Predicting phenotypic variation in growth and metabolism of marine invertebrate larvae

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Abstract. Understanding the mechanisms that establish variation in growth and metabolism is fundamental in evolutionary and physiological ecology. Although a genetic basis is frequently invoked to explain variation in performance, it remains challenging to study such processes in marine animals due to the lack of genetically-enabled “model” organisms. The Pacific oyster *Crassostrea gigas* is a species for which pedigreed genetic lines have been established. In this study, a series of larval families were produced by crossbreeding pedigreed lines to yield large-volume larval cultures to provide sufficient biomass for biochemical and physiological analyses. Major phenotypic contrasts in larval growth rate were evident. A primary goal of this study was to investigate the physiological bases for this variation in growth and to identify biomarkers that are predictive of growth potential. To that end, measurements were undertaken to define the relationship between rates of growth, respiration, and ion transport by the sodium-potassium pump (*in vivo* Na^+, K^+ -ATPase activity). The relationship of respiration and ion transport during larval growth showed that, on average, 17% of total energy demand was allocated to support ion transport. Further analyses of total Na^+, K^+ -ATPase activity (*in vitro* enzyme assay) revealed that 41% of the total metabolic rate could be accounted for by this single process if all of the enzyme was physiologically active. Significant biological variation was evident, however, when size-specific comparisons were made across different larval families. These differences were up to (i) 2.2-fold in ion transport rates; (ii) 2.8-fold in the allocation of energy to support the metabolic demand of ion transport; (iii) 3.5-fold in total enzyme activity; (iv) 3.9-fold in the physiologically active fraction of total enzyme; and (v) 3.1-fold in gene expression. These differences among families highlight the need to distinguish genetic from environmental causes of biological variation. Notably, for inferences of physiological changes based upon molecular biological analyses, the measured rates of ion transport were not predicted from concurrent

measurements of gene expression or enzyme activity. Size-corrected rates of ion transport were predictive of variation in growth rates among different larval families, supporting the application of physiological rates of ion transport as a predictor of growth differences. Evolutionary variation in physiological performance has important implications for understanding the ecology of larval forms. Developing physiological indices will be of value in predicting growth and metabolism and corresponding survival of larval forms of different genotypes in response to environmental change.

Keywords: Physiology, Bivalve, Larvae, Growth, Metabolism, Ion Transport

1. Introduction

Most marine animals have complex life cycles with larval phases of development. Understanding variability in growth and metabolism of dispersive larval stages is central to many marine ecological processes (Bergenius et al., 2002; Cushing, 1990; Hare and Cowen, 1997; Houde, 2008; Shima and Findlay, 2002; Widdows, 1991). Differences in size and maternal energy reserves, for instance, impact the duration of the larval phase and subsequent recruitment potential (Lannan et al., 1980; Marshall et al., 2010; Moran and Manahan, 2004). Such biological variation generally has both environmental and genetic components, the balance of which may determine evolutionary outcomes (Lynch and Walsh, 1998). Environmental manipulations (*e.g.*, food and temperature) of growth and metabolism, using wild-type animals, have been a major theme in studies of the physiological ecology of larval forms (Kroeker et al., 2013; Marshall et al., 2010; Moran and Manahan, 2004; Widdows, 1991). However, such approaches limit the ability to separate the effects of exogenous environmental factors from endogenous, genetically-determined interactions.

It is widely accepted that developmental rates and larval growth are highly variable (Gallager and Mann, 1981; Loosanoff and Davis, 1993; Marshall and Keough, 2007; Strathmann, 1987), even among sibling larvae reared under similar environmental conditions (Pace et al., 2006). Endogenous variation in growth rate at many stages of the life cycle has a metabolic basis, since differences can be attributed to differential energy use by biological processes that support growth (Bayne and Hawkins, 1997; Hawkins and Day, 1996; Koehn and Shumway, 1982; Pace et al., 2006; Pan et al., 2015a). In our previous work with larval stages, specific physiological processes were identified that account for major components of metabolic energy demand during

development and growth (Leong and Manahan, 1997; Marsh et al., 2001; Pace et al., 2006; Pace and Manahan, 2006; Pan et al., 2015b). One of these major energy-consuming processes is ion transport by the sodium pump (Na^+, K^+ -ATPase). The regulation of ion gradients is required for many essential physiological processes of ecological relevance, such as osmotic balance (Evans et al., 2005) and nutrient uptake (Manahan, 1990). Notably, the energy required to support this single enzymatic process of Na^+, K^+ -ATPase activity can account for up to 80% of the total energy requirements of a larva (Leong and Manahan, 1997; 1999).

While the major metabolic processes that establish the cost of living in larval forms have been identified, the relationship between growth variability and such metabolic processes is not well understood. This latter issue is the focus of the current study. A starting premise for the study of standing genetic variation is to be able to produce genetically determined variation in phenotype for experimental analysis. The lack of genetically-enabled “model” marine organisms (*cf.* terrestrial organisms, such as the fruit fly *Drosophila melanogaster*, Mackay et al., 2012) has been a major constraint to understanding genotype-phenotype variation in marine larval biology (Applebaum et al., 2014; Munday et al., 2013). In the current study, pedigreed lines of the Pacific oyster *Crassostrea gigas* were used to produce larval families that were reared under similar, controlled environmental conditions. Larval families that showed contrasting growth and physiological phenotypes were used to investigate variation in a major energy-consuming process at the level of physiology (ion transport), biochemistry (enzyme activity), and molecular biology (gene expression), and to develop a potential cellular-level biomarker that is predictive of differential growth.

2. Materials and Methods

2.1. Pedigreed lines and experimental crosses

Families with varying larval growth rates were produced by controlled crosses using pedigreed lines of the Pacific oyster *Crassostrea gigas*. The adult broodstock used in these crosses resulted from a 17-year breeding program carried out by Taylor Shellfish Farms, Quilcene, WA (J. P. Davis and D. Hedgecock, personal communication; Fig. 1A), in which genetic markers were used before each cross to confirm parentage (Hedgecock and Davis, 2007; Sun et al., 2015). Using males and females from seven different pedigreed lines (labeled 1-7 in Fig. 1A), a total of 14 larval families was produced (Fig. 1B). Within a given genetic line, males and females were selected depending upon the number of adults available from this long-term breeding program and, importantly, the spawning condition of the broodstock regarding gamete availability and viability. Herein, each larval family is named according to the parental lines used for crosses. For example, larval family 2A×4A (male×female) was the result of crossing an individual male and female from lines “2” and “4”, respectively. When more than one male or female from the same pedigreed line was used to conduct pairwise crosses and start different families of larvae, such individuals were designated with a letter coding (*e.g.* male 2A, 2B; female 4A, 4B, 4C).

