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Integrative biological analyses of responses to food deprivation reveal resilience mechanisms in sea urchin larvae

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Abstract

A fundamental question in ecology is how organisms survive food deprivation. In the ocean, climate change is impacting the phenology of food availability for early life-history stages of animals. In this study, we undertook an integrative analysis of larvae of the sea urchin *Strongylocentrotus purpuratus*—an important keystone species in marine ecology and a molecular biological model organism in developmental biology. Specifically, to identify the mechanisms of resilience that maintain physiological state and the ability of organisms to recover from food deprivation, a suite of molecular biological, biochemical, physiological and whole organism measurements was completed. Previous studies focused on the importance of energy reserves to sustain larvae during periods of food deprivation. We show, however, that utilization of endogenous energy reserves only supplied 15% of the metabolic requirements of long-term survival (up to 22 days) in the absence of particulate food. This large energy gap was not supplied by larvae feeding on bacteria. Estimates of larval ability to transport dissolved organic matter directly from seawater showed that such substrates could fully supply metabolic needs. Integrative approaches allowed for filtering of gene expression signatures, linked with gene network analyses and measured biochemical and physiological traits, to identify biomarkers of resilience. We identified 14 biomarkers related to nutrition-responsive gene expression, of which a specific putative amino acid transporter gene was quantified in a single larva experiencing continuous nutritional stress. Advances in applications of gene expression technologies offer novel approaches to determine the physiological state of marine larval forms in ecological settings undergoing environmental change.

KEYWORDS
environmental change, food availability, gene signatures, larvae, phenology

1 | INTRODUCTION

The ability to predict how organisms respond to environmental change is a long-standing goal in ecological physiology. An understanding of response mechanisms requires integrative approaches spanning different levels of biological hierarchy—from the whole organism, through physiological, biochemical and molecular biological analyses. The reproductive and recruitment ecology of the majority of marine animals is dominated by complex life-history strategies (Hjort, 1914; Marshall & Morgan, 2011; Thorson, 1950). It has

long been recognized that a range of biological responses are required for survival in the nutritionally dilute ocean (Conover, 1968). The phenology of food availability in the pelagic ocean is particularly crucial for successful recruitment of larvae to the adult stage (Cushing, 1990; Edwards & Richardson, 2004; Ferreira et al., 2023; Platt et al., 2003; Starr et al., 1990). These survival strategies are further impacted by climate change in marine food webs (Asch, 2015; Du Pontavice et al., 2021).

Several indices have been applied to measure the nutritional state of marine invertebrate larval forms. For example, morphological measurements and analysis of biochemical content have been applied to assay nutritional condition in response to food deprivation in a range of larval forms (Anger et al., 1985; Fenaux et al., 1994; Gallager et al., 1986; Meyer et al., 2007). For field-collected samples, however, the technical requirements for applications of these analyses are challenging, and particularly so for applications to small larvae in natural settings. Here, we apply well-established mRNA sequencing technology (Stark et al., 2019) to identify molecular biological signatures of resilience to food deprivation in larvae.

Advances in DNA sequencing and bioinformatics offer promising new approaches that can be applied to analyses of ecological and physiological state of individual larvae. A common assumption of gene expression analyses is that changes at the molecular biological level reflect changes at other levels of biological organization (e.g. biochemical, physiological and whole organism), yet validation of candidate gene biomarkers remains challenging (Dalziel & Schulte, 2012; Lemos, 2021). To address this question for marine larvae, we investigated nutrition-responsive gene expression to various experimental food treatments, by (1) integrating analysis of entire transcriptomes with parallel studies of (2) biochemical processes, (3) physiological rates and (4) growth morphology of the sea urchin *Strongylocentrotus purpuratus*. This is a keystone organism in marine ecosystems (Pearse, 2006), and a model species in developmental, evolutionary and genomic biology (Arshinoff et al., 2022; Wang et al., 2020). We identified potential biomarkers that are consistent with physiological resilience to food deprivation by larvae. In addition, we show that changes in a particular biomarker for nutrient transport can be quantified by gene expression analysis in a single larva experiencing continuous nutritional stress.

2 | MATERIALS AND METHODS

2.1 | Approach and rationale

This study had the major goal of understanding the ecologically important question of how larvae cope with algal food deprivation. Specifically, we tested for mechanisms of physiological resilience by measuring the ability of unfed larvae to: (1) sustain respiration, (2) conserve lipid and protein reserves, (3) maintain essential rates of protein synthesis and turnover and (4) recover once algal food becomes available. In addition, measurements were made to determine if larvae have the capacity to feed on alternative particulate sources, specifically bacteria. To identify mechanisms and potential biomarkers of resilience, gene expression analysis was linked with biochemical and physiological processes to provide an integrative, experimental analysis of potential responses to changes in food availability. Details of the sampling regimes are given in Appendix S1 (Part-A).

2.2 | Larval culturing and experimental treatment

Larvae were reared in filtered seawater (pore size: 0.2 μm, Nuclepore, Whatman, UK) at 15°C and a salinity of 33, using culturing facilities at the University of Southern California Wrigley Marine Science Center, Santa Catalina Island, United States. The high-volume, flow-through filtering systems used removed over 99% of bacteria from seawater (see Section 2.4 for DNA staining and Flow Cytometer methods). For example, filtered seawater contained 7080 ± 559 ($n = 6$) bacteria per millilitre—a 99.6% reduction of bacterial populations of over 1.8 million per mL (see Section 3.3 for bacterial enumeration). Gametes were obtained from adult sea urchins (purchased from Marinus Scientific, LLC, Long Beach, California, USA) by intracoelomic injection of 0.5 M KCl. Fertilized eggs were pooled, enumerated and distributed to a series of replicate 20-litre culture vessels. Developing animals in each culture vessel were maintained in suspension using motorized, polycarbonate stirring paddles. Temperature loggers (Onset HOBO Pro v2, USA) were placed in culture vessels to monitor the consistency of seawater temperature during the experimental period.

Once feeding stages (i.e. 4-day-old pluteus-stage larvae) were observed in cultures at 15°C, experimental treatments were initiated based on the presence or absence of algal food. Larvae in the 'fed' treatments were provided with 15,000 cells mL^{-1} of *Rhodomonas lens* (AlgaGen LLC, USA), with algae replenished daily to maintain ad libitum feeding conditions. Larvae from the same cohort (starting at 4-day-old) were reared under the same environmental conditions, except the 'unfed' larvae were not provided with algal food (filtered seawater only). To assess the ability of unfed larvae to recover when food was subsequently made available (i.e. delayed feeding treatments), 12- and 16-day-old unfed larvae were provided with 15,000 cells mL^{-1} of *R. lens*. Seawater in all larval cultures was changed every 2 days, at which time samples were collected for measurements of larval size, biochemical content, respiration rate, ammonia excretion rate, protein synthesis rate and gene expression.

