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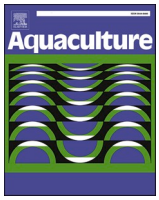

Integration of physiological and gene expression analyses to reveal biomarkers for protein dynamic mechanisms regulating higher growth and survival among larval oyster families (*Crassostrea gigas*)

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ABSTRACT

The oyster *Crassostrea gigas* is a major, global aquaculture species. As with any domestically-farmed species, the characterization of breeding lines that yield desired phenotypes is of immense value. An understanding of the fundamental biological bases of such phenotypes is needed to enhance aquaculture production. The aim of our study was to investigate the mechanisms of protein metabolic dynamics and energy allocation in oyster larvae. A series of controlled crosses yielded full-sibling larval families that allowed for measurements of integrative physiological processes during development. Experimentally, phenotypic contrasts between larval families were assayed by measuring: (1) growth and survival, (2) utilization of energy reserves of lipid and protein, (3) rates of protein synthesis and turnover, (4) respiration rates, and (5) transcriptome gene expression. Initially, newly-formed 2-day-old veliger larvae from four different families had similar sizes and physiologies, as measured by respiration, protein synthesis, turnover and content, the amount of energy allocated to protein synthesis, and gene expression pattern. Upon feeding, notable phenotypic contrasts became evident in different families. The larval family with faster growth had higher rates of protein synthesis and allocated a higher percentage of available energy to that single process. Based on family-specific differences, a series of samples was selected for developmental time-course analysis of changes in RNA pools. Principal component analyses of family-specific differential gene expression, combined with measured biochemical and physiological processes, led to the identification of two ribosomal gene biomarkers for protein synthesis. Such biomarkers could be potentially valuable tools for assessing complex traits that regulate physiological state, leading to optimization of breeding programs for oyster aquaculture.

1. Introduction

Many complex biological traits are controlled by genes of both small and large effects and their interactions with variable environmental factors (Lynch and Walsh, 1998). In analysis of species that are important for human food production (agricultural animals and plants), analysis of genetic architectures of complex traits have facilitated identification of causal biological factors impacting desired phenotypes (Mackay, 2004; Georges, 2007; Holland, 2007; Womack et al., 2012; Goddard et al., 2016). While quantitative trait locus mapping and genome-wide association studies have assisted with pinpointing locations of gene regions underlying complex traits, few gene variants have proven to be causative when tested as biomarkers for breeding purposes (Georges, 2007; Holland, 2007; Womack et al., 2012). Predictions of phenotypes are also complicated by variable transgenerational inheritance of complex traits (Mackay, 2004). Analysis of candidate genes that are based on biochemical and physiological processes offer additional approaches for identification of specific genes linked to complex traits (Georges, 2007; Zhu and Zhao, 2007). A focus on integrative biological approaches has the potential to advance understanding of fundamental mechanisms regulating complex traits (*e.g.*, resilience to biotic and abiotic stresses for enhancing food production and yield).

Aquaculture of "Blue Food" has been growing dramatically for decades and is making a substantial contribution to the global food supply (Kobayashi et al., 2015; Botta et al., 2020; Nature editorial, 2021; FAO, 2023). Of the many organisms cultivated in aquaculture, species of bivalve shellfish are globally cultivated in the coastal waters of six continents (except Antarctica). In addition to being of nutritional value

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as a human food source, such species serve as important ecosystem engineers in marine environments (Smaal et al., 2019).

The oyster *Crassostrea gigas* comprises a large category of total aquaculture production, as one of the most commonly farmed bivalves in the world (FAO, 2023). Many oyster production facilities rely largely on "seed" (larvae, or newly-settled juveniles reared from larvae) produced by hatcheries. As with agriculture, breeding technologies have the potential to improve yields in aquaculture (Hedgecock et al., 1995; Hedgecock and Davis, 2007; De Melo et al., 2016; Kong et al., 2017; Hollenbeck and Johnston, 2018; Han et al., 2020; Houston et al., 2020; Allen Jr et al., 2021; Liang et al., 2023). Pan et al. (2016, 2018) utilized pedigreed lines of *C. gigas* to analyze the biochemical and physiological traits of growth variation in larvae, and demonstrated that rates of protein synthesis and ion transport are predictive of growth rate. Furthermore, biological resilience to environmental change among marine organisms is a major concern (*e.g.,* rising temperature, ocean acidification, and disease) (Somero, 2010; Dégremont et al., 2015; Przeslawski et al., 2015; Divilov et al., 2023). Studies using pedigreed lines of *C. gigas* revealed promising evidence of standing genetic variation and physiological capacities to resist stress (Frieder et al., 2017; Pan et al., 2021; DellaTorre et al., 2022). Clearly, there is the potential to identify the genetic and physiological bases in pedigreed lines that grow faster and have superior phenotypes that are resilient to a wide range of environmental stressors.

Candidate gene analyses have also been conducted on larval stages produced from genetic crosses of pedigreed lines of *C. gigas* (Hedgecock et al., 2007; Meyer and Manahan, 2010). By utilizing experimentally designed crosses, gene expression profiling of genetically determined growth variation revealed the key roles of protein metabolism in regulating growth, and further identified ribosome proteins as the candidate biomarkers (Hedgecock et al., 2007; Meyer and Manahan, 2010). These previous findings highlighted the potential of coupling phenotypic comparisons of pedigreed lines with measurements of differential physiological capacities to screen for candidate genes underlying genetic-based complex traits. In the present study of oyster larvae, we (1) identify physiological mechanisms regulating differential growth and resilience, (2) quantify the biochemical basis of differential energy allocation underlying faster growth and higher survival, and (3) propose specific gene biomarkers for predicting these desired aquaculture traits. The experimental approach and rationale given below revealed protein synthesis and turnover, and resultant energy re-allocation thresholds, as key mechanisms regulating growth and survival.

2. Materials and methods

2.1. Approach and rationale

The general goal of this study was to increase understanding of biochemical and physiological processes that regulate growth and survival of oyster larvae. First, a series of distinct full-sibling larval families was generated for subsequent experimental identification of contrasting growth phenotypes of larvae reared under the same environmental conditions (algal food and temperature). Second, the same full-sibling larval families were reared in the absence of algal food to determine rates of basic foundational processes that are required to sustain larvae. Third, to identify mechanisms of metabolic regulation, a set of fully integrated measurements was applied to larval families that showed maximum contrasting phenotypes. These measurements spanned from whole-organism assays (size and biochemical content), physiological rates (respiration and energy allocation), biosynthetic dynamics (protein synthesis and turnover), to gene expression analyses (DNA sequencing of complete transcriptomes). Fourth, analysis of patterns of differential gene expression – directly linked by experiments with biochemical and physiological processes – was undertaken to identify gene biomarkers for the complex traits of growth and survival. Specific details of the sampling regimes and levels of replication are given in Section 2.4. Scale of larval measurements.

2.2. Broodstock genotyping and generation of larval families

Adult broodstock of the oyster *Crassostrea gigas* were obtained from our multi-generational breeding program (for descriptions see Pan et al., 2021; DellaTorre et al., 2022). Samples of mantle tissue were collected from individual oysters by relaxing the adductor muscle with treatment of a solution of $MgSO_4$ (Epsom Salt, added to seawater at 73 g l^{-1}). Tissue samples were then preserved in 70% ethanol for subsequent DNA extraction using DNeasy Blood & Tissue Kits (Qiagen, USA). The individuals used in a series of single male-by-female crosses were genotyped at 45 different DNA marker sites, using high-resolution melting of SNP-containing amplicons (Sun et al., 2015). Parentage was determined by comparisons with the genotypes of their putative parents (*i.e.,* the grandparents of larval families; details in DellaTorre et al., 2022), thus assuring that larval families were not related to each other.

Larval families were generated by fertilizing gametes from a single genotyped male with a single genotyped female. A series of four independent larval families were produced for this study. Briefly, sterile Pasteur pipettes were used to manually collect gametes from gravid adults. Sperm from each male was added to separate 1.7-ml microcentrifuge tubes and kept on ice. Eggs from each female were quantified and, once their abundance was determined, sperm was diluted (1:1000) into 0.2 μm (pore size) filtered seawater containing known numbers of eggs. Fertilization success was validated by visual confirmation of polar-body formation approximately 20 min post fertilization.

2.3. Larval culturing and experimental treatment

Fertilized eggs from each larval family were stocked at an initial number of 15 fertilized eggs per ml in 200-l culture vessels (Nalgene), containing 0.2 μm (pore-size) filtered seawater. Larval culturing was conducted using freshly-collected Pacific Ocean seawater offshore from Santa Catalina Island (Wrigley Marine Science Center, University of Southern California). Larval families were reared at 25 °C and a salinity of 33 in a temperature-controlled facility. Temperature loggers (Onset HOBO Pro v2, USA) were placed in culture vessels and in the culture room to monitor the consistency of temperature during the experimental period. Seawater in each culture vessel was mixed by gentle aeration using filtered air.