2.2. Larval culturing

Eggs and sperm were removed directly from the gonads (“strip spawning”) of gravid adults of known pedigree. Eggs were fertilized, checked for fertilization success, and placed at an initial concentration of 10 fertilized eggs ml⁻¹ in 200-l culture vessels, each containing 0.2-μm (pore-size) filtered seawater at 25°C. Larvae were reared in a dedicated culture facility at the University of Southern California Wrigley Marine Science Center (Santa Catalina Island,

California). Continuously flowing ambient seawater was filtered and heated with (inert) titanium heat exchangers to the required rearing temperature of 25°C. Throughout the many months required for these experiments, temperature data-loggers (HOBO U12, Onset Computer Corp., MA) were used to continuously monitor the seawater temperature in individual culture vessels. Over the experimental period tested for any given larval culture (range 10 to 20 days), the seawater temperature used to rear larvae was held constant at 25.2°C ± 0.18 (± S.E.M.) (data based on 24-h daily averages of temperature recorded every 30 min). When veliger larvae reached feeding competency on day 2 post fertilization, they were fed the algae *Isochrysis galbana* at 30 cells µl⁻¹. This feeding ration was increased to 50 cells µl⁻¹ as larvae grew (according to established protocols see Breese and Malouf, 1975; Helm et al., 2004). A complete replacement of the seawater in each of the 200-l culture vessels was performed every two days during all experiments.

2.3. Growth rate

The growth rates of 14 larval families were measured over a 10- to 20-day period (rearing duration and growth rate varied, depending on family). Larval sizes in each culture were documented by photomicroscopy. At least 50 randomly-selected larvae at each sampling interval were measured based on their shell length. Larval shell length was quantified as the distance from the anterior to posterior edge on calibrated photomicroscopic images using ImageJ (National Institutes of Health, MD). The precision of the size measurement was < 2 µm (replicate measures of the same individuals). Growth rate was calculated for each larval family from the slope of the linear regression model describing the relationship between shell length and age. A premise of this study was to produce a series of larval families that had contrasting

growth phenotypes. Figure 2 illustrates that this experimental design was successful in yielding cultures of larvae that had growth rates of low variance *within* a given family, compared to substantially different growth rate phenotypes *between* families. Major growth contrasts were evident across all 14 families. For instance, there was a 2.2-fold day⁻¹ difference between hybrid families 2A×4A and maternal line half-sibs 7×4C, resulting in a 54 µm difference in size at day 15 for these specific larval cultures (2A×4A: 126 µm and 7×4C: 180 µm).

2.4. Respiration

Respiration rates were measured as O₂ consumption as described previously (Marsh and Manahan, 1999), with modifications for larvae of *C. gigas* (Pace et al., 2006). In brief, a known number of larvae of *C. gigas* (250-500 individuals, dependent on size) was placed in a sealed micro-biological-oxygen-demand vial that contained air-saturated filtered seawater. For any given respiration measurement, 8-10 replicate respiration vials were used (volume ~600 µl; each vial was independently calibrated for volume). Incubation times ranged from 3-4 h per assay, depending upon respiration rates (size of larvae). At the end of each incubation period, oxygen in each respiration vial was measured by injecting a subsample of seawater into a temperature-controlled microcell (MC100, Strathkelvin) fitted with a polarographic oxygen sensor (Model 1302, Strathkelvin) and connected to an O₂ meter (Model 782, Strathkelvin). Oxygen sensor readings were converted to moles of oxygen using standard calibrations.

2.5. Na⁺,K⁺-ATPase activity

The activity of Na⁺,K⁺-ATPase was measured at both biochemical and physiological levels. Biochemically, the total enzyme activity was measured *in vitro* (V_{optimum} approximating to V_{max}).

Physiologically, the actual rate of ion transport by the enzyme was measured *in vivo*. The latter *in vivo* physiological activity is the key measurement to characterize the metabolic cost of ion transport by Na⁺,K⁺-ATPase. Details of the optimization of both the *in vitro* and *in vivo* assays are given below.

2.5.1 *In vitro* Na⁺,K⁺-ATPase activity

The total amount of Na⁺,K⁺-ATPase enzyme activity was measured as the ouabain-sensitive rate of inorganic phosphate (P_i) production when ATP is present (Esmann, 1988). The optimization of the assay for larvae of *C. gigas* was based on previous protocols for developing sea urchins (Leong and Manahan, 1997). For larvae of *C. gigas*, a known number of individuals was removed from each culture vessel that contained a different larval family. Each sample was placed in a 1.7-ml microcentrifuge tube, centrifuged at low speed, the seawater supernatant removed, and the larval pellet stored at -80°C pending subsequent biochemical assay. To measure *in vitro* Na⁺,K⁺-ATPase activity, larvae were sonicated (ultrasonicator fitted with a microprobe, Model VC50, Sonics & Materials, CT) in ice-cold homogenization buffer (10% sucrose, 5 mM EDTA, 50 mM imidazole, pH 7.7). Each larval homogenate was further treated by centrifugation at 4,000 g (Allegra X-30R Centrifuge, Beckman Coulter, CA) to remove shell fragments. Protein content of the supernatant was measured using a modified Bradford assay for molluscan larvae (Jaeckle and Manahan, 1989). Multiple aliquots (6-10), each containing a known amount of total protein, were used for the quantification of total enzyme activity in larvae of a given size and genotype. For each enzyme assay, an aliquot was added to the general reaction mixture (130 mM NaCl, 20 mM KCl, 10 mM MgCl₂, 5 mM ATP, 50 mM imidazole, pH 7.7), to which ouabain was added (ouabain was not added for controls). After an appropriate

incubation time (see below), each reaction assay was terminated by the addition of 5% ice-cold trichloroacetic acid. Ammonia molybdate and 8-anilino-1-naphthalenesulfonic acid were added to the reaction mixture (Peterson, 1978). (All chemicals were obtained from Sigma-Aldrich, MO.) After a 30-min incubation, the amount of inorganic phosphate was determined from absorbance measured at 700 nm on a microplate reader (SpectraMax M2, Molecular Devices, CA). The difference in rates of inorganic phosphate production between control (no ouabain) and ouabain-treated samples represents total *in vitro* Na⁺,K⁺-ATPase activity.