2.3 | Survivorship and growth

Survivorship was determined by counting replicate aliquots of individuals sampled from each culture vessel for the duration of experiments. Individual larvae (~50 per sampling day) were photographed with a digital camera attached to a microscope for morphometric measurements of midline body and post-oral arm lengths (ImageJ; Schneider et al., 2012).

2.4 | Assays of feeding rates by larvae on algae and bacteria

To assess the possibility that larvae may gain energy from feeding on bacteria in seawater, a series of assays was conducted with 4-day-old, feeding stage larvae. Larvae were enumerated and placed at 50 individuals mL^{-1} in three replicate 20-mL glass vials, each containing known numbers of bacteria (populations collected from Pacific Ocean waters off Santa Catalina Island, California). This high number of larvae was selected to maximize the possibility of detecting larval feeding on bacteria. A parallel algal feeding control experiment was established in a separate series of three 20-mL vials, each containing 50 cells μL^{-1} of *R. lens* to demonstrate that larvae were capable of active feeding under the experimental conditions tested. An additional series of triplicate vials containing only algae (no larvae present) were used to correct for any possible algal growth during the course of the 2–3 h experimental assays. (Aluminium foil was used to wrap each vial to prevent light impacting algal growth.) Triplicate vials, containing only bacteria, were assayed to correct for any possible bacterial growth during feeding assays. Gentle hand-mixing every 10–15 min was used to keep larvae, algae and bacteria in suspension throughout each assay. The rate of particle depletion of algal cells (feeding by larvae) was measured by quantifying chlorophyll fluorescence signal with a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, USA). After staining bacteria with SYBR Green (Biotium, USA) for 40 min, the number of bacteria was measured with a BD Accuri C6 Plus Flow Cytometer. A series of seawater samples containing bacteria, but diluted with known amounts of filtered seawater (pore size: 0.2 μm), were used to assess the sensitivity of the flow cytometer to changes in bacterial numbers in seawater. To assay for the viability and health of the larvae used in this feeding experiment, their respiration rates were measured. Fluorescence microscopy (Olympus BX60, Japan) under UV excitation (U-MWU filter cube) was used to image the presence of algae in the digestive system of actively feeding larvae.

2.5 | Respiration rate

Respiration rate was measured as oxygen consumption using volume-calibrated micro-respiration chambers (~500 μL), as previously described (Marsh & Manahan, 1999; with optode technology from Pan et al., 2021). Briefly, known numbers of individuals ($n \geq 250$, depending on treatment and developmental stage) were placed in sealed micro-respiration chambers. The amount of oxygen consumed was measured during time-course assays, using a fibre-optic sensor connected to a Wintrox single-channel oxygen meter (Loligo Systems, Denmark). Respiration chambers with no animals, containing only filtered seawater (0.2 μm pore size), were assayed as controls. Respiration rates were calculated as the slope of the regression of oxygen depletion with time in each individual respiration chamber, corrected for the number of larvae present.

2.6 | Determination of ammonia excretion rate and oxygen-to-nitrogen ratio

Samples of the same seawater that were used to measure respiration rate were also assayed for ammonia content (Solorzano, 1969). This parallel sampling regime allowed for calculations of oxygen-to-nitrogen ratios (O-to-N ratio; Mayzaud & Conover, 1988), a measure of which biochemical components were used to sustain respiration. An O-to-N ratio <20 indicated a protein- and amino acid-dominated catabolism; higher values indicated catabolism of other components (e.g. lipid; Mayzaud & Conover, 1988).

2.7 | Biochemical content

Known numbers of individuals ($n \geq 1200$, depending on treatment and developmental stage) were aliquoted into 1.7 mL microcentrifuge tubes, briefly centrifuged, excess seawater removed and frozen at −80°C until further analysis. Whole-body protein content, lipid classes and carbohydrate content were measured (see method details in Moran & Manahan, 2004). ANOVA was performed to compare biochemical content among different ages of unfed larvae.

2.8 | Rates of amino acid transport and protein synthesis

Rates of amino acid transport and protein synthesis were measured (details in Pan et al., 2015; Lee et al., 2016) in newly formed 4-day-old pre-feeding larvae and among fed and unfed larvae, sampled from the contrasting feeding treatments during larval development. In brief, ^{14}C alanine (Perkin Elmer, USA) was used as the tracer to quantify protein synthesis rates in larvae of different ages and sizes, with appropriate calculations to account for changes in the intracellular specific activity of ^{14}C alanine in the free amino acid pool of larvae (*op. cit.*).

2.9 | RNA extraction, library preparation and transcriptome sequencing (RNA-seq)

Pre-feeding, unfed and fed larvae ($n \geq 1200$ larvae per replicate, depending on treatment and developmental stage) were collected from culture vessels, rinsed with filtered seawater, immediately homogenized in 300 μL of TRIzol Reagent (Ambion, USA) and stored at −80°C. All RNA preparation protocols used disposable RNase-free polypropylene pestles. Sample RNA was extracted with Direct-zol RNA MicroPrep Plus Kit (Zymo Research, USA) (following manufacturer's extraction protocols). DNase I treatment was conducted to remove genomic DNA in separation columns. Nuclease-free water (30 μL) was added to elute RNA from each column. RNA integrity was assessed on a 2% agarose gel and concentration quantified by Qubit RNA HS Assay Kit (Invitrogen,

USA). A total of 120 ng RNA per sample were used for preparation of transcriptome sequencing libraries by TruSeq Stranded mRNA Library Prep Kit (Illumina, USA). Standard single-index adapter kit (24 Illumina Index Adapters: AR001 to AR016, AR018 to AR023, AR025 and AR027) was utilized as barcodes for sample multiplexing. DNA concentration for each of the libraries prepared was quantified by Qubit dsDNA HS Assay Kit (Invitrogen, USA). Size distribution of sequence fragments was determined by Agilent 2100 bioanalyser (Agilent, USA). In total, 36 different libraries were constructed and randomly mixed by proportion of equal molar mass of DNA for sequencing in two lanes of an Illumina HiSeq 4000 system to obtain an average of 43 million, 150 bp reads for each library (Fulgent Genetics, Temple City, California, USA). One sample of 4-day-old larvae was sequenced on both lanes to monitor potential bias from different sequencing lanes (no sequencing lane bias was observed).

2.10 | Gene expression analysis

Illumina universal TruSeq adapters and low-quality bases (base quality score < 20) were trimmed from individual sequencing reads. In addition, short reads (length < 36 bp) were removed by Trimmomatic v0.38 with paired end mode (Bolger et al., 2014). FastQC v0.11.8 was used to evaluate for quality control (Andrews, 2010). Trimmed reads were aligned by HISAT2 v2.1.0 (Kim et al., 2015) to the reference genome of the sea urchin *S. purpuratus* (Spur_5.0; GenBank accession number: GCA_000002235.4; Arshinoff et al., 2022). Nucleotide sequence read counts, calculated as relative expression values (TPM: Transcript Per Million), were determined for each annotated gene using StringTie v1.3.4d (Pertea et al., 2015). Pre-filtering of sequences was carried out to retain both moderately and highly expressed genes (21,732 genes; Table S1) that had read counts of >10 in 10 or more libraries. Principal component analysis (PCA) was conducted to display gene expression profiles for larvae of different ages and sizes. DESeq2 (Love et al., 2014) was utilized to analyse differential gene expression between feeding treatments (pre-feeding, unfed, fed and delayed feeding). *p*-values were adjusted by the Benjamini and Hochberg procedure (Benjamini & Hochberg, 1995) to correct for a false discovery rate. Genes with adjusted *p*-value <.05, and with an absolute magnitude of fold change >2, were categorized as differentially expressed genes (DEGs). Gene Ontology (GO) enrichment analysis of DEGs was conducted by clusterProfiler v3.10.1 (Yu et al., 2012) to identify putative functions of up- and down-regulated genes.