Once the first larval feeding-stage developed under these culturing conditions (*i.e.*, 2-day-old D-hinge veliger larva), six replicate 20-l culture vessels (Polycarbonate) each containing 350,000 larvae were set up for each larval family. For each family, larvae in two culture vessels were fed at 30,000 cells ml^{-1} with the alga *Tisochrysis lutea* (T-ISO, CCMP1324, National Center for Marine Algae and Microbiota, USA). These larvae represented "fed" *ad libitum* treatments. Larvae from the same family, held in four additional culture vessels, were not provided with algal food and constituted an "unfed" treatment. In all culture vessels, seawater was replaced every 48 h by gently filtering larvae onto size-appropriate mesh sieves, and then re-suspending the larvae in newly-filtered seawater (with algal food added for fed treatments).

2.4. Scale of larval measurements

Larval samples were collected from each family for a suite of integrative measurements spanning (1) shell length, (2) rates of oxygen consumption (respiration), (3) ammonia excretion (for calculation of oxygen-to-nitrogen ratios), (4) biochemical content of carbohydrate, lipid, and protein, (5) rates of protein synthesis and turnover, and (6) gene expression. The approximate numbers (rounded to the nearest thousand) were: 3000 individual larvae measured for shell length; 350,000 larvae for 420 measurements of oxygen consumption; 204 measurements of ammonia excretion (used for calculations of oxygen-to-nitrogen ratios); 919,000 larvae for 345 measurements of

carbohydrate, lipid, and protein content; 640,000 larvae for 64 measurements of protein synthesis/turnover rate; 135,000 larvae for transcriptome sequencing of 48 different DNA libraries used to generate 986 million paired-end, 150 base-pair (bp) DNA sequence reads.

2.5. Larval survivorship and growth

Survival in culture vessels was determined by replicate enumerations of aliquots of individuals from each culture vessel (n = 3–5 aliquots to yield coefficients of variation <10%). Larvae were photographed using a Nikon Digital Camera coupled to a compound microscope (at 10× magnification). Individual shell lengths were measured (n = 50 larvae per size assay) using Fiji digital software (formerly named ImageJ; Schneider et al., 2012). Increases in shell length over time for fed larvae were used to calculate family-specific growth rates for fed larval treatments. ANCOVA was used to compare growth rates among families, with age as a covariate. Survival rates were fitted to a Cox-proportional hazard model with mixed effects using the coxme package v2.2–16 in R version 3.5.1.

2.6. Rates of oxygen consumption and ammonia excretion

Oxygen consumption rates (respiration rates) of larvae were measured in sealed, volume-calibrated glass micro-respiration chambers (~500 μl) as described in Pan et al. (2021). Each respiration chamber contained a known number of larvae (300 to 1400 individuals, depending on size and age). Oxygen content in each chamber was measured at least four times during a time-course assay of approximately 3 h. A non-invasive optode sensor (Witrox single-channel oxygen meter, Loligo Systems, Denmark), coupled to an oxygen sensor spot placed in each chamber, was used for six replicate respiration chamber measurements of oxygen consumption of larvae in each culture vessel. Linear regression analysis of the decline in oxygen content was used to calculate respiration rate per larva. Chambers containing no larvae (only 0.2 μm pore-size filtered seawater) were used as controls for each measurement of respiration.

Seawater samples from the chambers used for respiration measurements of larvae were collected for ammonia content, assayed as previously described (Solorzano, 1969; DellaTorre and Manahan, 2023). Rates of oxygen consumption (pmol O_2 larva^{-1} h^{-1}) and ammonia excretion (pmol NH_3 larva^{-1} h^{-1}) were used to calculate the oxygen-to-nitrogen ratios (Mayzaud and Conover, 1988). An O:N ratio of <20 indicated that larvae catabolized protein and amino acids; ratios >40 indicated that larvae catabolized lipid and/or carbohydrate (Mayzaud and Conover, 1988).

2.7. Biochemical content

Whole-body protein content was measured using a modified Bradford assay for marine invertebrate larvae as described in Jaeckle and Manahan (1989). Lipid content was extracted, and lipid classes were profiled in larvae as previously described (Moran and Manahan, 2004). Carbohydrate content was quantified using a modified method of Holland and Gabbott (1971; with further details in Moran and Manahan, 2004).

2.8. Protein synthesis rate

Rates of protein synthesis in larvae of *C. gigas* were determined using *in vivo* assays with radioactively-labeled glycine (^{14}C glycine; Perkin Elmer, USA) as described previously (details in Lee et al., 2016; Pan et al., 2018). In brief, ^{14}C glycine was used as a tracer of protein synthesis, based upon the known composition of the intracellular free amino acid pool to accurately quantify synthesis rates in larvae of *C. gigas* of different ages and sizes (Lee et al., 2016; Frieder et al., 2018; Pan et al., 2018; Pan et al., 2021; DellaTorre et al., 2022). Duplicate time-course assays (n = 6 time points for each *in vivo* assay), each containing 10,000 larvae per 20-ml assay vial, were conducted for larvae in each culture vessel sampled.

2.9. Calculation of energy allocation to protein synthesis

The total amount of energy as measured by respiration rate was converted to energy equivalents using an oxyenthalpic average value of 484 kJ (mol O_2)$^{-1}$ (Gnaiger, 1983). For calculation of the energy cost of synthesizing proteins, a value of 2.1 ± 0.2 kJ g^{-1} protein synthesized was utilized (Lee et al., 2016). For example, the calculation of a 46 ± 5% energy allocation to protein synthesis in 2-day-old pre-feeding larvae from family 1 is as follows (see Table 1). The respiration rate of these larvae was 5.7 ± 0.5 pmol O_2 larva^{-1} h^{-1}. This equates to 2.8 μJ larva^{-1} h^{-1} (484 kJ (mol O_2)$^{-1}$). The protein synthesis rate for these same larvae was 0.6 ± 0.2 ng protein synthesized larva^{-1} h^{-1}. This equates to 1.3 μJ larva^{-1} h^{-1} (2.1 ± 0.2 kJ g^{-1} protein synthesized). Hence, the energy demand to support protein synthesis was 46% of the energy supplied by respiration (1.3 ÷ 2.8 = 0.46).

2.10. Transcriptome sequencing and gene expression analysis

Two biological samples of pooled larvae taken from each culture vessel (600 to 3000 larvae, depending on developmental stage and feeding treatment) were collected and placed in 300 μl TRIzol Reagent (Ambion, USA). Samples were extracted with Direct-zol RNA MicroPrep Plus Kit (Zymo Research, USA) following manufacturer's protocols. Integrity of total RNA was assessed on a 2% agarose gel and concentration determined by Qubit RNA HS Assay Kit (Invitrogen, USA). TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) was utilized for construction of sequencing libraries. DNA concentration in each library was determined by Qubit dsDNA HS Assay Kit (Invitrogen, USA), and size distribution of sequence fragments was assessed by Agilent 2100 Bioanalyzer (Agilent, USA). A total of 48 different DNA libraries were sequenced (RNA-seq) by an Illumina HiSeq 4000 platform at Fulgent Genetics (Temple City, California, USA).

Analysis of DNA sequence reads was conducted by first removing (Trimmomatic v0.38: Bolger et al., 2014) adapters, low-quality bases of reads (base quality score < 20), as well as short sequence reads (length < 36 bp). Trimmed reads were then aligned to the reference genome of the oyster *C. gigas* (GenBank accession number GCA_902806645.1) by HISAT2 v2.1.0 (Kim et al., 2015). StringTie v1.3.4d (Pertea et al., 2015) was used to quantify sequence read counts for each annotated gene. Prefiltering of sequences was carried out to retain genes that had read counts >10 in 24 samples, resulting in 20,908 remaining genes for further analysis (Supplementary Table S1). Principal component analysis (PCA) was performed to illustrate relationships among larvae (family, age, and feeding treatment). DESeq2 (Love et al., 2014) was utilized for analysis of differential gene expression between larval families. *P*-values were adjusted by the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995). Genes with adjusted *P*-value <0.05, and with an absolute value of fold-change greater than two, were identified as differentially expressed genes.

3. Results

3.1. Larval growth

The growth rates of larvae for the different families tested when fed the alga *Tisochrysis lutea* were measured morphologically (Fig. 1A, B: shell length) and biochemically (Fig. 2A, B: protein content). There was a predictive relationship between shell length and protein content (Fig. 2C: r^2 = 0.90; n = 36) for all four larval families. There were, however, differences in the rates of protein accretion between the four families (Fig. 2D). As expected, unfed larvae from all families did not grow (Figs. 1, 2).

Table 1
Larval family, age, and biochemical and physiological rates for larvae from four families of *Crassostrea gigas*. Fractional synthesis rate was calculated as a ratio of protein synthesis rate to whole-body protein content. Energy allocation to protein synthesis was calculated a ratio of protein synthesis rate to respiration rate in energy equivalents (see Materials and methods section 2.9 for detailed calculations). Data are shown as Mean ± 1 S.E.M.