This enzyme assay for larvae of *C. gigas* was optimized in a series of preliminary experiments with multiple combinations of the amount of enzyme in each assay (2-20 µg total protein), the concentration of the inhibitor ouabain (Fig. 3A: 0.5-12 mM ouabain), the duration of the assay (10-30 min), and the incubation temperature (Fig. 3B: 15-31°C). Based on this series of tests, an incubation time of 30 min at 25°C with 10 mM ouabain was chosen for all subsequent measurements of total *in vitro* Na⁺,K⁺-ATPase activity in larvae of *C. gigas*.

2.5.2. *In vivo* Na⁺,K⁺-ATPase activity

The total enzyme activity measured *in vitro* using the above optimization steps represents the maximum capacity of Na⁺,K⁺-ATPase, but does not describe the *in vivo* physiological activity in living cells. The actual physiological rate of ion transport (*in vivo* Na⁺,K⁺-ATPase activity) by larvae was measured as the ouabain-sensitive transport rate of rubidium ion from seawater (⁸⁶Rb⁺ is a physiological analog of K⁺, Hilden and Hokin, 1975). A known number of larvae was placed in a 20-ml glass vial containing 10 ml of filtered seawater, with or without ouabain added. For assays with ouabain, a pre-incubation period of at least 30 min was allowed before the start

of each transport assay (*i.e.*, before the addition of $^{86}\text{Rb}^+$ isotope). To start the assay, 0.9 MBq of $^{86}\text{Rb}^+$ (Perkin Elmer, CA) was added to the incubation medium. A ~10-min time-course transport assay was conducted, during which a series of 5-6, 1-ml aliquots was taken (Fig. 4A). Each aliquot of seawater containing larvae was passed through an 8- μm membrane filter (Whatman, GE Healthcare, NJ) and washed with 10 ml of filtered seawater to remove excess radioactivity. The filter with larvae was then placed in a 7-ml scintillation vial containing 500 μl of tissue solubilizer (Solvable, Perkin Elmer, CA). After an overnight solubilization period, liquid scintillation cocktail (Ultima Gold, Perkin Elmer, CA) was added, and the amount of radioactivity was quantified using liquid scintillation counting (Model LC 6000SC, Beckman Coulter, CA). The rate of $^{86}\text{Rb}^+$ transport was obtained from the slope of the linear regression describing the relationship between $^{86}\text{Rb}^+$ transport and assay time (Fig. 4A). Potassium transport rate was calculated by correcting the rate of $^{86}\text{Rb}^+$ transport by the specific activity of K^+ in 33.5‰ ambient Pacific Ocean seawater (Todd et al., 2009) used in the larval culture facility on Santa Catalina. For these *in vivo* assays on living larvae, a concentration of 2 mM ouabain was sufficient to inhibit Na^+, K^+ -ATPase activity based on tests using different concentrations of ouabain to optimize the *in vivo* assay (Fig. 4B). The difference in rates of K^+ transport between control (no ouabain) and ouabain-treated samples represents *in vivo* Na^+, K^+ -ATPase activity.

2.6. Na^+, K^+ -ATPase gene expression

The expression of the gene Na^+, K^+ -ATPase α subunit in larvae of *C. gigas* was determined using quantitative, real-time polymerase chain reaction (qPCR) assays. Three replicate samples of larvae were collected from each culture vessel that contained a given larval family. Each of the

three replicate samples was then assayed in duplicate by qPCR. Total RNA was extracted using TRIzol reagent (Life Technologies, CA) according to the manufacturer's instructions; extracts were treated with RNase-free DNase and purified with a RNA Clean and Concentrator kit (Zymo Research, CA). Samples were eluted from purification columns in nuclease-free water. RNA concentration in the eluate was determined from absorption at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, TX). DNA-free RNA was reverse transcribed to cDNA using SuperScript III reverse transcriptase (Life Technologies, CA) and random hexamers. Reverse-transcribed cDNA was then diluted 1:5 with nuclease-free water. Steady-state amounts of the gene *Na⁺,K⁺-ATPase α* subunit (GenBank accession no. **XM_011442971**) were determined by qPCR using primers with sequences as follows: Forward – ACACACTGACCTCCAACATCCC; Reverse – TTGGCAGCACTGGTATCCTG. These primers were designed based upon the published sequence for the *Na⁺,K⁺-ATPase α* subunit in *C. gigas* (Zhang et al., 2012). Expression of *Na⁺,K⁺-ATPase α* subunit was normalized to expression of a reference gene, *60S ribosomal protein L7 (RL7)* (GenBank accession no. **AJ557884**). This reference gene has been validated as a “housekeeping gene” (Du et al., 2013) for use as an internal control for qPCR studies of gene expression in developmental stages of *C. gigas*. The following primer sequences were used to amplify *RL7*: Forward – TCCCAAGCCAAGGAAGGTTATGC; Reverse – CAAAGCGTCCAAGGTGTTTCTCAA. SYBR Advantage qPCR Premix (Clontech, CA) was mixed with primers (0.2 μ M each), cDNA template, and nuclease-free water added to a final volume of 25 μ l. Samples were cycled on a MX3005P thermocycler (Agilent Technologies, CA) for an initial denaturation of 120 sec at 95°C, followed by 40 cycles of 5 sec at 95°C, and 30 sec at 60°C. Standard curves created from plasmids containing clones of *C. gigas Na⁺,K⁺-ATPase α* or *RL7* were used to determine the

number of transcript copies per sample. Relative gene expression is presented as copies of Na^+, K^+ -ATPase α per copy of *RL7*.

2.7 General statistical analysis and calculation of ion pump activity

Standard statistical tests, using analysis of variance (ANOVA), were applied to determine differences in growth rates ($n = 200$ -250 individual measurements per larval family), respiration rates ($n = 8$ -10 per developmental stage), *in vitro* Na^+, K^+ -ATPase activity ($n = 6$ -10 per family), and levels of gene expression ($n = 3$ per developmental stage).

The *in vitro* enzyme activity of total Na^+, K^+ -ATPase was calculated as the difference between rates measured with and without the specific inhibitor ouabain. For example, the rates measured with and without ouabain at 31°C were 20.4 ± 1.50 (S.E.M., $n = 5$) and 25.2 ± 1.57 ($n = 5$) $\mu\text{mol P}_i \text{ mg protein}^{-1} \text{ h}^{-1}$, respectively (Fig. 3B). The difference between these two ATPase assays is the activity of Na^+, K^+ -ATPase, calculated by subtraction to be $4.8 \mu\text{mol P}_i \text{ mg protein}^{-1} \text{ h}^{-1}$ (indicated by an asterisk symbol in Fig. 3B, 3C).