2.11 | Weighted gene co-expression network analysis (WGCNA)

WGCNA (Langfelder & Horvath, 2008) version 1.69 was used to identify co-expressed network modules in larvae, based on an analysis of gene expression profiles for 23,061 genes after filtering out genes with counts of <10 across 90% of the 36 libraries (Table S1). This number of 23,061 genes from WGCNA is slightly larger than the number of 21,732 genes from the differential expression analysis above because of different pre-filtering methods from the WGCNA and DESeq2 software packages. In the current analysis, each individual gene was assigned as a member of a specific, single module. Briefly, a matrix of pairwise correlations between all pairs of genes across all samples was calculated, which was then computed into a Topological Overlap Matrix. This latter analysis showed the interconnection between genes that was then used for hierarchical clustering analysis. Each hierarchical gene cluster with high topological overlap was then grouped into a single module. WGCNA network modules were constructed with the software parameters of 'networkType = signed, softPower = 4 and minModuleSize = 30'. Genes that were not assigned by WGCNA to any of the 36 modules were placed in an additional module (module 37). Modules were calculated by the first principal component of the overall module expression profiles within WGCNA analysis. The correlation between modules and larval traits was further analysed by Pearson's correlation coefficient to identify modules potentially associated with the biology of resilience to food deprivation. The 10 traits included in this analysis were respiration rate, oxygen-to-nitrogen ratio, protein content, amino acid transport rate, fold change of protein synthesis rate relative to pre-feeding stage, midline body length, post-oral arm length and size-specific (midline body length) respiration rate, protein content and amino acid transport rate. WGCNA-defined gene significance between the level of expression of a given gene and a specific biochemical or physiological trait was measured by Pearson's correlation analysis (details in Glossary of WGCNA Terminology, see Langfelder & Horvath, 2008).

2.12 | Reverse transcription-quantitative PCR (RT-qPCR) validation of putative amino acid transporter gene LOC575259

RT-qPCR was utilized to validate the findings from gene expression analysis of the transcriptomes. A different cohort of larvae was used for this independent RT-qPCR validation. Total RNA was extracted from known numbers of pooled unfed larvae and quantified as previously described. RNA was also successfully extracted from single larvae, representing different ages. The RT-qPCR protocol for each of these individual larvae was conducted as described below for samples of pooled larvae. RNA was reverse transcribed to cDNA using GoScript Reverse Transcription system following the manufacturer's instructions (Promega, USA). The qPCR assay was performed by PerfeCTa SYBR Green FastMix (low ROX; Quantabio, USA) on a AriaMx Real-Time PCR system (Agilent, USA). The DNA primer sequences for gene LOC575259 (Forward: CCAGGAGGTTCCTCAACGAC; Reverse: TTGCCCAGTTG AAGCCAAGA) and reference gene elongation factor 1α (*EF1α*; F:

CAACGAAATCGTCAGGGAGGTC; R: AGATTGGGATGAAGGGC ACAG; Meyer & Manahan, 2009) were synthesized by Integrated DNA Technologies (Iowa, USA). The thermal cycling profiles consisted of an initial denaturation at 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by dissociation curve analysis to validate the specificity of amplified PCR products. The PCR efficiency of these primers was >90%. The expression of gene LOC575259 was normalized to an elongation factor reference gene (*EF1α*) and presented as the number of LOC575259 copies per copy of *EF1α*. The magnitudes of fold change for LOC575259 in unfed larvae at different ages relative to 4-day-old pre-feeding larvae were calculated based on the measured values of cycle threshold (Ct) from qPCR.

3 | RESULTS

3.1 | Resilience to food deprivation

Larvae were exposed to three experimental treatments of pre-feeding, unfed and fed. From these treatments, a series of molecular biological, biochemical, physiological and morphometric analyses was conducted. In the absence of algal food, unfed larvae were still capable of: (1) sustaining respiration rate (Figure 1a), conserving whole-body lipid and protein content (Figure 1b,f), and (3) maintaining rates of protein synthesis and turnover (Figure 1c,d) for the duration assayed (to 20-day-old larva). Relative to newly formed 4-day-old larvae (pre-feeding from embryogenesis), 8-day-old unfed larvae had a slight decrease in protein synthesis rate (0.2-fold: Figure 1d; slope comparison: $p = .0003$), a difference that was maintained for the duration of protein synthesis assays (day 16). Since unfed larvae exhibited no protein accretion (Figure 1b), these protein synthesis rates were a measure of protein turnover under food deprivation. In the control feeding treatment, 8-day-old fed larvae had a 4.9-fold increase in protein synthesis rate, relative to the 4-day-old pre-feeding stage (Figure 1d; slope comparison: $p = .004$). Across all feeding treatments (fed, unfed and recovery), the relationship between respiration rate and protein content was highly correlated ($r^2 = .82$: Figure 1e).

Notably, unfed larvae were capable of surviving for 22 days (at which point, the experiment was terminated: Figure 2a). Furthermore, unfed larvae were capable of recovering from prolonged food deprivation. Recovery for 12- and 16-day-old unfed larvae when provided with algal food was evident with a slight delay in response, as measured by increases in respiration, protein accretion (Figure 1a,b), midline body length and post-oral arm length (Figure 2a,b). These recovery rates were within 0.9-fold of the respiration rate and 0.8-fold of protein accretion rate of control larvae (fed as soon as the feeding stage was reached, 4-day-old; Figure 1a,b).

Maternally provided reserves of carbohydrate, lipid and protein were quantified to determine their relative role in the observed lifespan and support of metabolism in unfed larvae. The biochemical composition of eggs was dominated by protein at 26.0 ± 0.9 ng, with 9.1 ± 1.2 ng phospholipid, and 2.3 ± 0.1 ng triglyceride per egg (Figure 1f). Other lipid classes of cholesterols, free fatty acids, hydrocarbons and wax esters were measured and found to be below the limit of analytical detection. The carbohydrate content was also negligible, below the limit of detection (<0.2 ng carbohydrate per individual) for all developmental stages tested. During development from a fertilized egg to the newly formed 4-day-old pre-feeding larva, the triglyceride content was fully consumed, resulting in the availability of 18.2 ± 1.1 ng protein and 3.2 ± 0.3 ng phospholipid (Figure 1f) to support subsequent metabolism (Figure 1a). Over the period tested, unfed larvae did not utilize their remaining phospholipid content (ANOVA of all data between 4- and 16-day-old larval phospholipid contents: $p = .93$; $n = 20$: Figure 1f). In addition, in unfed larvae protein content was conserved from 8- to 20-day-old larvae (ANOVA of all protein content data between 8- and 20-day-old unfed larvae, $p = .25$; $n = 21$: Figure 1b, f).