Family	Age	Treatment	Protein content	Respiration	Protein synthesis	Fractional synthesis rate	Energy allocation to protein synthesis
	(Day)		(ng larva^{-1})	(pmol O_2 larva^{-1} h^{-1})	(ng protein synthesized larva^{-1} h^{-1})	(%)	(%, ± 1 S.E.M.)
1	2	Pre-feeding	8.9 ± 0.5	5.7 ± 0.5	0.6 ± 0.2	7%	46 ± 5%
2	2		7.0 ± 0.5	5.4 ± 0.3	0.5 ± 0.1	7%	40 ± 6%
3	2		6.6 ± 0.7	4.4 ± 0.2	0.6 ± 0.1	9%	59 ± 5%
4	2		7.9 ± 0.5	4.8 ± 0.4	0.4 ± 0.1	5%	36 ± 9%
Average			**7.6**	**5.1**	**0.5**	**7%**	**45%**
1	7	Fed	22.3 ± 0.9	16.3 ± 0.5	2.5 ± 0.1	11%	67 ± 3%
2	7		11.8 ± 0.6	11.0 ± 0.4	1.0 ± 0.1	8%	39 ± 6%
1	7	Unfed	4.7 ± 0.5	4.0 ± 0.2	0.5 ± 0.1	11%	54 ± 13%
2	7		1.2 ± 0.2	2.8 ± 0.3	0.5 ± 0.1	42%	77 ± 15%
1	11		2.7 ± 0.4	3.6 ± 0.3	0.8 ± 0.2	30%	96 ± 13%
3	10		2.0 ± 0.1	4.2 ± 0.3	0.9 ± 0.1	45%	93 ± 12%

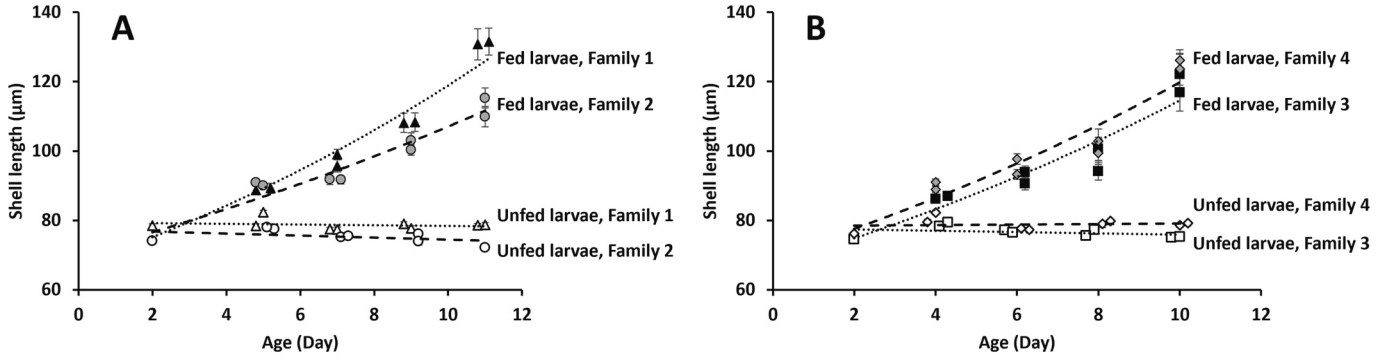

Fig. 1. Growth (shell length) for larvae of *Crassostrea gigas* from four larval families. Each family is indicated by different symbols (triangle, family 1; circle, family 2; square, family 3; diamond, family 4). Each treatment is indicated by solid (fed) and open (unfed) symbols in panels-A, and —B. Except for 2-day-old pre-feeding larvae (one 200-l experimental starting point culture vessel), larvae from two replicate 20-l culture vessels were measured for each family and each feeding treatment. For visual clarity, certain data points are graphically offset on the x-axis.
(A) Relationship of shell length with age for fed (solid symbols) and unfed (open symbols) larvae in family 1 (black triangle) and family 2 (grey circle). Each data point represents Mean ± 1 S.E.M. of $n \geq 50$ independent shell length measurements of individual larvae.
(B) Relationship of shell length with age for fed (solid symbols) and unfed (open symbols) larvae in family 3 (black square) and family 4 (grey diamond). Each data point represents Mean ± 1 S.E.M. of $n \geq 50$ independent shell length measurements of individual larvae.

3.1.1. Shell length of fed larvae

In each larval family, growth rate was calculated from an analysis of the relationship between shell length and age (Fig. 1). An ANCOVA ($P =$ 5.3e-11 for four families), followed by post-hoc statistical analyses, revealed that larval family 1 was the fastest-growing at 5.7 ± 0.3 µm day^{-1}. Larval family 2 was the slowest-growing, at 4.0 ± 0.2 µm day^{-1}.

3.1.2. Protein content of fed larvae

Protein is the dominant biochemical component in larvae of *Crassostrea gigas* (Moran and Manahan, 2004; Pan et al., 2018). An ANCOVA ($P <$ 2.2e-16 for four larval families), followed by post-hoc statistical analyses, revealed that larval family 1 had the fastest rate of protein accretion (Tukey test $P \leq 0.001$ for comparisons with families 2, 3, and 4), and larval family 2 was the slowest (Tukey test $P < 0.001$ for comparisons with families 1, 3, and 4).

The rationale for the experimental design of producing multiple larval families was to identify the fastest- and slowest-growing larvae for subsequent analysis of mechanisms of differential growth, based on an integrative study of physiology, biochemistry, and molecular biology. In summary, from Generalized Linear Model analyses of growth rates of shell length (Fig. 1) and protein accretion (Fig. 2D), larval family 1 was identified to be the fastest, in contrast to family 2 which was the slowest.

3.2. Larval survival

Initially, each of the six 20-l replicate culture vessels contained 350,000 newly-formed, 2-day-old D-hinge veliger larvae (*i.e.*, an initial stocking number of 18 larvae per ml). Four of those culture vessels were used for an analysis of the physiology of unfed larvae (absence of algal food). Within a larval family, rates of survival for unfed larvae were consistent across all replicate culture vessels (Fig. 3). Unfed larvae from family 1 showed the highest resilience to algal food deprivation. Live larvae in this family were observed even after 13 days without algal food (Fig. 3A). In contrast, larvae from family 2 had the lowest resilience to algal food deprivation, with live larvae not being observed after 7 days (Fig. 3B). Statistically, a Mixed Effects Cox Model of survival (with culture vessel as a random effect) revealed that unfed larvae from family 1 had the highest survival under algal food deprivation ($P \leq$ 3.1e-10 for comparisons with each of the other three families), and family 2 had the lowest survival ($P <$ 2.0e-16 for comparisons with each of the other three families). Larval family 4 had a better survival than family 3 ($P =$ 0.002); both of these families showed intermediate levels of resilience to algal food deprivation. In summary, these Mixed Effects Cox Model analyses revealed the family-specific survival ranking was: 1 > 4 > 3 > 2 (Fig. 3).