Statistical differences for *in vivo* Na^+, K^+ -ATPase activity between larval families were evaluated based on comparisons using 95% confidence intervals. The *in vivo* ion transport assays of physiological Na^+, K^+ -ATPase activity were calculated as the difference between the regression slopes of two time-course experiments conducted with and without ouabain (Fig. 4A). Error associated with each slope was propagated for the calculation of 95% confidence intervals using standard methods based on propagating variances and sample sizes (Taylor, 1997), with modifications for regression-based errors (use of S.E. of slopes *cf.* S.E.M, with the appropriate

statistical significance level for a given sample size, $\alpha = 0.05$).

3. Results

3.1 Growth rates

Crossbreeding pedigreed lines (Fig. 1A) from a 17-year breeding program produced 14 larval families (Fig. 1B). Daily growth rate for each larval family was calculated from the slope of a regression model of increases in shell length with time ($\pm$ S.E., $n = 200$ -250 individual measurements, depending upon length of time each larval family was reared, range 10-20 days).

A wide range of contrasting growth phenotypes is evident in Figure 2 for larval families grown under similar environmental conditions, with a 2.2-fold difference in daily growth rates for maternal line half-sib families ($3.7 \pm 0.18 \mu\text{m d}^{-1}$ in family 2A $\times$ 4A, to $8.2 \pm 0.21 \mu\text{m d}^{-1}$ in family 7 $\times$ 4C). The total dataset analyzed in Fig. 2 was based on more than 5,500 individual larval size measurements across all 14 families (slope comparisons: $F_{13, 5523} = 80.7$, $p < 0.0001$). The ANOVA followed by *post hoc* analyses of all primary data revealed that many of the larval families have different growth rates (illustrated by the letter-groupings shown on Fig. 2) when cultured under similar rearing conditions. Another noteworthy difference in ranking of growth rates occurred among four larval families from crosses of a single male from line 7 with four different females (females 3, 4B, 4C, and 6) (Fig 1B; Fig. 2).

3.2 Optimization of *in vitro* enzyme assay

Optimal conditions of the Na^+, K^+ -ATPase enzyme assay were determined for larvae of *C. gigas* by varying assay time, ouabain concentration, temperature, the amount of enzyme, and the amount of a demasking chemical agent (resulting in Fig. 3A, B). A ouabain concentration of 10

mM was required to saturate inhibition of total Na^+, K^+ -ATPase activity. As Na^+, K^+ -ATPase is only one of many ATPases in an organism, Figure 3A shows that 35% of the total ATPase is Na^+, K^+ -ATPase. The value of Na^+, K^+ -ATPase activity was confirmed with additional assays, using the demasking chemical agent sodium deoxycholate (concentration range: 0.01-0.80 mg ml^{-1}) to check for the possibility of concealed activity due to different orientations (outside-in; inside-out) of membrane vesicles in larval tissue homogenates (Leong and Manahan, 1997). Preliminary assays for total Na^+, K^+ -ATPase with homogenates of larvae of *C. gigas* revealed that sodium deoxycholate did not result in differences in activity and, hence, this additional chemical step was not necessary. Total Na^+, K^+ -ATPase activity increased with incubation temperature between 15°C and 31°C (Fig. 3B). The sensitivity of the enzyme to temperature increase is shown in Fig. 3C, from which a Q_{10} value of 2.6 is calculated. A temperature of 25°C was chosen for the *in vitro* assay of Na^+, K^+ -ATPase because this temperature differentiated between Na^+, K^+ -ATPase and other ATPases and permitted direct comparison with measures of *in vivo* ion transport rate and respiration rate (also conducted at 25°C). Further, this choice of temperature allowed for a direct calculation of the physiologically active fraction of Na^+, K^+ -ATPase (the ratio of *in vivo* to *in vitro* activity) and the percent of metabolism attributable to ion transport.

3.3 *In vivo* activity of Na^+, K^+ -ATPase

Figure 4A is an example of *in vivo* ion transport assays undertaken for larvae of *C. gigas*. To determine *in vivo* K^+ transport (measured as $^{86}\text{Rb}^+$ transport), duplicate time-course assays ($n = 5-6$ time points per assay) were conducted. A statistical analysis was performed to compare the slope of regression model of K^+ transport and time for each duplicate. When no significant difference was found, these two regressions were pooled into a single rate as shown in Fig. 4A.

This procedure was repeated for each set of measurements, using a different concentration of ouabain. A concentration of 2 mM ouabain was found to saturate inhibition of *in vivo* Na^+, K^+ -ATPase, which represented ~80% of the total K^+ transport by larvae (Fig. 4B).

3.4 Comparison of *in vitro* and *in vivo* assays of Na^+, K^+ -ATPase

Ouabain is a well-known, specific inhibitor of Na^+, K^+ -ATPase (Laursen et al., 2013; Ogawa et al., 2009). For larvae of *C. gigas*, there were differences between the concentrations required to inhibit Na^+, K^+ -ATPase under *in vitro* and *in vivo* conditions. A concentration of 10 mM ouabain was required to saturate inhibition under *in vitro* conditions (Fig. 3A). Under *in vivo* conditions, saturation of inhibition occurred at 2 mM ouabain (Fig. 4B). A difference in ouabain concentrations has also been reported between *in vivo* and *in vitro* for amphibian brain tissue (Morris et al., 1997). There were also differences in the percent inhibition by ouabain in larvae of *C. gigas* under *in vitro* (35%) and *in vivo* conditions (80%). These inhibition values are in agreement with transport assays, using fertilized eggs of mice (Van Winkle and Campione, 1991) and developmental stages of sea urchin (Leong and Manahan, 1997; Pan et al., 2015b). These results from assay optimization steps in *C. gigas* demonstrate the need to pre-establish the dose-response relationships of the inhibitor used.

3.5 Ontogenetic changes in physiological rates

Respiration and ion transport (*in vivo* Na^+, K^+ -ATPase activity) were measured during growth, for larvae with shell lengths ranging from 100 to 200 μm (Fig. 5). Respiration and ion transport (expressed in ATP equivalents) increased significantly during growth (respiration: regression ANOVA, $F_{1,25} = 42.7$, $p < 0.001$, $r^2 = 0.63$; *in vivo* Na^+, K^+ -ATPase: $F_{1,25} = 40.8$, $p < 0.001$, $r^2 =$

0.62). The relationship of respiration and ion transport during growth is described by parallel regression lines (slope comparison: $F_{1, 52} = 0.1, p = 0.75$) with different intercepts (intercept comparison: $F_{1, 53} = 50.4, p < 0.001$). This demonstrates that the fraction of total metabolic rate of a larva accounted for by the energy demand of ion transport is constant during growth: on average, 17% of the total ATP pool was allocated to support the physiological activity of ion transport by Na^+, K^+ -ATPase (Fig. 5).