The importance of protein- or amino acid-based substrates to sustain respiration rate in unfed larvae was evident from an analysis of oxygen-to-nitrogen ratios (O-to-N ratios). That ratio was 47.5 ± 8.0 in newly formed 4-day-old larvae, consistent with the measured biochemical utilization of both lipid and protein (Mayzaud & Conover, 1988) at this early stage of development (Figure 1f). In contrast, 8-day-old unfed larvae had an O-to-N ratio of 11.6 ± 1.1—a ratio that decreased to 9.1 ± 0.1 in 12-day-old and further decreased to 6.9 ± 1.9 in 16-day-old unfed larvae. These low O-to-N ratios support the conclusion that protein- and amino acid-based metabolism (Mayzaud & Conover, 1988) sustained the respiration of unfed larvae (Figure 1a).

3.2 | Bioenergetic calculations of energy reserve depletion and respiration in unfed larvae

Bioenergetic analyses revealed that during extended periods of survival without algal food, maternally provided energy reserves supplied only 15% of respiratory costs. Hence, 85% (552 μJ, based on 98 μJ actual energy available and 650 μJ required) of the energy cost of sustaining respiration was not accounted for by utilization of energy reserves for the survival duration of unfed larvae (Figure 1a), pending their subsequent ability to physiologically recover once algal foods became available. Details for these bioenergetic conclusions are given below.

3.2.1 | Total energy available

The major biochemical reserves in newly formed, 4-day-old pre-feeding pluteus larvae were protein at 18.2 ± 1.1 ng and phospholipid at 3.2 ± 0.3 ng (Figure 1f). Converting these biochemical components into energy equivalents yields 437 μJ from protein (18.2 ng × 24.0 μJ ng^{-1}) and 126 μJ from phospholipid (3.2 ng × 39.5 μJ ng^{-1}). Combined, the available energy was 563 μJ in