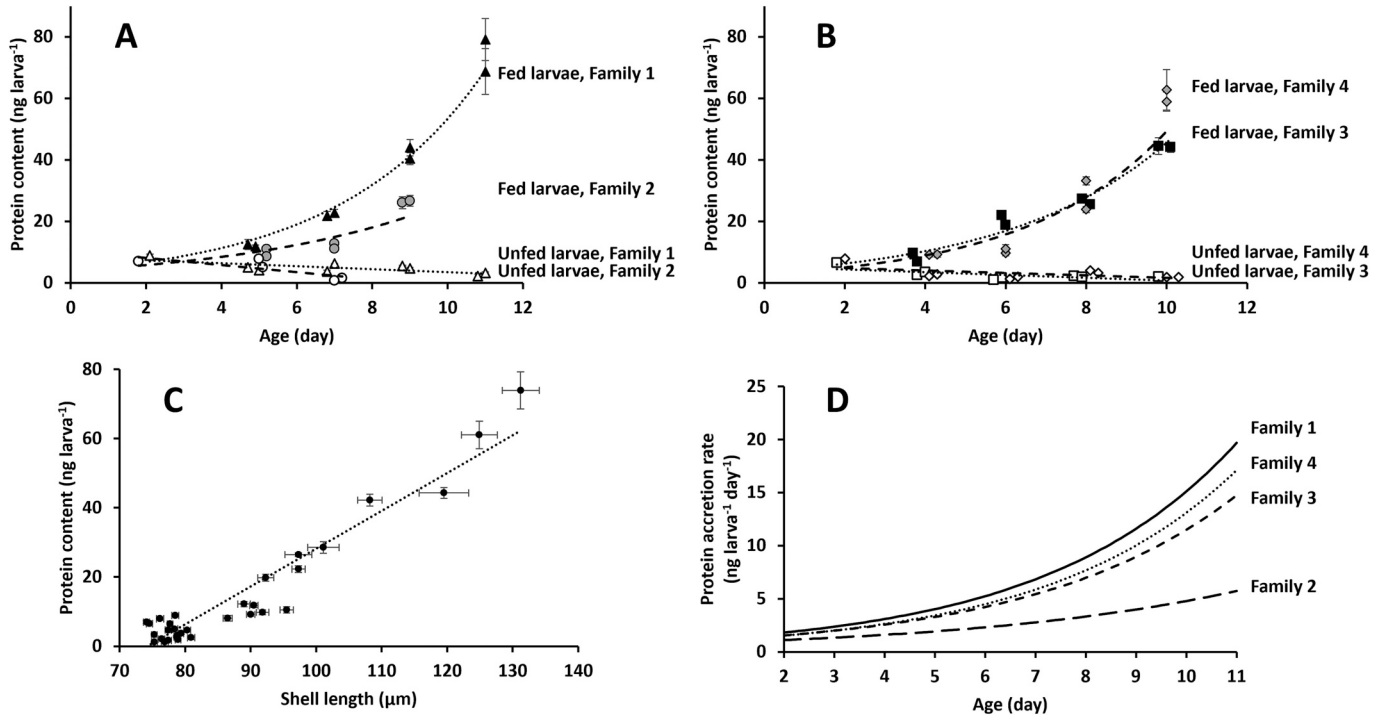

Fig. 2. Growth (protein accretion) for larvae of *Crassostrea gigas* from four larval families. Each family is indicated by different symbols (triangle, family 1; circle, family 2; square, family 3; diamond, family 4). Each treatment is indicated by solid (fed) and open (unfed) symbols in panels-A, and —B. Except for 2-day-old pre-feeding larvae (one 200-l experimental starting point culture vessel), larvae from two replicate 20-l culture vessels were measured for each family and each feeding treatment. For visual clarity, certain data points are graphically offset on the x-axis.

(A) Relationship of protein content with age for fed (solid symbols) and unfed (open symbols) larvae in family 1 (black triangle) and family 2 (grey circle). Each data point represents Mean ± 1 S.E.M. of n = 3–5 independent protein content measurements.

(B) Relationship of protein content with age for fed (solid symbols) and unfed (open symbols) larvae in family 3 (black square) and family 4 (grey diamond). Each data point represents Mean ± 1 S.E.M. of n = 3–5 independent protein content measurements.

(C) Relationship between protein content (data from panels-A and —B) and shell length (data from Fig. 1). r^2 = 0.90; n = 36; error bars represent 1 S.E.M. for both protein content and shell length. All data points are graphed as solid circles with error bars.

(D) Rates of protein accretion as a function of age in the 4 larval families, calculated from the equations of the primary data for protein accretion rates given in panels-A and —B.

3.3. Respiration and protein synthesis

Rates of energy supply (from respiration) and energy demand (for protein synthesis) were measured to assess the physiological basis of the contrasting differences in family-specific growth and survival phenotypes (Fig. 1-3).

3.3.1. Fed larvae

ANCOVA was used to quantify increasing differences in rates of respiration with age for the four larval families tested (Fig. 4A-B; $P <$ 2.2e-16). Post-hoc statistical analyses revealed that larvae from families 1 and 3 had the highest respiration rates (growth- and size-dependent) ($P < 0.05$ for comparisons with families 2 and 4; $P = 0.06$ between families 1 and 3). Family 2 had the lowest rate of respiration ($P = 0.005$ for comparison with family 4). The contrasting respiratory phenotypes of fed larvae from families 1 and 2 were consistent with their larval growth rates, as evident from the positive correlation between respiration rate and protein content (Fig. 4C; $r^2 = 0.91$; $n = 36$). (Note that the relationship in Fig. 4C can be used to calculate size-specific respiration rates for larvae of *C. gigas*, independent of family.)

For comparisons of protein synthesis rates between larval families 1 and 2, a single rate calculation for each family was determined from four time-course assays (*i.e.,* there were no statistical differences in rates between two duplicate assays for each of two different culture vessels). As an example (Fig. 5A), one regression line was fitted for the culture vessel and assay replicates for each larval family measured for 7-day-old fed larvae for both larval families 1 and 2. For newly-formed 2-day-old pre-feeding larvae, there were no differences in protein synthesis rates between larval families 1 and 2 (Fig. 5B, slope comparison: $P = 0.86$). Major differences in protein synthesis rates became evident, however, once feeding was initiated, with 7-day-old fed larvae from family 1 having a 2.5-fold greater synthesis rate than larvae from family 2 (Fig. 5B; Table 1).

3.3.2. Unfed larvae

Unfed larvae from each family sustained similar rates of respiration (Fig. 4A-B; ANOVA of regression: $P > 0.2$ for all families). For protein synthesis rates, there were no differences observed in 7-day-old unfed larvae between families 1 and 2 (Fig. 5B, slope comparison: $P = 0.96$). These low rates of synthesis measured in 7-day-old unfed larvae were similar to the initial rates in 2-day-old pre-feeding larvae (Fig. 5B).

3.4. Energy allocation to protein synthesis

3.4.1. Pre-feeding larvae

The 2-day-old pre-feeding larval stage in each of the four families had similar amounts of whole-body protein (from Table 1, an average of 7.6 ng protein larva^{-1}; $P = 0.44$; $n = 9$). Also, larvae of this age in these families had similar rates of respiration (average, 5.1 pmol O_2 larva^{-1} h^{-1}) and protein synthesis (0.5 ng protein synthesized larva^{-1} h^{-1}). Given that rate of protein synthesis and protein content were similar for pre-feeding larvae, their fractional rates of protein synthesis were also similar (7%). (Fractional rates of protein synthesis were calculated based on the percent-ratio of measured rates of protein synthesis and

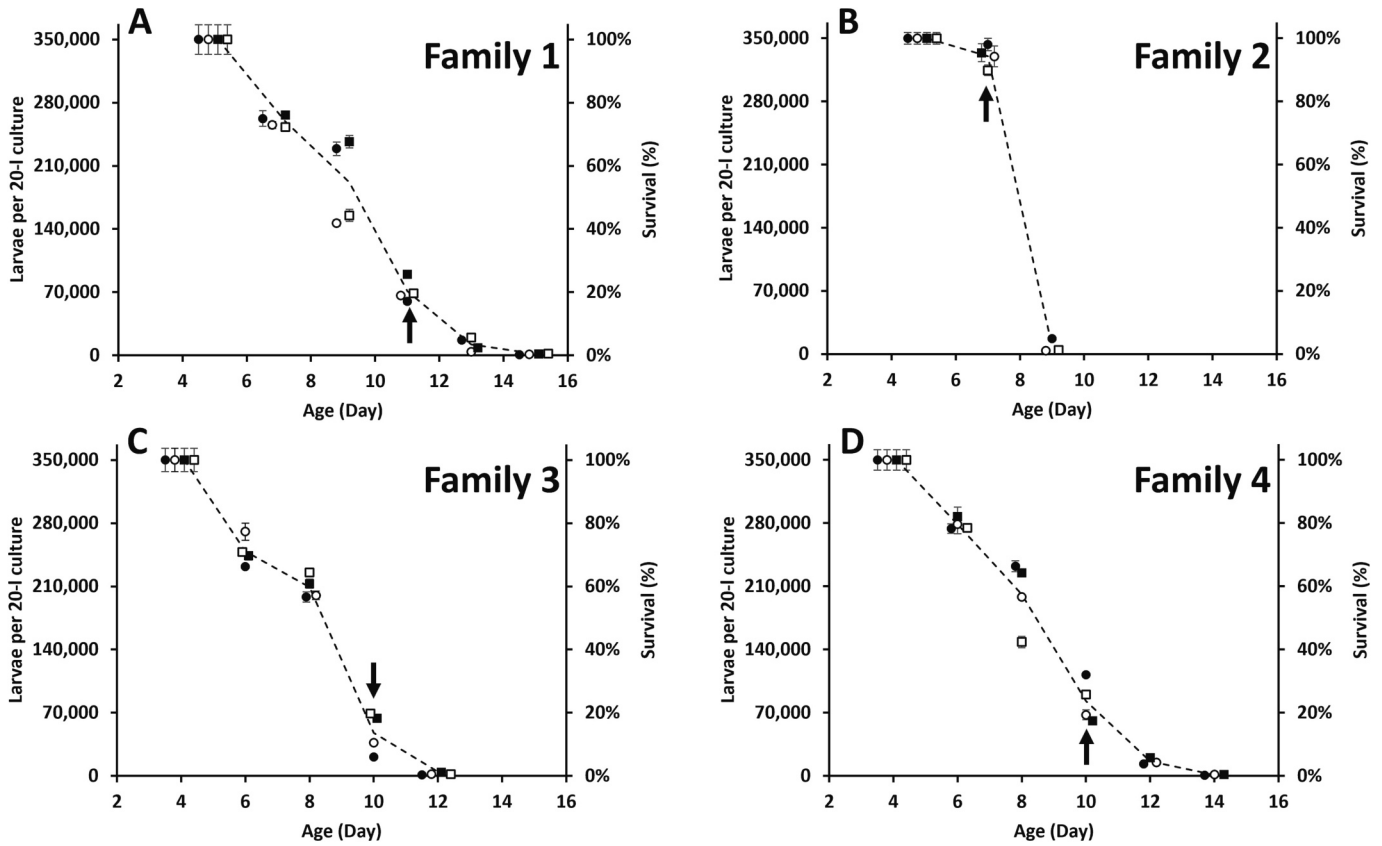

Fig. 3. Survival of unfed larvae of *Crassostrea gigas* from larval family 1 (A), family 2 (B), family 3 (C), and family 4 (D). Data points represent Mean ± 1 S.E.M. of the visual (under a microscope) enumeration of the number of live larvae on a given day (left y-axis), each from a different 20-l culture vessel for a larva family (four symbols represent four replicate culture vessels in each larval family). For ease of graphical presentation, data are also presented as percent survival (right y-axis). Data shown on each day includes 3–4 independent enumerations from each of the 4 culture vessels (n = 12–16 in total), except measurements from the first day [at day 4 or 5, the starting value (n = 350,000) was the initial number of larvae present in each culture vessel], and the last day of sampling each larval family (depending on the number of culture vessels still containing live larvae). For visual clarity, certain data points are graphically offset on the x-axis. Arrows indicated specific age-dependent survival where the next sampling time (day, x-axis) measurement had a near-zero survival for larvae in a given family.

protein content.) In this case, for newly-formed pre-feeding larvae, a protein synthesis rate is a measure of protein turnover, because this larval stage has not yet grown (*i.e.*, accreted protein). The average allocation of energy (joules) to support rates of protein turnover in pre-feeding veliger larvae was 45% (Table 1).