3.6 Family-specific variation at different levels of biological organization

As above, the rates of respiration and ion transport were measured in larvae from all 14 families (Fig. 5). A subset of nine of these 14 families was chosen for more detailed analyses of family-specific differences in respiration, *in vivo* rates of ion transport, *in vitro* total enzyme activity of Na^+, K^+ -ATPase, and gene expression. To account for a possible influence of differences in size on physiological rates, these nine larval families (data points between vertical dash lines in Fig. 5) were selected based on their near-identical shell lengths ($145.5 \pm 1.31 \mu\text{m}$, mean $\pm$ S.E.M.).

Table 1 lists for each of the nine families the *in vivo* activity of Na^+, K^+ -ATPase (column I), the *in vitro* total activity of the enzyme (column II), the relative expression of the Na^+, K^+ -ATPase gene (column III), the rate of respiration expressed as ATP equivalents (column IV), the percent of the total enzyme activity that was physiologically active *in vivo* (column V), the percent of the total ATP pool (respiration) that could be accounted for by the *in vitro* total enzyme activity (column VI), and the percent of the total ATP pool that could be accounted for by the *in vivo* activity of Na^+, K^+ -ATPase (column VII). The ranking of the nine families shown is based on the *in vivo* activity rates of Na^+, K^+ -ATPase given in column I. It is evident from the data in Table 1

that the maximum fold-differences in activity for several important processes at the levels of molecular biology, biochemistry, and physiology are family-specific. These findings and tests for statistically significant differences are given in Table 1 and are summarized as follows:

1. There is up to a 2.2-fold difference in the size-specific rate of ion transport between families (slowest rate in family 4B×3 = 26.3 ± 4.41 pmol P_i larva⁻¹ h⁻¹; fastest rate in family 2B×4C = 58.6 ± 6.84 pmol P_i larva⁻¹ h⁻¹).
2. Variance in the total amount of enzyme (column II, maximum 3.5-fold) does not predict physiological rate of ion transport (column I) (Pearson Correlation: $r = 0.29$, $p = 0.46$, $n = 9$).
3. Changes in the relative gene expression of Na^+, K^+ -ATPase α (column III, maximum 3.1-fold) do not predict the total amount of enzyme (column II, $r = -0.36$, $p = 0.34$, $n = 9$), nor the physiological rate of ion transport difference among families (column I, $r = -0.12$, $p = 0.75$, $n = 9$).
4. There is up to a 1.8-fold difference between maximum and minimum respiration rates for different larval families (slowest rate in family 2B×4C = 171.5 ± 6.46 pmol ATP larva⁻¹ h⁻¹; fastest rate in family 7×6 = 314.0 ± 14.53 pmol ATP larva⁻¹ h⁻¹).
5. While the average of total enzyme that is physiologically active is 53% across different families, the percent activity between maternal half-sib families varied 3.9-fold (column V: lowest in family 4B×3 = 22%; highest in family 2B×3 = 86%).
6. Metabolically, if all of the enzyme was physiologically active, on average 41% of the total ATP pool could be accounted for by this single process. Again, considerable variation is evident across different families, with *in vitro* Na^+, K^+ -ATPase activity potentially accounting for 21% to 63% of total metabolism (column VI).

7. In terms of actual metabolic cost of ion transport, the metabolic allocation to *in vivo* activity of Na^+, K^+ -ATPase across these nine families averaged 20%, with a biological variance ranging from 12% to 34% in families (2B×4B and 2B×4C, respectively) (column VII). Notably, this 2.8-fold difference was a result of crossing one male with two females from the same pedigreed line (genetic “sisters”), which warrants consideration of the relative importance of genetic and environmental influences on physiological processes.

4. Discussion

Understanding variability in physiological rates and the mechanisms of differential allocation of cellular energy is central to the study of metabolic regulation during development and growth. In summary, the approach taken in the present study was to produce family-specific growth contrasts to investigate potentially endogenous biological bases of growth variation in larvae. Identifying key mechanisms could lead to the identification of biomarkers for those processes that predict variation in larval growth. Experimental crossbreeding of pedigreed lines of the Pacific oyster (*Crassostrea gigas*) (Fig. 1) resulted in a series of larval families that showed substantial variation in larval growth (Fig. 2) and ion transport by the Na^+, K^+ -ATPase (Table 1) – a major energy-consuming physiological process in animals (Rolfe and Brown, 1997). The wide range of growth and physiological contrasts observed across different larval families allowed for an assessment of ion transport rate as a biomarker for growth potential (Fig. 6). Importantly, a quantitative analysis of possible underlying mechanisms of family-specific differences in rates of ion transport revealed that physiological variation was not predicted from changes in the amount of total enzyme activity nor gene expression (Table 1, Fig. 7).

4.1 Rates of transport as predictors for larval growth potential

The 14 families analyzed in this study exhibited more than a 2-fold difference in growth rates (Fig. 2). Given the universal importance of Na^+, K^+ -ATPase in animals cells and the significant amount of cellular energy consumed by this single enzyme, the relationship between *in vivo* Na^+, K^+ -ATPase activities and differences in growth rates of larvae was examined further. A regression between size-specific *in vivo* Na^+, K^+ -ATPase activity and growth rates showed that 43% of the variation in growth was explained by changes in ion transport rate by Na^+, K^+ -ATPase (Fig. 6: $r^2 = 0.43$, $F_{1, 25} = 18.9$, $p < 0.001$). We reported previously (Pan et al., 2015a) that increased amino acid transport capacity in larvae of *C. gigas* predicted genetically determined higher growth rates. In that study, amino acid transport capacity of early-stage larvae from seven different families accounted for 70% of the variation in growth rate. In marine larvae, sodium/amino acid co-transport across the body wall is driven by the electrochemical gradient between seawater and cellular epithelia, maintained by Na^+, K^+ -ATPase (Leong and Manahan, 1997; Wright and Manahan, 1989). For early developmental stages of *C. gigas*, ouabain has been shown to inhibit amino acid transport (Manahan, 1983a). Physiologically, the link now identified between sodium-potassium transport and growth differences suggests that higher rates of ion transport in faster-growing larvae may be related to increased rates of amino acid transport. Additionally, higher size-specific feeding rates on algae by larvae of different genotypes are known to be related to faster growth (Pace et al., 2006). Combined measurements of ion flux, amino acid transport, and feeding rates offer new approaches to predict the physiological state and growth potential of larvae under a range of environmental conditions.