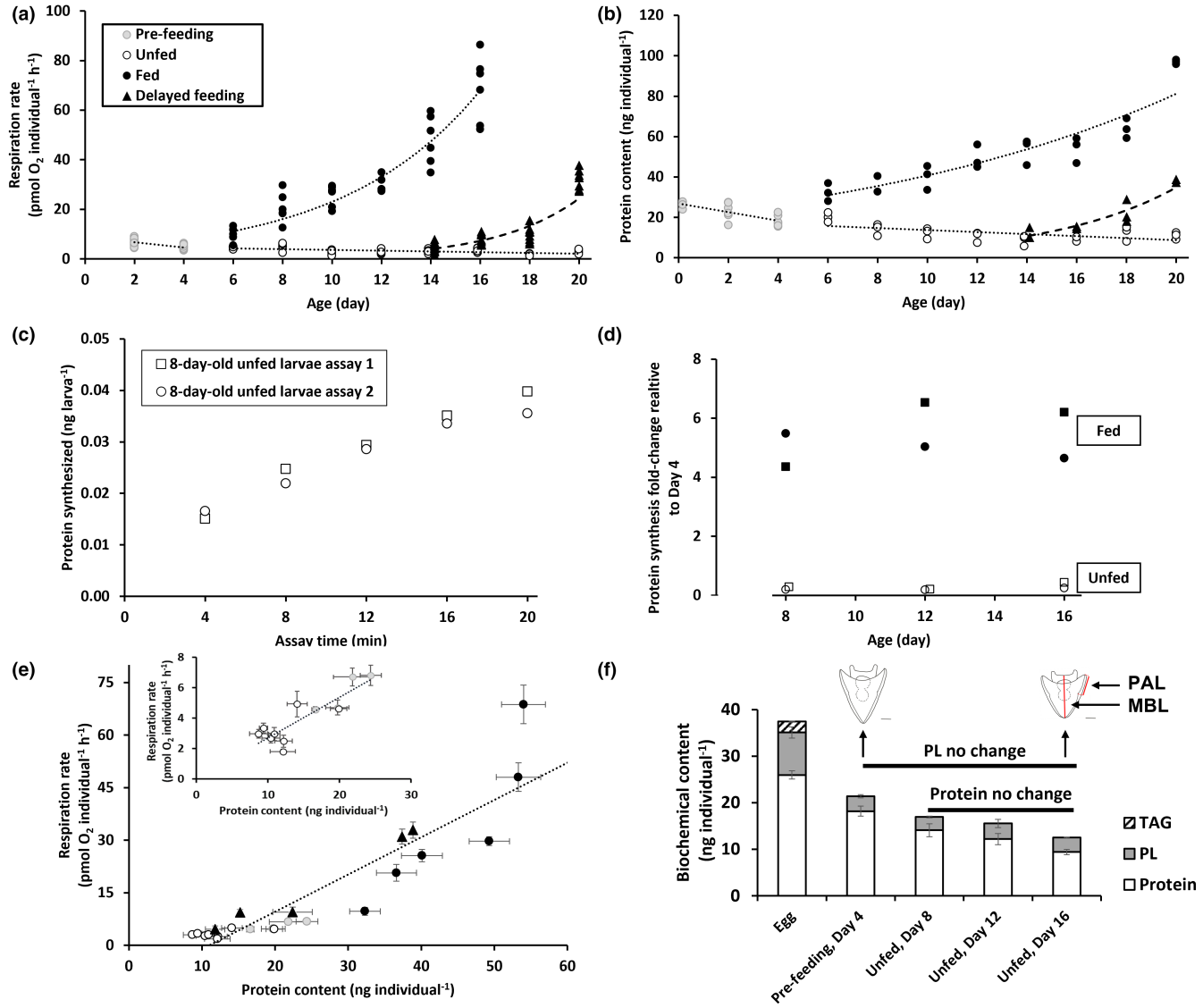

FIGURE 1 Physiology and biochemistry of developmental stages of *Strongylocentrotus purpuratus* (eggs, pre-feeding, unfed, fed and delayed feeding larval treatments). Each treatment is indicated by different symbols in panels a, b, e and e inset (data points legend in panel a). For visual clarity, certain data points are graphically offset on the *x*-axis. (a) Rates of oxygen consumption. Each data point represents a separate measurement of larvae in a replicate micro-respiration chamber. (b) Protein content, corresponding to sampling intervals in panel a (analysis of eggs included). Each data point represents a replicate measurement of larvae. (c) Protein synthesis rate in 8-day-old unfed larvae. Circles and squares represent two independent assays. Protein synthesis rate calculated as the slope of increase in the amount of synthesized protein with time, as measured by the incorporation rate of ^{14}C-alanine into protein (see Section 2 for details). (d) Fold change in rate of protein synthesis for unfed (open circles and open squares, corresponding to symbols in panel c) and fed larvae (solid circles and solid squares). Protein synthesis rates expressed relative to the rate in 4-day-old pre-feeding larvae. Fold change calculated from each independent assay for each age and feeding treatment. A value of 1.0 on *y*-axis indicates no change in protein synthesis rate relative to 4-day-old pre-feeding larvae. (e) Relationship between respiration rate (data from panel a) and protein content (corresponding sampling time intervals from data in panel b). ANOVA of regression: Respiration rate = 1.0644 × Protein content–11.741; $p < .001$; $r^2 = .82$; $n = 23$; error bars represent 1 SEM for both respiration rate and protein content. (Inset graph) Expanded *y*-axis from panel e to illustrate the relationship between respiration rate and protein content in pre-feeding and unfed larvae. ANOVA of regression: Respiration rate = 0.2656 × Protein content + 0.0573; $p = .0002$; $r^2 = .76$; $n = 12$; error bars represent 1 SEM. (f) Biochemical components of eggs, 4-day-old pre-feeding larvae and 8-, 12- and 16-day-old unfed larvae. Triglyceride lipid (TAG) content was only observed in eggs. Phospholipid (PL) content did not change significantly from 4-day-old pre-feeding larvae to 16-day-old unfed larvae (ANOVA, $p = .93$, $n = 20$). Protein content did not change significantly from 8- to 16-day-old unfed larvae (ANOVA, $p = .11$, $n = 9$). Error bars represent 1 SEM for each biochemical component. Larval diagrams illustrate the relative size of a 4-day-old pre-feeding larva compared to a 16-day-old unfed larva (scale bar: 50 μm). Morphometric measurements are illustrated (red lines) for post-oral arm length (PAL) and midline body length (MBL).

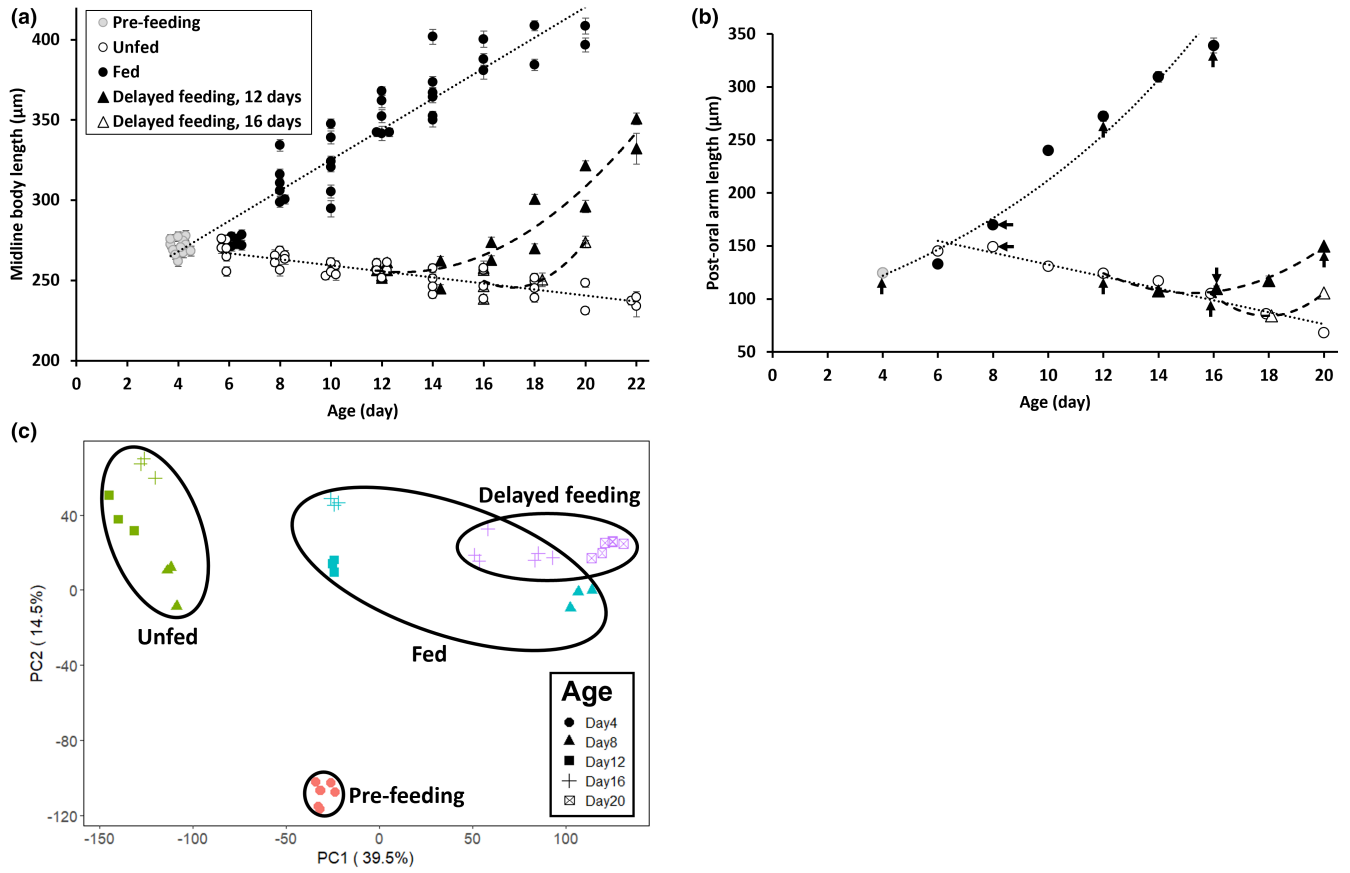

FIGURE 2 Morphological changes and gene expression (transcriptomic analysis) in different ages and feeding treatments for larvae of *Strongylocentrotus purpuratus*. Each treatment is indicated by different symbols in panels a and b. For visual clarity, certain data points are graphically offset on the *x*-axis. (a) Midline body length for fed larvae, and unfed larvae exposed to extended periods of algal food deprivation (to day 22). All measurements started from newly formed 4-day-old pre-feeding larvae (12 culture vessels, each indicated by a separate data point). For each data point, 50 larvae were measured in each culture vessel. For the delayed feeding treatments, algal food was made available on day 12 and on day 16 (timing of delay in recovery shown as calculated best-fit lines). Error bars represent 1 SEM (b) Post-oral arm length for fed, unfed and delayed feeding treatments. Each data point represents the measurements of 50 larvae from representative culture vessels (all culture vessels shown in panel a). Arrows beside each of nine data points illustrate samples taken for transcriptomic analysis. Error bars represent 1 SEM. Where error bars are not shown, error fell within the graphical representation of the data point. (c) Expression patterns of 21,732 genes analysed by principal component analyses (PC1 and PC2) in larvae exposed to different feeding treatments. The proportions of variance explained by PC1 and PC2 are indicated beside each axis. Different feeding treatments illustrated by symbol colour; different ages illustrated by symbol shape. Statistical groupings were based on gene expression, highlighted by ellipses (pre-feeding, red symbols; unfed, green; fed, blue; delayed feeding, purple).

a 4-day-old pre-feeding larva to support further respiration under conditions of food deprivation.