3.4.2. Fed larvae

Upon the initiation of growth from feeding on algal food, same-aged larvae from the faster-growing family 1 synthesized protein at a 2.5-fold higher rate than larvae in the slower-growing family 2 (Table 1: 7-day-old larvae from family 1 = 2.5 ± 0.1 ng protein synthesized larva^{-1} h^{-1}; *cf.* 7-day-old larvae from family 2 = 1.0 ± 0.1). From data for rates of protein synthesis and protein accretion, a protein depositional efficiency was calculated for each larval family. Specially for family 1, the protein depositional efficiency of 7-day-old larvae was 11% (the percent ratio of 6.8 to 60.0). This percent ratio was calculated from the daily rate of protein synthesis of 60.0 ng larva^{-1} day^{-1} (from Table 1: 2.5 ± 0.1 ng larva^{-1} h^{-1}, converted to a 24-h daily rate), and the corresponding rate of protein accretion of 6.8 ng larva^{-1} day^{-1} (Fig. 2D). The protein depositional efficiency for 7-day-old larvae from family 2 was 12% (from Table 1: 1.0 ± 0.1 ng larva^{-1} h^{-1}, converted to a 24-h daily rate; from Fig. 2D: protein accretion of 2.8 ng larva^{-1} day^{-1}). These calculations revealed that the difference in protein accretion between larval families 1 and 2 was driven by different rates of protein synthesis, not by different rates of protein turnover because protein depositional efficiency was similar (11%–12%). Calculations for larval families 3 and 4 showed that protein depositional efficiencies ranged from 12%–16% (family 3: 14% in 10-day-old larvae; family 4: 16% in 6-day-old larvae and 12% in 10-day-old larvae). An additional set of measurements was made for 11-day-old larvae from family 1, which had a protein depositional efficiency of 14%. On average, the protein depositional efficiency across all families and ages tested was 13%.

Regarding insights into energy allocation to growth, larvae from the fastest-growing family 1 allocated more energy to protein synthesis (67 ± 3%, 1 S.E.M.; with 95% confidence intervals, 61–73%) than did larvae from the slowest-growing family 2 (39 ± 6%; with 95% confidence intervals, 27–51%). A greater energy investment in protein synthetic capacity in larvae of family 1 supported the observation of faster growth in that family (Figs. 1, 2). Even though these larvae of the same age from families 1 and 2 were different in size, the calculations of energy allocation given in Table 1 are independent of size (*i.e.*, allocations were scaled to metabolic rates).

3.4.3. Unfed larvae

When deprived of food, 7-day-old unfed larvae from both families 1 and 2 maintained similar rates of protein turnover at 0.5 ± 0.1 ng protein synthesized larva^{-1} h^{-1}(Fig. 5B; Table 1). Their respective fractional rates of protein synthesis were, however, 3.8-fold different, with unfed larvae from family 2 having the higher fractional synthesis rate of 42%, compared to larvae from family 1 at only 11% (Table 1). This higher fractional synthetic rate (42%) in larvae from family 2 required 77% ± 15% of a 7-day-old unfed larva's available energy to support protein turnover, in contrast to only 40% ± 6% in a newly-formed 2-day-old pre-feeding larva (7% fractional synthesis rate) from

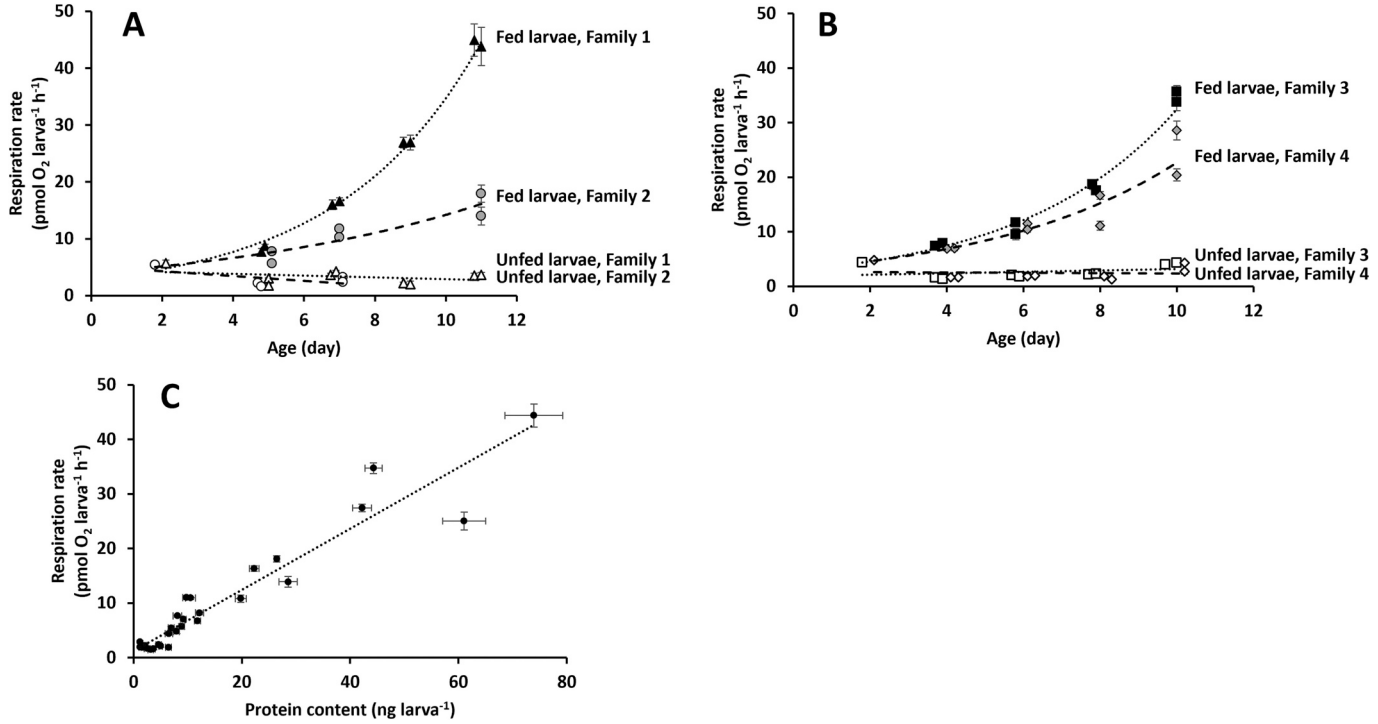

Fig. 4. Respiration rates for larvae of *Crassostrea gigas* from four larval families. Each family is indicated by symbols (triangle, family 1; circle, family 2; square, family 3; diamond, family 4). Each feeding treatment is indicated by solid (fed) and open (unfed) symbols in panels-A, and —B. Except for 2-day-old pre-feeding larvae (one 200-l experimental starting point culture vessel), larvae from two replicate 20-l culture vessels were measured for each family and each feeding treatment. For visual clarity, certain data points are graphically offset on the x-axis.
(A) Relationship of respiration rate with age for fed (solid symbols) and unfed (open symbols) larvae in family 1 (black triangle) and family 2 (grey circle). Each data point represents Mean ± 1 S.E.M. of $n = 6$ independent measurements in replicate micro-respiration chambers.
(B) Relationship of respiration rate with age for fed (solid symbols) and unfed (open symbols) larvae in family 3 (black square) and family 4 (grey diamond). Each data point represents Mean ± 1 S.E.M. of $n = 6$ independent measurements in replicate micro-respiration chambers.
(C) Relationship between respiration rate (primary data from panels-A and —B) and protein content (data from Fig. 2). $r^2 = 0.91$; $n = 36$. Error bars on each data point represent 1 S.E.M. for both respiration rate (vertical error bars) and protein content (horizontal error bars).

