Membr. Biol. 91 (1), 33–41.
Olson, R.R., Olson, M.H., 1989. Food limitation of planktotrophic marine invertebrate larvae—does it control recruitment success? Ann. Rev. Ecolog. Syst. 20, 225–247.
Pace, D.A., Manahan, D.T., 2006. Fixed metabolic costs for highly variable rates of protein synthesis in sea urchin embryos and larvae. J. Exp. Biol. 209, 158–170.
Paulay, G., Boring, L., Strathmann, R.R., 1985. Food limited growth and development of larvae—experiments with natural seawater. J. Exp. Mar. Biol. Ecol. 93 (1–2), 1–10.
Pechenik, J.A., 1987. Environmental influences on larval survival and development. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (Eds.), Reproduction of Marine Invertebrates, vol. IX. The Boxwood Press, Pacific Grove, CA, pp. 551–608.
Pechenik, J.A., Fisher, N.S., 1979. Feeding, assimilation, and growth of mud snail larvae, *Nassarius obsoletus* (Say), on three different algal diets. J. Exp. Mar. Biol. Ecol. 38, 57–80.
Platt, T., Fuentes-Yaco, C., Frank, K.T., 2003. Marine ecology: spring algal bloom and larval fish survival. Nature 423, 398–400.
Reinfelder, J.S., Fisher, N.S., 1994. The assimilation of elements ingested by marine planktonic bivalve larvae. Limnol. Oceanogr. 39, 12–20.
Rossier, B.C., Geering, K., Kraehenbuhl, J.P., 1987. Regulation of the sodium pump—how and why. Trends Biochem. Sci. 12 (12), 483–487.
Schmidt-Nielsen, K., 1997. Animal Physiology: Adaptation and Environment, 5th ed. Cambridge University Press, Cambridge, U.K. p. 612.
Shull, G.H., 1948. What is "heterosis"? Genetics 33, 439–446.
Srere, P.A., 1969. Citrate synthase. In: Lowenstein, J.M. (Ed.), Methods in Enzymology, vol. 13. Academic Press, New York, pp. 3–11.
Starr, M., Himmelman, J.H., Therriault, J.C., 1990. Direct coupling of marine invertebrate spawning with plankton blooms. Science 247 (4946), 1071–1074.
Strathmann, R.R., Leise, E., 1979. Feeding mechanisms and clearance rates of molluscan veligers. Biol. Bull. 157 (3), 524–535.
Strathmann, R.R., Fenaux, L., Sewell, A.T., Strathmann, M.F., 1993. Abundance of food affects relative size of larval and postlarval structures of a molluscan veliger. Biol. Bull. 185, 232–239.
Strickler, J.R., 1982. Calanoid copepods, feeding currents, and the role of gravity. Science 218, 158–160.
Thorson, G., 1950. Reproductive and larval ecology of marine bottom invertebrates. Biol. Rev. 5, 1–45.
Torres, G., Gimenez, L., Anger, K., 2002. Effects of reduced salinity on the biochemical composition (lipid, protein) of zoea 1 decapod crustacean larvae. J. Exp. Mar. Biol. Ecol. 277 (1), 43–60.
Vavra, J., Manahan, D.T., 1999. Protein metabolism in lecithotrophic larvae (Gastropoda: *Haliotis rufescens*). Biol. Bull. 196, 177–186.
Welborn, J.R., Manahan, D.T., 1995. Taurine metabolism in larvae of marine mollusks (Bivalvia, Gastropoda). J. Exp. Biol. 198 (8), 1791–1799.
Winberg, G.G., 1956. Rate of energy and food requirements of fishes. Fish. Res. Board Can Trans. Ser. 194, 1–202.
Zouros, E., Pogson, G.H., 1994. Heterozygosity, heterosis and adaptation. In: Beaumont, A.R. (Ed.), Genetics and Evolution of Aquatic Organisms. Chapman & Hall, London, pp. 135–146.