3.2.2 | Energy required

Once the newly formed 4-day-old larva developed, respiration rate was observed to continue to day 20 under conditions of food deprivation (Figure 1a). During this 16-day period, the average respiration rate of unfed larvae was 3.5 ± 0.2 pmol O_2 larva^{-1} h^{-1} (Figure 1a). The total oxygen consumed was 1344 pmol O_2 (3.5 pmol O_2 larva^{-1} h^{-1} × 24 h day^{-1} × 16 days). Conversion (Gnaiger, 1983) of oxygen (moles) to energy (J) was calculated with an oxyenthalpic value of 484 kJ (mol O_2)$^{-1}$. Justification for using this oxyenthalpic average value is based on protein and lipid content [484 kJ (mol O_2)$^{-1}$] (no carbohydrate content was measurable in all stages tested), and that both protein and lipid decreased from the initial content in the 4-day-old larva as development proceeded for an unfed larva (Figure 1f). Starting from the 4-day-old larva, subsequently the total amount of oxygen consumed for the next 16 days (to a 20-day-old larva) equates to 650 µJ [1344 pmol O_2 × 0.484 µJ (pmol O_2)$^{-1}$].

3.2.3 | Actual energy available

This was calculated from the measured rate of depletion of endogenous energy reserves by unfed larvae, starting from the newly formed 4-day-old larva. Protein decreased by 4.1 ng, from

18.2 ± 1.1 ng in a 4-day-old larva to 14.1 ± 1.4 ng in an 8-day-old unfed larva (Figure 1b). There was no further decrease in protein content beyond 8-day-old unfed larva (Figure 1f). This age-dependent depletion threshold was calculated based on an ANOVA analysis that showed no depletion of protein between 8- and 20-day-old unfed larvae ($p = .25$; $n = 21$). This depletion of 4.1 ng protein is equivalent to 98 μJ (4.1 ng × 24.0 μJ ng^{-1}). The decrease in phospholipid in unfed larvae was not significant ($p = .93$; $n = 20$: Figure 1f). The actual energy available from the depletion of protein and phospholipid was 98 μJ over 16 days (i.e. from 4- to 20-day-old larvae).

3.2.4 | Energy gap

From Figure 1a, unfed larvae were capable of sustaining respiration for 20 days without algal food. Following embryogenesis, the total energy available as lipid and protein in a newly formed 4-day-old larva was 563 μJ (see Section 3.2.1). If those energy reserves were fully depleted to support respiration in food-deprived unfed larvae, that amount of energy would have been sufficient to support the energy demand for respiration of 650 μJ for a further 16 days of food deprivation (to 20-day-old unfed larvae). From biochemical analysis of lipid and protein, however, only protein was utilized after the 4-day-old pre-feeding larval stage developed (Figure 1b,f). For protein, the utilization threshold was only 23% depletion of the available energy reserves in a newly formed 4-day-old larva (Figure 1b). This depletion threshold was calculated based on an ANOVA analysis of the depletion of protein (Figure 1f). Beyond this 23% depletion threshold, protein energy reserves were not further utilized to support respiration, which was observed to continue to the end of the experimental period tested (20 days). The measured threshold for conservation of biomass was reached at day 8. Hence, larvae sustained respiration and maintained protein turnover under food deprivation to a 20-day-old unfed larva—a further 12 days beyond the observed bioenergetic limit supported by the measured depletion of energy reserves. The total cost of living under food deprivation to 20 days was 650 μJ (see Section 3.2.2). These calculations reveal a striking resilience of larvae to survive extended periods of food deprivation, beyond assumptions based solely on utilization of maternally provided energy reserves.

3.3 | Sea urchin larvae are not bacterivores

Bioenergetic analyses (see, Section 3.2 above) revealed that 85% of the energy cost of sustaining respiration in the absence of algal food was not accounted for by utilization of endogenous energy reserves. Here, we tested that possibility that unfed larvae could feed on bacteria to supply the energy gap for survival in the experimental absence of algal food. We show that healthy larvae (i.e. capable of feeding on algae and maintaining normal respiration rates) did not feed on bacteria in seawater. Details for this conclusion are given below.

The 4-day-old larvae used in feeding assays were active and healthy, as indicated by: (1) a respiration rate of 5.6 ± 0.2 pmol O_2 larva^{-1} h^{-1} (value similar to previous respiration measurements for larvae of *S. purpuratus*: Pan et al. (2015), also Figure 1a in this study, measured on another cohort of 4-day-old larvae), and (2) feeding rates on the alga, *Rhodomonas lens* (Figure 3a). This algal species is most commonly used to feed and grow larvae of *S. purpuratus* (Leahy, 1986). Algae were also observed (fluorescence photomicrograph: Figure 3a) in the digestive system of larvae that were provided with algal food. Larvae of the same cohort that were used for algal feeding assays (controls, as above) were not observed to feed on natural assemblages of bacteria (Figure 3c). During experiments, the number of bacteria remained constant in experimental vials (20 mL), either when larvae were not present (control for bacterial growth: Figure 3b; ANOVA of regression, $p = .87$) or when larvae were present (Figure 3c; ANOVA of regression, $p = .52$). These assays support the conclusion that larvae of *S. purpuratus*––actively feeding on algae––did not feed on the populations of bacteria occurring in seawater samples collected from the Pacific Ocean (off Santa Catalina Island, California, USA).

3.4 | Gene expression profiling

Gene expression analyses of transcriptomes were conducted for larvae of specific age, developmental stage and nutritional state (Figure 2b). These analyses, based on 21,732 genes in *S. purpuratus* (Table S1), resulted in several distinct gene expression groupings (Figure 2c). Fed larvae had a distinct grouping from unfed larvae as shown by principal component analysis, with both fed and unfed larvae having a substantially different gene expression pattern from newly formed 4-day-old larvae. Also, of note from this analysis is that larvae unfed for 12 and 16 days—and then subsequently fed—exhibited a recovery gene expression profile with partial overlap to control larvae (Figure 2c, comparison of fed and delayed feeding ellipses) that had been immediately fed once the feeding stage developed (4-day-old larvae).

A gene expression analysis was conducted to explore the possibility of an integrative linking between observed biochemical and physiological processes, with predictive molecular biological markers from transcriptomic analysis. Relative to 4-day-old pre-feeding larvae, 8-, 12- and 16-day-old unfed larvae had a shared pattern of differential expression of 2487 genes, representing 11% of the whole transcriptome (ratio of 2487–21,732: Figure 4a). The shared 2487 genes that were differentially expressed across the three age groups of unfed larvae (8-, 12- and 16-day-old) represent a set of common genes affected by food deprivation. Gene Ontology (GO) enrichment analysis revealed genes that were down-regulated for biosynthesis, cell cycle processes and other cellular responses related to DNA damage (Figure 4d). No enrichment of up-regulated GO terms was found in unfed larvae.