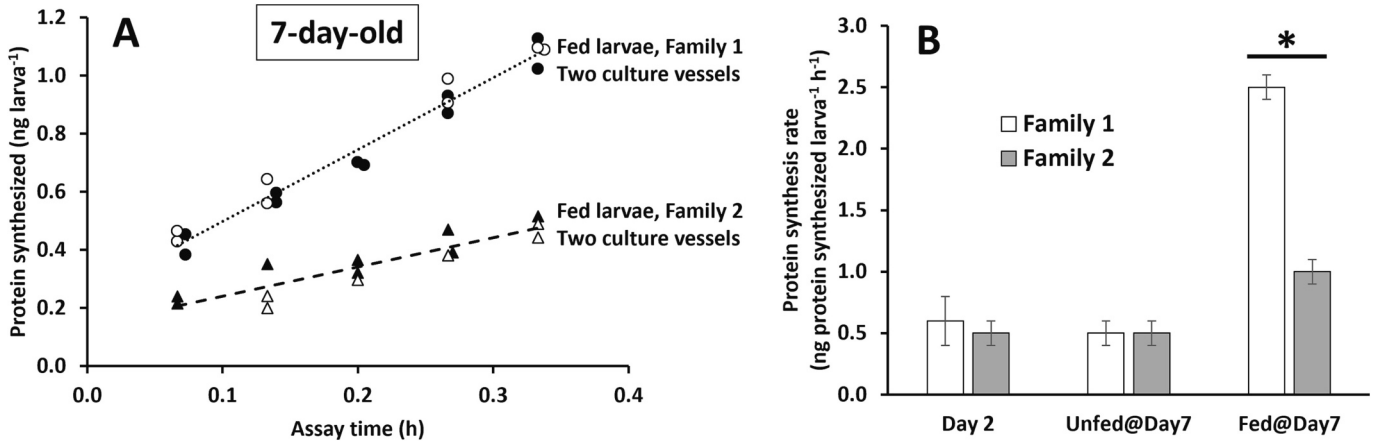

Fig. 5. Protein synthesis rates for larvae of *Crassostrea gigas* from family 1 and family 2. See Table 1 for additional physiological measurements for these two families.
(A) Protein synthesis rate in 7-day-old fed larvae from family 1 (circle) and family 2 (triangle). Solid and open symbols represent larvae from two replicate culture vessels, and two independent measurement assay of protein synthesis conducted for samples of larvae within each culture vessel ($n = 4$ assays in total for each larval family). Protein synthesis rate was calculated as the slope of increase in the amount of synthesized protein with assay time.
(B) Protein synthesis rate for larvae from family 1 and family 2. Each bars represent Mean ± 1 S.E.M. ($n = 2$ for 2-day-old pre-feeding larvae; $n = 4$ for 7-day-old larvae). *P-value <0.01.

the same family 2. In summary, there is an apparent relationship between the high percent of energy allocated to maintain protein turnover in unfed larvae and their subsequent survival. Specifically, once a threshold in excess of ~75% of energy allocation is reached (Table 1) in unfed larvae, high rates of mortality soon followed (Fig. 3). It is notable that there is a survival-age relationship difference between unfed larvae from different families when this energy allocation threshold is reached (Fig. 3; Table 1). This suggests a bioenergetic explanation, related to the requirement of supporting the dynamics of protein metabolism, for observations of differential resilience under conditions of extended algal

food deprivation.

3.5. Utilization of biochemical reserves in unfed larvae

Unfed larvae from families 1 and 2 showed the largest contrast in resilience to algal food deprivation (Fig. 3A, B: measured by percent survival with time). Further biochemical analyses were undertaken to determine the bioenergetic basis for sustaining respiration and maintaining protein synthesis and turnover under conditions of algal food deprivation.

3.5.1. Protein

As noted above in section 3.4, all 2-day-old newly-formed larvae had similar amounts of whole-body protein (Table 1). Unfed larvae from families 1 and 2 had contrasting depletion rates of their endogenous protein reserves when algal food deprivation continued from day 2 to day 7 (Fig. 6A). There was a significant decrease in protein content between 2- and 5-day-old unfed larvae from family 1, but not a further decrease in 7-day-old unfed larvae. In contrast, there was a significantly lower ($P = 0.01$, $n = 13$) amount of protein in 7-day-old unfed larvae from family 2 (1.2 ± 0.2 ng larva^{-1}), compared to similar-aged 7-day-old unfed larvae from family 1 (4.7 ± 0.5 ng larva^{-1}). An analysis of oxygen-to-nitrogen ratios revealed values lower than 20 (Mayzaud and Conover, 1988) in families 1 (4.7 ± 0.5 on day 5, and 9.1 ± 0.7 on day 7) and 2 (7.4 ± 0.3 on day 5, and 4.2 ± 0.4 on day 7), showing that protein- and amino acid-based catabolism is dominant in larvae of these two families.

3.5.2. Lipid

Larvae from families 1 and 2 conserved phospholipid (ANOVA of regression: $P > 0.05$ for each family) and exhibited no difference in the amount of phospholipid over time (slope comparison: $P = 0.62$). On average, family 1 had 1.2 ± 0.1 ng larva^{-1} of phospholipid from day 2 to day 7 ($n = 21$), and family 2 had a similar amount of 1.0 ± 0.1 ng larva^{-1} ($n = 24$). The 2.7 ± 0.03 ng of triglyceride in a newly-formed 2-day-old pre-feeding larva from family 1 was greater (Fig. 6B; $P < 0.01$) than the triglyceride content of similar-aged newly-formed larvae in family 2 (1.9 ± 0.03 ng larva^{-1}). By day 7, unfed larvae from both families contained the same 2.0 ± 0.03 ng larva^{-1} of triglyceride, with larvae from family 2 not utilizing their initial triglyceride reserves present in 2-day-old pre-feeding larvae (ANOVA of regression: $P = 0.96$). Unfed larvae from family 1 initially had a higher amount of triglyceride in the newly-formed 2-day-old pre-feeding larvae, with 7-day-old unfed larvae consuming 0.7 ng of that lipid reserve.

3.5.3. Carbohydrate

The carbohydrate content was negligible, below the limit of analytical detection (<0.2 ng carbohydrate per larva), for different ages ($n = 5$) in all four families tested in this study.

3.5.4. Threshold of biochemical content

Larvae from family 1 retained a higher amount of protein during development than did larvae from family 2. Larvae from family 1, however, used a portion of their greater amount of initial triglyceride reserves that were present in 2-day-old pre-feeding larvae. This differential utilization of energy reserves by unfed larvae from different families supports a suggestion that there is a biochemical threshold for remaining protein content. When that protein content was further depleted under conditions of algal food deprivation, as for 7-day-old unfed larvae from family 2 (Fig. 6A), lower survival was observed (Fig. 3B). These biochemical thresholds (see arrows highlighting specific age-dependent survival in Fig. 3) for survival of unfed larvae across all families were 2.0 ± 0.3 ng larva^{-1} for protein and 2.0 ± 0.04 ng larva^{-1} for triglyceride (Fig. 6). The value for phospholipid was 1.0 ± 0.04 ng larva^{-1}.

3.6. Transcriptome sequencing and gene expression analysis

The transcriptomes of both fed and unfed larvae from families 1 and 2 were sequenced for comparisons of gene expression across contrasting phenotypes of growth and survival (Figs. 1, 2, 3, and 7A). The transcriptomes of larvae from all four families were sequenced in early, newly-formed larval stages (2-day-old pre-feeding larvae) for identification of gene signatures (biomarkers) for fast growth and high resilience to algal food deprivation. Arrows by specific data points in Fig. 7A indicate the larval families, ages, and feeding treatments that were sampled for transcriptomic analysis. An average of over 15 million paired-end DNA sequence reads per library (data points in Fig. 7A) were aligned to a reference genome for *C. gigas* (Penaloza et al., 2021). Principal Component Analysis (PCA) in two dimensions was used to visualize the relationships among the different larval treatments (Fig. 7B: PC1 and PC3; PC2 did not display any patterns). PC1 accounted for 26.5% of the expression variance among treatment patterns, with 2-day-old pre-feeding larvae located in the near-middle of the PC1 range, separating unfed and fed larvae on either side of the x-axis. Pairwise comparisons of 2-day-old pre-feeding larvae among the four larval families revealed that an average of 80% of the 20,908 genes analyzed showed similar gene expression patterns (Supplementary Table S2). PC3 accounted for 14.3% of the expression variance. This analysis clustered larvae from family 2 for all treatments in the top ellipse; in contrast,