Even after decades of work, it still remains technically challenging to make precise physiological

measurements of solute transport rates in larval forms (Manahan, 1983b; Pan et al., 2015a). The small size of larvae necessitates detection methods that have high sensitivity (*e.g.*, use of radioactively-labeled substrates), and large-scale culturing of larvae is required to provide sufficient biomass for *in vivo* transport assays. A possible simplification is the application of gene- or protein-based markers *in lieu* of needing to make *in vivo* physiological assays. Such biomarkers would be of assistance for field-based measurements on individual larvae and – given the global importance of Pacific oyster culture (FAO, 2014) – would be of immense value in commercial hatchery operations that need to predict growth and other complex traits (*cf.* assays for seeds and crop yield in agriculture).

4.2 Size- and family-specific variation in Na^+, K^+ -ATPase physiology, enzyme activity, and gene expression

The availability of a sequenced genome for *C. gigas* (Zhang et al., 2012) facilitated identification of potential biomarkers at the molecular biological level that could be used to infer physiological rates. In our previous study on family-specific growth and amino acid transport (Pan et al., 2015a), the potential to use gene-based markers for transport physiology was complicated at the genomic level by the large number of different transporter genes in *C. gigas*, even for a single family of neutral amino acid transporters (23 genes identified: Table 1, Pan et al., 2015a). In the present study of sodium-potassium transport, only one gene (GenBank accession no. [XM_011442971](#)) that encodes the Na^+, K^+ -ATPase α subunit protein (the major functional subunit) is present in the genome of *C. gigas* (Zhang et al., 2012). The relative genomic simplicity (*i.e.*, a single gene) of sodium-potassium transport in *C. gigas*, combined with the protocol developed in the present study for *in vitro* measurement of total Na^+, K^+ -ATPase

enzyme activity, allows an evaluation of the relationship between different levels of biological organization (gene, protein, physiology) to identify possible biomarkers for growth.

A wide range of larval sizes (Fig. 5) studied allowed for selection of a specific size class for multiple families to compare variation in ion transport at different levels of biological organization (Table 1). Even within the narrow size range selected (average $145.5 \pm 1.31 \mu\text{m}$, to remove size variation as a confounding factor), substantial variation is evident in rates of ion transport (Table 1: column I), in total enzyme activity (column II), and in gene expression (column III). Notably, the pattern of variation at the level of gene expression and total enzyme activity (amount of protein) did not predict the actual physiological rate of ion transport (Fig. 7). These data strongly suggest that inferences of physiological rates based on measurements of transcripts or amounts of individual proteins (enzyme activity, in this case) should be interpreted with caution. Interestingly, gene expression did correlate negatively with growth rate for the nine families tested ($r = -0.90$, $p = 0.001$, $n = 9$). Since gene expression patterns did not, however, explain variation in ion transport rate or enzyme activity, there is not an obvious functional mechanism at the biochemical or physiological level that can account for this negative correlation between gene expression and growth rate. Nonetheless, further evaluation of differential expression of the sodium pump gene may offer the possibility of an additional biomarker for variation in growth (see feeding rate and amino acid transport as discussed above: Pace et al., 2006; Pan et al., 2015a).

If neither gene expression nor enzyme activity explains the variation in ion transport rate, what might be the potential mechanism that regulates Na^+, K^+ -ATPase physiology? Table 1 shows that

regulation is likely to be kinetic, with changes in the amount of physiologically active Na^+, K^+ -ATPase in different larval families. Comparison of *in vivo* transport rate (Table 1, column I) with total enzyme activity (column II) shows that, on average, about half (53%) of the total Na^+, K^+ -ATPase activity in all nine families was physiologically active (Table 1, column V). This average of 53% for larvae of *C. gigas* agrees well with the value of 51% for larvae of the sea urchin, *Strongylocentrotus purpuratus* (Leong and Manahan, 1997). A striking consistency between these ~50% values indicates that the physiological activity of Na^+, K^+ -ATPase is about half of maximum velocity ($V_{\max}$) – *i.e.*, the enzyme is operating near K_m of a Michaelis-Menten model during development and growth of marine invertebrates. This finding is consistent with the long-standing analyses of enzyme activity operating near K_m (Hochachka and Somero, 2002). An important new insight that emerges from the current study is that, underlying the average value of 53%, is a significant, 3.9-fold range (22% to 86%) of physiologically active enzyme in different larval families (Table 1, column V). This finding extends previous studies of ion transport physiology with wild-type animals (Foskett and Scheffey, 1982; Hwang and Lee, 2007; Leong and Manahan, 1997; Melzner et al., 2009; Shumway, 1977) by showing that the “error” in the range of reported rates is not all analytical. Another example is the substantial 2.2-fold (220%) variation in the rate of ion transport across all families (Table 1, column I). Most of that variation, however, was biological, since within a single family the maximum analytical variation was only 19% (family 7×6: ratio of error to mean, 9.35/50.1). For physiological studies, the use of pedigree lines in experimental crosses offers a distinctive approach to distinguish between analytical error and important biological variation, and also to partition such variance into genetic and environmental components.

The standing genetic variation in physiology of *C. gigas*, shown by these crossbreeding experiments, likely has implications for adaptive responses to environmental change. The differences between *in vivo* and total Na^+, K^+ -ATPase activity represent regulatory potential to meet increased physiological demands. In developmental stages of *S. purpuratus*, for instance, the addition of an ionophore (monensin), or an amino acid (alanine), results in increased *in vivo* Na^+, K^+ -ATPase activity from ~51% (near K_m) to ~90% (near V_{max}) of total activity (Leong and Manahan, 1997). For the same species of sea urchin, the physiological response to seawater acidification resulted in an 1.4-fold increase of *in vivo* Na^+, K^+ -ATPase activity that occurred in the absence of any change in total enzyme activity, demonstrating the importance of regulatory responses to environmental change (Pan et al., 2015b). Genotype-dependent “fine-tuning” of physiological rates likely has evolutionary implications for understanding variation of organismal fitness in response to rapid rates of environmental change.