A similar GO analysis of the transcriptome of fed larvae (control) showed that 1039 genes were shared by growing larvae, based on a

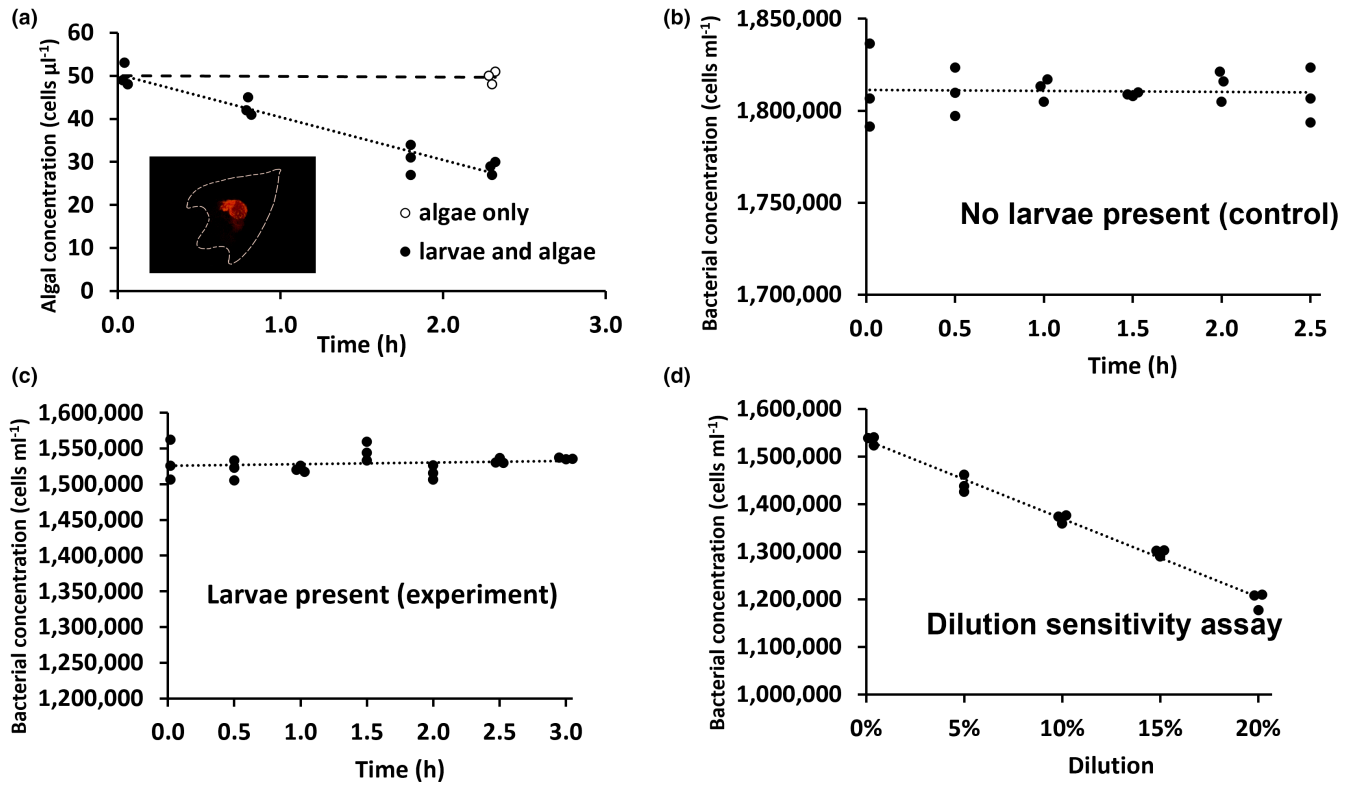

FIGURE 3 Feeding rate assays for 4-day-old larvae of *Strongylocentrotus purpuratus* on the alga *Rhodomonas lens* and on ocean-collected bacteria. For visual clarity, certain data points are graphically offset on the *x*-axis. (a) Time course measurement of removal of algal cells by feeding larvae. Open circles: control vials (20 mL) with algae, but with no larvae present ($n = 3$ at each time point shown). Solid circles: experimental vials with algae and larvae present ($n = 3$ at each time point shown). Each data point represents a separate assay vial. Dash line, control: ANOVA of regression showed no significant decrease, $p = .86$; Solid line, experimental: ANOVA of regression showed significant decrease, $p < .001$, $r^2 = .93$. Photomicrograph image shows the digestive system of a larva containing algae (red colour under the illumination chosen; dashed line added for visual clarity to show larval form). (b) Changes in bacterial concentration in control vials (20 mL) with no larvae present ($n = 3$ at each time point shown). Each data point represents a separate assay vial. ANOVA of regression showed no significant decrease, $p = .87$. (c) Changes of bacterial concentration in the presence of larvae ($n = 3$ at each time point shown). Each data point represents a separate assay vial. ANOVA of regression showed no significant decrease, $p = .52$. (d) Sensitivity assay to quantify the detection limit of the flow cytometer method used to assay changes in bacterial concentration ($n = 3$ at each time point shown). Changes in bacterial concentration were experimentally attained by dilution of ocean-collected bacterial samples with sterile-filtered seawater (0.2 µm pore size). Each data point represents a separate assay vial (no larvae present). ANOVA of regression showed significant experimental decrease, $p < .001$, $r^2 = .99$.

comparison of 8-, 12- and 16-day-old fed larvae with 4-day-old pre-feeding larvae (Figure 4b). This number of differentially expressed shared genes during growth represented only 5% of the transcriptome (ratio of 1039–21,732). These genes showed a GO expression pattern (Figure 4e) related to the measured up-regulation of protein biosynthesis in fed larvae (Figure 1d).

An analysis was undertaken for larvae recovering from food deprivation to identify genes linked to physiological recovery. The transcriptomes of 16- and 20-day-old larvae that had been provided with algal food after an extended food-deprived duration was compared to the starting point of the recovery experiments (i.e. 12-day-old unfed larvae). In contrast to the comparisons of unfed larvae (2487 genes: Figure 4a) and fed larvae (1039 genes: Figure 4b), three- to sevenfold more genes were affected during physiological recovery from delayed feeding (6832 genes: Figure 4c). GO analysis revealed a highly complex up- and down-regulation response for gene expression involving, for example, RNA processing, biosynthesis, metabolism and cell cycle processes (Figure 4f). The inference from this GO analysis highlighted the underlying molecular biological complexity for unfed larvae to recover to a growth physiological state following an extended period of food deprivation. The 6.6-fold increase in the number of differentially expressed genes in recovering larvae relative to control larvae (ratio of 6832–1039: Figure 4b,c) provided insights into the transcriptomic complexity of recovery from food deprivation.