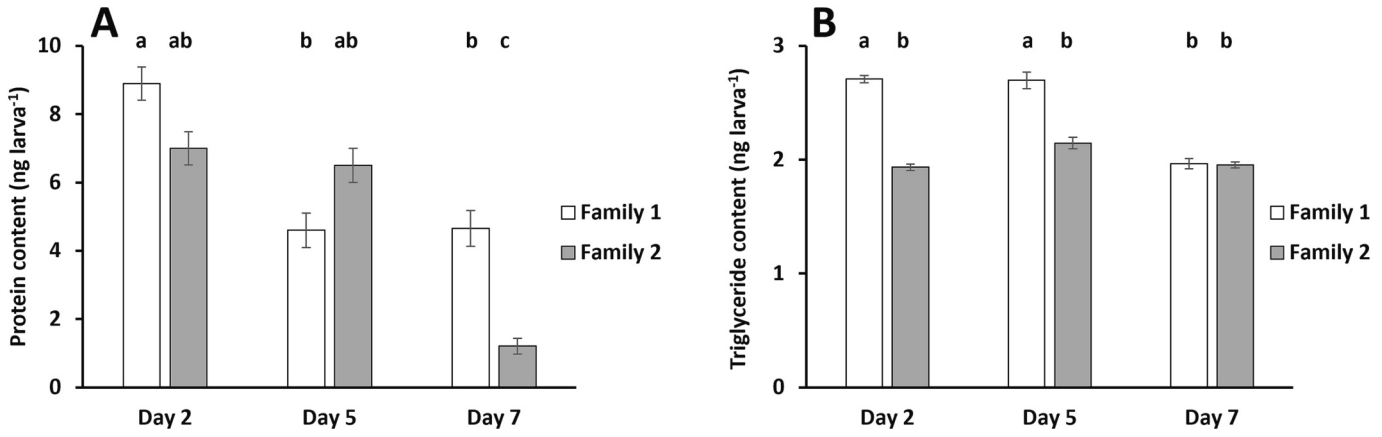

Fig. 6. Protein (A) and triglyceride (B) contents of 2-day-old pre-feeding larvae, and 5- and 7-day-old unfed larvae of *Crassostrea gigas* in family 1 and family 2. Error bars represent 1 S.E.M. for each biochemical component. Different letters (a, b, c) indicate significant differences in the amount of biochemical components between larval families and larval age (ANOVA and subsequent post-hoc analysis, *P*-value <0.05).

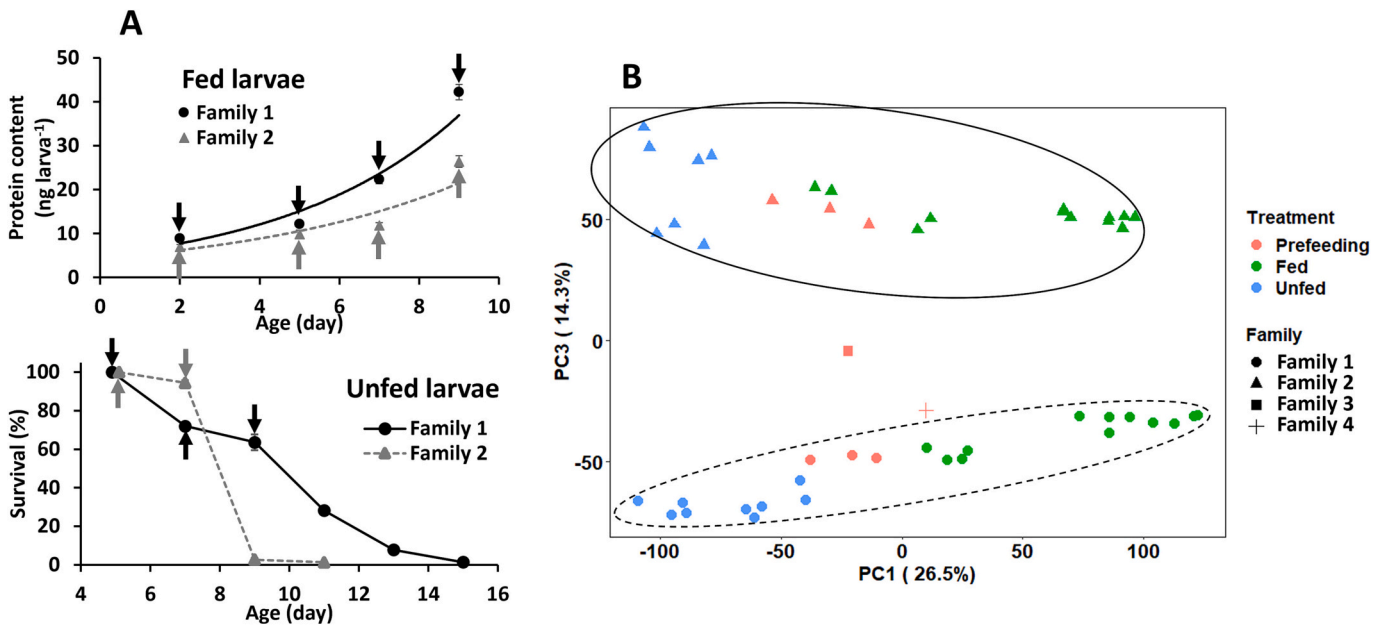

Fig. 7. Gene expression (transcriptomic analysis) in larvae of *Crassostrea gigas* of different ages and feeding treatments.
(A) Sampling scheme for transcriptome sequencing. Panels were modified from Figs. 2 and 3. Each family is indicated by different symbols (black circle: family 1; grey triangle: family 2). Arrows beside each of the data points illustrate samples taken for transcriptomic analysis. Top panel indicated samples of fed larvae; bottom panel indicated samples of unfed larvae. Error bars represent 1 S.E.M. Where error bars were not shown, error fell within the graphical representation of the data point.
(B) Expression patterns of 20,908 genes analyzed by principal component analysis (PC1 and PC3) in larvae of all four families reared under different treatments. The proportions of variance explained by PC1 and PC3 are indicated beside each axis. Feeding treatments illustrated by symbol color; families illustrated by symbol shape. Statistical groupings were based on gene expression analysis, indicated by cluster in top ellipse (family 2) and bottom ellipse (family 1).

Table 2
The number of differentially expressed genes between family 1 and family 2 (F1 *cf.* F2) for larvae of *Crassostrea gigas* of different ages (days). Column labeled "Shared genes" gives the number of genes shared across different age groups in both fed and unfed larvae. Two ribosomal genes (LOC105335239: 40S ribosomal protein S19, and LOC117682815: 60S ribosomal protein L23) were identified as biomarkers related to protein synthesis, because both genes had significantly higher expression in family 1 compared to family 2 for all pre-feeding, unfed, and fed larval treatments. See primary data in Supplementary Table S1.

Treatment	Family	Age	#Up-regulated genes	#Shared genes	Biomarker gene ID (protein synthesis)
Fed	F1 *cf.* F2	2	1878	790	LOC105335239, LOC117682815
		5	1813		
		7	1678		
		9	1787		
Unfed	F1 *cf.* F2	2	1878	900	
		5	2274		
		7	3431		

larvae from family 1 for all treatments were clustered in the bottom ellipse (Fig. 7B). The conclusion from these principal component analyses is that specific families had distinct gene expression patterns along PC1 in response to the environmental treatments of fed algae *ad libitum* and algal food deprivation.

For the phenotype of protein accretion, although larvae from family 1 eventually grew faster than larvae from family 2 (ANCOVA, $P = 1.3e\text{-}6$), their growth differences only became apparent after 5 days (Fig. 2A: 5-day-old fed larvae, $P = 0.9$; 7-day-old fed larvae, $P = 4e\text{-}6$). Consequently, the transcriptomes of 5- and 7-day-old fed larvae were compared to reveal possible patterns of differential gene expression associated with the contrasting growth phenotypes for larvae in families 1 and 2. Of the 20,908 genes analyzed in this study (Supplementary Table S1), there were 2.4-fold more genes differentially expressed in fed larvae in the slower-growing family 2 (4325 genes) than for fed larvae in the faster-growing family 1 (1805 genes). This finding suggests a more complex transcriptional regulation in the slower-growing larval family.

For the survival phenotype, unfed larvae from family 2 had a ~ 90% survival at day 7, followed by a near-zero survival at the next day of sampling (Fig. 3B: day 9). Hence, day 7 is a critical age for analysis of contrasting survival phenotypes for larvae in families 1 and 2. In total, 3.7-fold more genes were differentially expressed in unfed larvae of family 2 (4649 genes) than for unfed larvae of family 1 (1250 genes). Analysis of the survival phenotype indicated the complexity of transcriptional processes in larvae of the lower-surviving family.

To identify predictive biomarkers for growth across different ages, the transcriptomes for larvae from the faster-growing family 1 were compared with the slower-growing larvae from family 2 (Table 2). Notably for fed larvae, comparisons of families 1 and 2 of different ages consistently revealed ~1800 up-regulated genes. A total of 790 up-regulated genes were shared across the four age groups measured (2-, 5-, 7-, and 9-day-old fed larvae). Of these 790 genes, 345 (44%) had an annotation in a reference genome for *C. gigas* (Penaloza et al., 2021; gene identified as an uncharacterized protein is not considered as an annotation). Of these annotated genes, two ribosomal proteins (LOC105335239: 40S ribosomal protein S19, and LOC117682815: 60S ribosomal protein L23) were identified as possible biomarker candidates for faster growth. This selection was based on biochemical and physiological evidence (Table 1), which revealed that higher rates of protein synthesis and greater energy allocation to synthesis were associated

with the faster-growth phenotype (Figs. 1, 2: larvae in family 1).