4.3 Family-specific variation in metabolic costs of Na^+, K^+ -ATPase

During larval growth, the relationship between size, respiration, and ion transport reveals the average metabolic allocation of the ATP pool to ion transport (Fig. 5). This finding highlights the importance of maintaining a fixed allocation of cellular energy to ion transport, even when many complex traits and physiological processes are changing during development and growth. The average value for ATP allocation to ion transport agrees well with published values for cultured cells and isolated tissues of vertebrates ranging from 10% to 25% (Buttgereit and Brand, 1995; Deigweiher et al., 2010; Hulbert and Else, 2000; Rolfe and Brown, 1997; Siems et al., 1992). The values we report here for larvae of *C. gigas* are consistent with other developmental stages of marine invertebrates (Leong and Manahan, 1997; Pan et al., 2015b). Particularly

noteworthy in this regard is that the average metabolic allocation of 20% to ion transport in 145 μm larvae (Table 1), which is based on a new ouabain inhibition assay for larvae of *C. gigas* (Fig. 3, 4), is consistent with our previous report (Pace et al., 2006) for ion transport in similar-sized larvae (150 μm), which was based on a ouabain-independent assay. Using the rates of ion transport and respiration measured in Pace et al. (2006), we calculate that 21% of the ATP pool was allocated to support ion transport. This cross comparison of studies with and without ouabain validates the protocols used in the current study, as does the Q_{10} value of 2.6 for *in vitro* studies of the Na^+, K^+ -ATPase (Fig. 3C).

Our use of crossbreeding experiments has now extended these prior studies, by showing that the range around the average value of ATP allocation to ion transport in similar-sized larvae has a family-specific basis, accounting for a 2.8-fold variation (12% to 34%) in the amount of the ATP pool allocated to ion transport (Table 1, column VII). Added to the average value of 20% ATP supporting ion transport across all families, our recent analysis shows that protein synthesis accounts for ~50% of the ATP pool in larvae of *C. gigas* (Lee et al., 2016). Combined, these estimates show that over 70% of metabolic rate in larvae of this species can be accounted for by just two processes – ion transport and protein synthesis. Major changes in the allocation of energy from a fixed ATP pool to ion transport and protein synthesis are important homeostatic response mechanisms under environmental stress (Pan et al., 2015b). Our analyses of ion transport in larvae of *C. gigas* demonstrate that biological variation in rates and costs of a fundamental physiological process may be genetically determined. Understanding how combinations of genetic and environmental factors impact trade-offs and alter metabolic allocation strategies will help predict resilience and potential for adaptation to environmental

change.

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Figure Legends

Figure 1. (A) Pedigreed lines resulting from a 17-year breeding program were used for controlled crosses. Closed squares indicate individual males; open circles indicate females. (B) Controlled crosses using males and females from the seven pedigreed lines produced 14 viable larval families. Within a given line, individuals were selected depending upon the number of adults available from this long-term breeding program and the spawning condition of the broodstock (gamete abundance and viability). When more than one male or female from the same pedigreed line was used to start a family of larvae, such individuals were designated with a letter coding (*e.g.* male 2A, 2B; female 4A, 4B, 4C).

Figure 2. Growth rates of 14 larval families produced from experimental crosses using pedigreed parental lines. Larval families were named according to the parental lines used. For example the fastest-growing larval family was a cross of parents 7×4C (sire×dam), the result of crossing an individual male and female from lines “7” and “4”, respectively. When more than one male or female from the same pedigreed line was used to start families of larvae, such individuals were designated with a letter coding (*e.g.*, female 4A, 4B, and 4C). Growth rate ($\mu\text{m d}^{-1}$) for each larval family represents slope $\pm$ S.E. of the linear regression model describing the relationship between shell length and age.

Figure 3. Optimization of assay for *in vitro* total Na^+, K^+ -ATPase activity. (A) Inhibition of total ATPase activity as a function of ouabain concentration. Percent inhibition was calculated based on rates measured at different concentrations of ouabain ($n = 3-6$ assays at each concentration). A concentration of 10 mM ouabain was required to saturate the inhibition of Na^+, K^+ -ATPase

activity. (B) Effect of temperature on ATPase activity measured in the presence (closed bars) or absence (open bars) of 10 mM ouabain. Means $\pm$ S.E.M. ($n = 3-6$) are shown. The symbol “*” indicates the difference in activity measured with and without ouabain, *i.e.*, *in vitro* total Na^+, K^+ -ATPase activity. (C) Determination of temperature coefficient (Q_{10}). The data point with the symbol “*” indicates Na^+, K^+ -ATPase activity calculated from values in Panel B. Regression model: $\text{Log}(\text{Na}^+, \text{K}^+\text{-ATPase activity}) = 0.420 \times \text{temperature} + 0.002$ ($F_{1,4} = 305.85$, $p < 0.001$, $r^2 = 0.99$). The value of Q_{10} is 2.6, based on the slope of the regression (Schmidt-Nielsen, 1997), where T_n is the enzyme activity measured at 18, 21, 24, 27, and 31°C, and T_1 is the activity measured at 15°C.

Figure 4. *In vivo* Na^+, K^+ -ATPase activity assay. (A) Time-course of K^+ transport measured as $^{86}\text{Rb}^+$ uptake corrected for the specific activity of K^+ in seawater. For each rate determination, duplicate time-course assays were conducted, each using a separate aliquot of larvae. Comparison of slopes was performed, and these two regressions were pooled into a single rate when no significant difference was found. No ouabain (control): K^+ transport = $0.67 \times \text{time} + 1.78 \times 10^{-15}$ ($F_{1,10} = 89.05$, $p < 0.001$, $r^2 = 0.90$); 2 mM ouabain: K^+ transport = $0.14 \times \text{time} + 0.03$ ($F_{1,9} = 46.31$, $p < 0.001$, $r^2 = 0.84$). (B) Percent inhibition of K^+ transport was calculated using rates measured at different concentrations of ouabain. Each rate (closed circle: no ouabain, control; closed triangle: 2 mM ouabain) was calculated from the slope of K^+ transport assays as shown in Panel A. A concentration of 2 mM ouabain was required to saturate the inhibition of Na^+, K^+ -ATPase-mediated K^+ transport.