The transcriptomes of unfed larvae from families 1 and 2 were also compared for identification of common up-regulated biomarker genes associated with differential survival in response to algal food deprivation. In this case, a total of 900 genes were shared across three age groups (2-, 5-, and 7-day-old unfed larvae). Of these 900 genes, 409 (45%) had an annotation in the reference genome for *C. gigas*. Strikingly, the same two ribosomal proteins (40S ribosomal protein S19 and 60S ribosomal protein L23) were also present in this subgroup of annotated genes, where the physiological processes driving protein turnover dynamics were associated with the higher-survival phenotype (*e.g.*, comparison of 7-day-old unfed larvae in families 1 and 2; Table 1).

4. Discussion

Efficient growth and high survival are desired traits in aquaculture yield. The central goal of the current study was to understand the fundamental physiology of development and to determine the biochemical and physiological mechanisms underlying growth and survival in oyster larvae (*Crassostrea gigas*). To achieve this goal, a series of controlled crosses of unrelated genetic lines produced full-sibling larval families for contrasting phenotypes, spanning morphological size of larval progeny, biochemical content, rates of physiological processes (respiration and protein synthesis), and gene expression (transcriptome sequencing). The integration of these measurements revealed that mechanisms of protein metabolic dynamics and energy allocation strategies underlie variation in growth and survival among larval families. The integrative analyses, linking gene expression with measured biochemical and physiological processes, further identified two specific biomarkers related to protein synthesis as potential indicators of desired complex traits.

Newly-formed, 2-day-old pre-feeding larvae across four families tested had, initially, no substantial biological differences in size, protein content, respiration, protein synthesis, the amount of energy allocated to protein synthesis (Table 1), and gene expression patterns (Table S2). The proportion of energy allocated to biosynthesis averaged 45% across all families, a value similar to the energy allocation reported for other larval families of *C. gigas* (Lee et al., 2016; Pan et al., 2018). Once feeding was initiated, there were notable phenotypic contrasts between the growth rates of different larval families (Figs. 1, 2). Larvae from family 1 had the fastest growth and allocated the majority of energy to protein synthesis. For instance, for 7-day-old larvae from family 1 (fastest growth), 67% of energy was allocated to protein synthesis, in contrast to larvae from family 2 (slowest growth) which allocated only 39% of energy to syntheses (Table 1). Notably, protein depositional efficiency (the ratio of protein accretion to protein synthesis) did not vary among families, averaging 13%, *i.e.*, 87% of synthesized protein was turned over and not accreted for growth. Hence, the key major biochemical process regulating growth of larvae was synthesis, not turnover. These data are consistent with Pan et al. (2018), who showed, in a study of 12 larval families, that protein depositional efficiency was consistently low (average of 14%), whereas energy allocation to protein synthesis varied up to 3.4-fold and was predictive of larval growth (*e.g.*, the fastest growing larval family allocated 74% of energy to protein synthesis). For unfed larvae, differences in survival were also evident (Fig. 3), along with physiological differences that changed with age (Table 1). These data support a suggestion that there are genetic differences across families that only become evident with age as contrasting phenotypes observed in growth and survival.

The low oxygen-to-nitrogen ratios reported here further supported the critical role of protein dynamics in maintaining larval metabolism of *C. gigas* (see section: 3.5). A focus on the genes regulating protein synthesis and turnover could provide predictive biomarkers of growth. Previous analyses of quantitative trait loci and transcriptomic studies identified hundreds-to-thousands of possible candidate genes related to production traits in the oyster, *C. gigas* (Hedgecock et al., 2007; Guo et al., 2012; Wang and Li, 2017; Pan et al., 2023). In this study, by integrating analyses of differential gene expression with phenotypic measurements of biochemical and physiological processes, two ribosomal proteins were identified to be up-regulated in faster-growing larvae (family 1) at all ages tested (Table 2 and Supplementary Table S1). This finding of ribosomal proteins as candidate biomarkers for the larval families in this study is consistent with previous gene expression analyses of genetically-determined growth variation in larvae of *C. gigas* (Hedgecock et al., 2007; Meyer and Manahan, 2010). The notable consistency across studies of multiple larval families highlights the importance of ribosomal proteins as key regulators of growth and potential biomarkers for aquaculture. In other fields of biology, ribosomal proteins have been identified as indicators of rapid cell proliferation in cancer (Kang et al., 2021), and cellular-level stress responses in "Minute" (slow-growing) phenotypes of fruit flies, *Drosophila melanogaster* (Kiparaki and Baker, 2023). For marine organisms, however, since the highly complex biological processes regulating protein synthesis involve several hundred genes (Gillespie et al., 2022: Reactome Pathway Database), further work is needed to identify the reliability of using a few key genes to predict complex traits, such as fast growth in aquaculture species.

Variability in the amount of food is a major theme in aquaculture and ocean ecology (Conover, 1968; Cranford et al., 2011; Ferreira et al., 2023; Matsubara et al., 2023). Understanding the basic biochemistry of protein metabolic processes will be helpful for optimizing aquaculture operations related to enhanced growth and survival. Under the experimental conditions tested in this study, there were striking differences in the physiological response to algal food availability among different larval families (Table 1). The analysis of larvae from families 1 and 2 also illustrated the contrasting phenotypes of response to algal food deprivation. Unfed larvae (7-day-old) from family 1 sustained a low rate of respiration (compared to fed larvae), with an allocation of 54% (Table 1) to protein synthesis from the energy gained from respiration. Since unfed larvae were not growing by accreting protein, this rate of protein synthesis is a measure of the fundamental biology of protein turnover during development of *C. gigas*. In contrast to unfed larvae from family 1, unfed larvae from family 2 had a higher fractional protein synthesis rate on day 7 (Table 1: family 1, 11%; family 2, 42%), required to maintain protein turnover in these different families. This difference in energy requirements would constrain the amount of energy available to support other essential physiological processes. For example, ion transport by Na^+/K^+-ATPase can account for an average of 20% of a larva's energy budget (Pan et al., 2016). Beyond the dynamics of protein synthesis and turnover, and the maintenance of ion gradients, larvae from family 2 would have little remaining energy to support physiological homeostasis of other essential processes, resulting in larval death (Fig. 3B: family 2).

The analyses of biochemical composition provided insights into the differential use of lipids and proteins to support larval metabolism in the absence of exogenous sources of algal food (Fig. 6; Table 1). Lipids have long been recognized as an important biochemical measure of larval health (Holland and Gabbott, 1971; Gallager et al., 1986; Moran and Manahan, 2004; Da Costa et al., 2016). In the current study, larvae from family 1 depleted triglyceride reserves instead of solely relying on protein to support energy metabolism. Across the four larval families tested, a biochemical threshold was observed of approximately 2.0 ng protein per larva. Larvae with protein amounts below that threshold had ~20% or lower survival (Fig. 3).

It is notable that considerably more genes were differentially expressed in the slower growing (4325 genes) and lower surviving (4649 genes) larvae from family 2, compared to the faster growing (1805 genes) and higher surviving (1250 genes) larvae from family 1. These averaged ~3-fold differences in gene expression among families suggest that a more complex transcriptional regulation may be present in slower-growing and lower-surviving larvae. This finding reveals the possibility of additional bioenergetic consequences from the metabolic

cost of differential transcription that could impact growth and resilience.

Bioenergetic analysis has highlighted the value of defining trade-offs between growth and other complex traits, such as resilience to environmental stressors, cost of reproduction, immune responses, *etc.* (Mangel and Stamps, 2001; Allen et al., 2016; Sae-Lim et al., 2017). Such trade-offs have important consequences for aquaculture yields (*e.g.*, summer heat mortality in triploid *C. gigas*: Li et al., 2022; George et al., 2023). In response to future climate change scenarios, selection for aquaculture yield would benefit from including considerations for selection of biological resilience to varying environmental conditions (Sae-Lim et al., 2017; Frieder et al., 2018; DellaTorre et al., 2022). Resilient animals with physiological capacities to maintain biological functions against perturbations caused by environmental change, are clearly desired for future aquaculture. In that regard, the identification in this study of larval family 1 that had physiological traits for faster growth and higher resilience, suggests the possibility of identifying "winners" under climate change scenarios. For successful applications of biomarkers, verification is required that a trait in a larval stage is retained in the juvenile and adult stage, since high variability between larval and later growth stages has been reported for bivalves (Newkirk et al., 1977; Losee, 1979; Ernande et al., 2003; Durland et al., 2019). Pending such verification, the possibility of using biomarkers (Table 2) offers the promise of screening for desired traits early in larval development for enhancing subsequent aquaculture yields.

CRediT authorship contribution statement

Ning Li: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – original draft. **Francis T.C. Pan:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Andrew W. Griffith:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Melissa B. DellaTorre:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Donal T. Manahan:** Supervision, Conceptualization, Formal analysis, Validation, Writing – original draft.

Declaration of Competing Interest

The authors declare no competing or financial interests.

Data availability

The raw Illumina DNA sequencing reads were deposited at the U.S. National Center for Biotechnology Information (NCBI) BioProject PRJNA970931 under the Sequence Read Archive (SRA) accession numbers SRR24489147 – SRR24489194.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739918.

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