Figure 5. Ontogenetic changes in respiration (closed circles) and *in vivo* Na^+, K^+ -ATPase activity

(open circles). Respiration measured as O₂ consumption was converted to ATP equivalents calculated using 5.2 moles ATP (mole O₂)⁻¹. Regression models: Log (respiration) = 2.39 × log (shell length) - 2.83 ($F_{1, 25} = 42.74$, $p < 0.001$, $r^2 = 0.63$). Log (*in vivo* Na⁺, K⁺-ATPase activity) = 2.28 × log (shell length) - 3.37 ($F_{1, 25} = 40.76$, $p < 0.001$, $r^2 = 0.62$). Each data point for respiration (closed circles) represents 8-10 independent rate measurements for larvae of the specified size (for graphical simplicity, error bars are not shown). Each data point for *in vivo* Na⁺,K⁺-ATPase activity (open circles) is based on time-course assays shown in Fig. 4A. Vertical dash lines represent the size range (145.5 ± 1.31 μm, mean ± S.E.M.) of larvae used for the analyses in Table 1.

Figure 6. Physiological index of growth. Relationship of growth rate and size-specific *in vivo* Na⁺,K⁺-ATPase activity for 14 larval families. Each data point represents an *in vivo* Na⁺,K⁺-ATPase activity measurement (Fig. 4A), determined from a total of four ion transport assays, two with ouabain and two without ouabain, on larvae of known size from a given family. Solid symbols represent a set of assays on each of the 14 families; open circles represent replicate assays on those same families performed at a later stage of development (2 to 10 days later, depending upon family). The rate of ion transport was size corrected (per μm shell length). Linear regression: Growth rate = 11.2 × (size-specific rate) + 2.6; $r^2 = 0.43$, $F_{1, 25} = 18.9$, $p < 0.001$.

Figure 7. Biological variation in Na⁺,K⁺-ATPase activity measured concurrently at the levels of physiology (ion transport rate), enzyme (total *in vitro* activity), and gene (relative expression). The height of each bar is based on measured values from Table 1 (column I to III) for nine larval

families. As in Table 1, the physiological ion transport rate is ranked, from left to right, lowest to highest (solid bars). Corresponding changes in enzyme activity and gene expression are indicated for each larval family, with highest and lowest ranking shown as solid bars. This figure diagrammatically illustrates that the physiological rankings of actual ion transport rates are not predicted from the corresponding changes in amount of enzyme or gene expression for all larval families tested. Larval families 1-9 represent from Table 1 families 4B×3, 2B×4B, 2B×3, 7×3, 2A×4A, 2A×5, 7×6, 2B×6, 2B×4C, respectively.

Table 1. *In vivo* and total activity of Na⁺,K⁺-ATPase, relative gene expression of *Na⁺,K⁺-ATPase α*, respiration, and allocation of ATP to Na⁺,K⁺-ATPase activity of similar-sized larvae from nine families

	I	II	III	IV	V	VI	VII
Family	<i>In vivo</i> activity (pmol P _i ind ⁻¹ h ⁻¹)	Total activity (pmol P _i ind ⁻¹ h ⁻¹)	Gene expression (<i>NKAα/RPL7</i>)	Respiration (pmol ATP ind ⁻¹ h ⁻¹)	Physiologically active fraction (%)	ATP allocation to total activity (%)	ATP allocation to <i>in vivo</i> activity (%)
4B × 3	26.3 ± 4.41	119.5 ± 15.14	4.8 ± 0.41	189.9 ± 11.29	22	63	14
2B × 4B	28.4 ± 5.08	53.5 ± 3.13	7.3 ± 1.41	238.9 ± 19.88	53	22	12
2B × 3	36.6 ± 2.32	42.4 ± 15.89	4.9 ± 0.42	198.5 ± 10.62	86	21	18
7 × 3	39.6 ± 5.61	110.9 ± 10.02	7.6 ± 0.95	(220)	36	50	18
2A × 4A	41.4 ± 3.63	72.0 ± 6.05	10.6 ± 1.09	181.7 ± 22.51	57	40	23
2A × 5	47.2 ± 3.79	75.1 ± 16.37	8.7 ± 0.86	230.6 ± 14.89	63	33	20
7 × 6	50.1 ± 9.35	89.8 ± 5.19	3.4 ± 0.62	314.0 ± 14.53	56	29	16
2B × 6	53.4 ± 6.33	149.8 ± 12.49	4.1 ± 0.16	254.3 ± 20.33	36	59	21
2B × 4C	58.6 ± 6.84	89.0 ± 16.57	6.3 ± 0.93	171.5 ± 6.46	66	52	34
Mean	42.4	89.1	6.3	220.0	53	41	20
Maximum fold-difference	2.2	3.5	3.1	1.8	3.9	3.0	2.8

Table legend: Nine larval families were selected based on individuals having near-identical shell length ($145.5 \pm 1.31 \mu\text{m}$, mean $\pm$ S.E.M. combined $n = 5615$) for comparisons of family-specific differences. Values for each measured variable are: Column I. *In vivo* activity = combined slope $\pm$ propagated S.E. ($n = 4$ five-point time-course assays, *i.e.*, 20 ion transport samples); Column II. Total activity = mean $\pm$ S.E.M. ($n = 6-10$); Column III. Relative gene expression ($NKA\alpha$: Na^+, K^+ -ATPase α normalized to expression of *RL7: ribosomal protein L7*) = mean $\pm$ S.E.M. ($n = 3$); Column IV. Respiration = mean $\pm$ S.E.M. ($n = 8-10$). Respiration measured as O_2 consumption was converted to energy equivalents using $5.2 \text{ moles ATP (mole } O_2)^{-1}$ based on the biochemical constituents of *C. gigas* larvae (Moran and Manahan, 2004). Columns V-VII are calculated variables: Column V. *In vivo* activity as percent of total activity; Column VI. Total activity as percent of respiration (*i.e.*, total ATP pool); Column VII. *In vivo* activity as percent of respiration. All measured variables were significantly affected by larval family: *in vivo* analyses of Na^+, K^+ -ATPase activity (column I), statistical comparisons were based on means $\pm$ 95% confidence intervals; total activity (column II): ANOVA, $F_{8, 31} = 3.85$, $p = 0.003$; gene expression (column III): ANOVA, $F_{8, 18} = 9.78$, $p < 0.001$; respiration (column IV): ANOVA, $F_{7, 60} = 8.61$, $p < 0.001$. Based on these statistical results and *post hoc* analyses, the maximum fold-difference given at the end of each column is statistical significant. The value for respiration for family 7 $\times$ 3 of $220 \text{ pmol ATP ind}^{-1} \text{ h}^{-1}$ is taken from the mean of all families to allow for calculation of data given in columns VI and VII for this specific family, which lacked a respiration value.

Figure 1

B

		Female (○)							
		1	4A	5	3	4B	4C	6	
Male (■)	2A	x	x	x					
	4A		x						
	2B				x	x	x	x	
	4B				x				x
	7				x	x	x	x	

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Biological variation