The transcriptomic sampling scheme (Figure 2b) was also designed to allow for a comparison of age- and size-specific patterns of gene expression. Regarding an age comparison, 16-day-old larvae that had experienced a period of delayed feeding for 12 days (including embryogenesis) were compared with 16-day-old larvae that had been fed as soon as the first feeding stage was reached (4-day-old pre-feeding larvae). In this comparison of small (268 ± 2 µm midline body length) and large (389 ± 3 µm) larvae of the same 16-day-old age, a total of 4417 genes (20% of transcriptome) were differentially

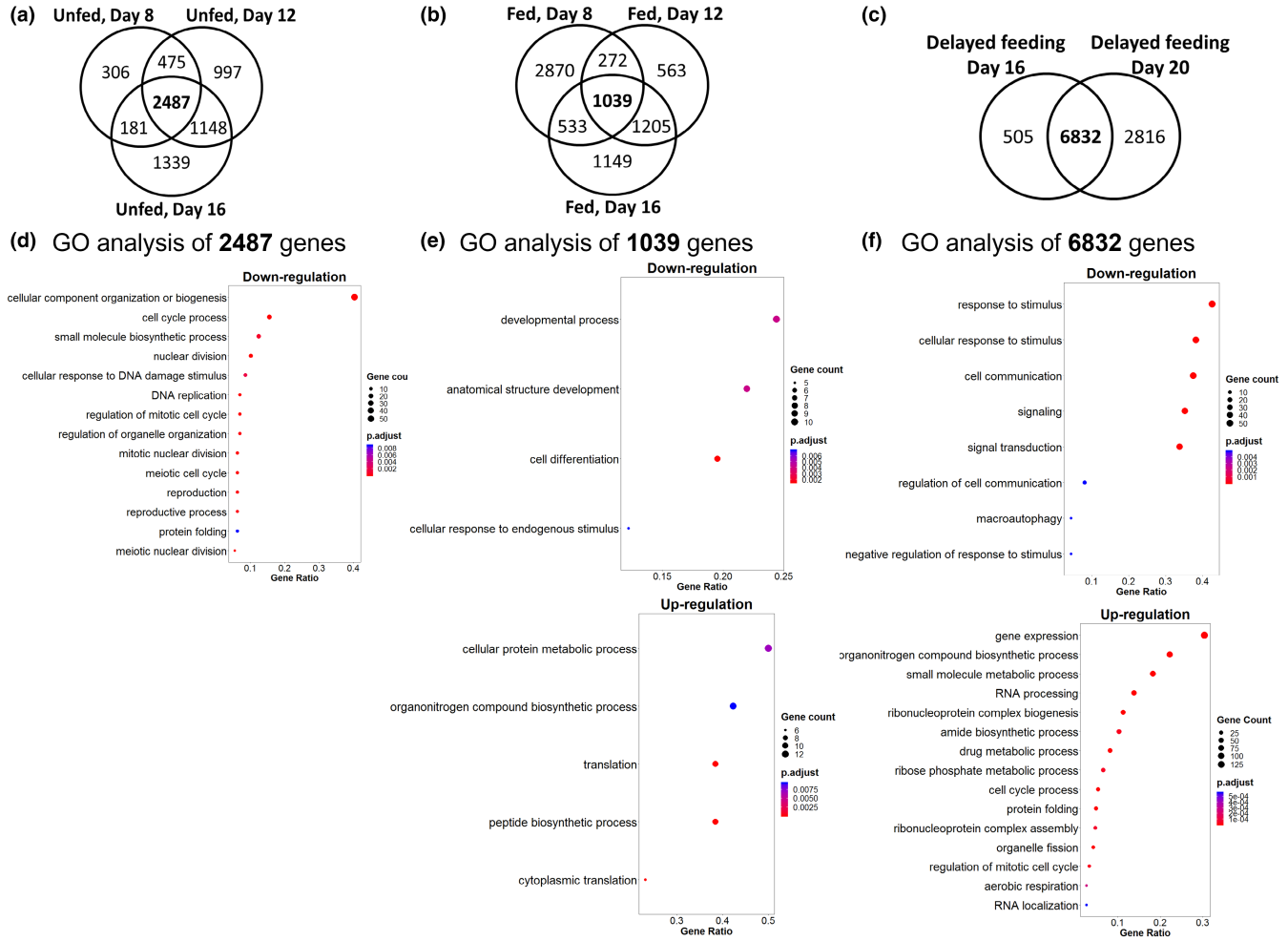

FIGURE 4 Patterns of differentially expressed genes (DEGs) in larvae of *Strongylocentrotus purpuratus* of different ages and feeding treatments. (a) Unfed treatment. DEGs in 8-, 12- and 16-day-old unfed larvae, relative to 4-day-old pre-feeding larvae. Venn diagrams show number of DEGs shared. The number of shared genes as food deprivation extended in unfed larvae was 2487 (highlighted by bold). (b) Fed treatment. DEGs in 8-, 12- and 16-day-old fed larvae, relative to 4-day-old pre-feeding larvae. Venn diagrams show number of DEGs shared. The number of shared genes as growth progressed in fed larvae was 1039 (highlighted by bold). (c) Feeding recovery treatment. DEGs in 16- and 20-day-old larvae after algal food was made available following 12 days of food deprivation. The number of shared genes following delayed feeding recovery was 6832 (highlighted by bold). (d) Gene Ontology (GO) analysis of the 2487 shared genes in unfed larvae from panel a. No enrichment of up-regulated GO terms was found in unfed larvae. (e) GO analysis of the 1039 shared genes in fed larvae from panel b. (f) GO analysis of the 6832 shared genes in delayed feeding larvae from panel c. In panels d, e and f gene count for each GO term indicated by circle size.

expressed (2117 genes up-regulated and 2300 down-regulated in small relative to large larvae). For a size comparison of transcriptomes in larvae under different feeding treatments, 20-day-old larvae that had experienced a period of delayed feeding for 12 days (311 ± 3 μm) were compared with same-sized 8-day-old larvae (311 ± 1 μm; control, fed starting at 4-day-old: Figure 2a). In this case, only 420 genes (2% of transcriptome) were differentially expressed (338 genes up-regulated; 82 genes down-regulated in 20-day-old relative to 8-day-old larvae of the same size). This 10-fold smaller fraction (2% for size-specific comparison vs. 20% for age-specific comparison) of differentially expressed genes in the transcriptome of larvae highlights the importance of understanding age–size gene expression relationships during development and growth in an environmental context.

3.5 | Gene networks

A Weighted Gene Co-expression Network Analysis clustered each individual gene as a member of a specific, single module to identify associations with measured biochemical and physiological traits of pre-feeding and unfed larvae (Figures 1 and 5). The following suite of complex traits was included in this analysis: (1) respiration rate, (2) oxygen-to-nitrogen ratio, (3) protein content, (4) amino acid transport rate, (5) fold change of protein synthesis rate, (6) midline body length (size), (7) post-oral arm length, size-specific (8) respiration, (9) protein content and (10) amino acid transport. Regarding morphological measurements in pre-feeding and unfed larvae, there was not a statistically significant relationship between post-oral arm length and protein content ($p = .18$; $n = 9$). There was, however,

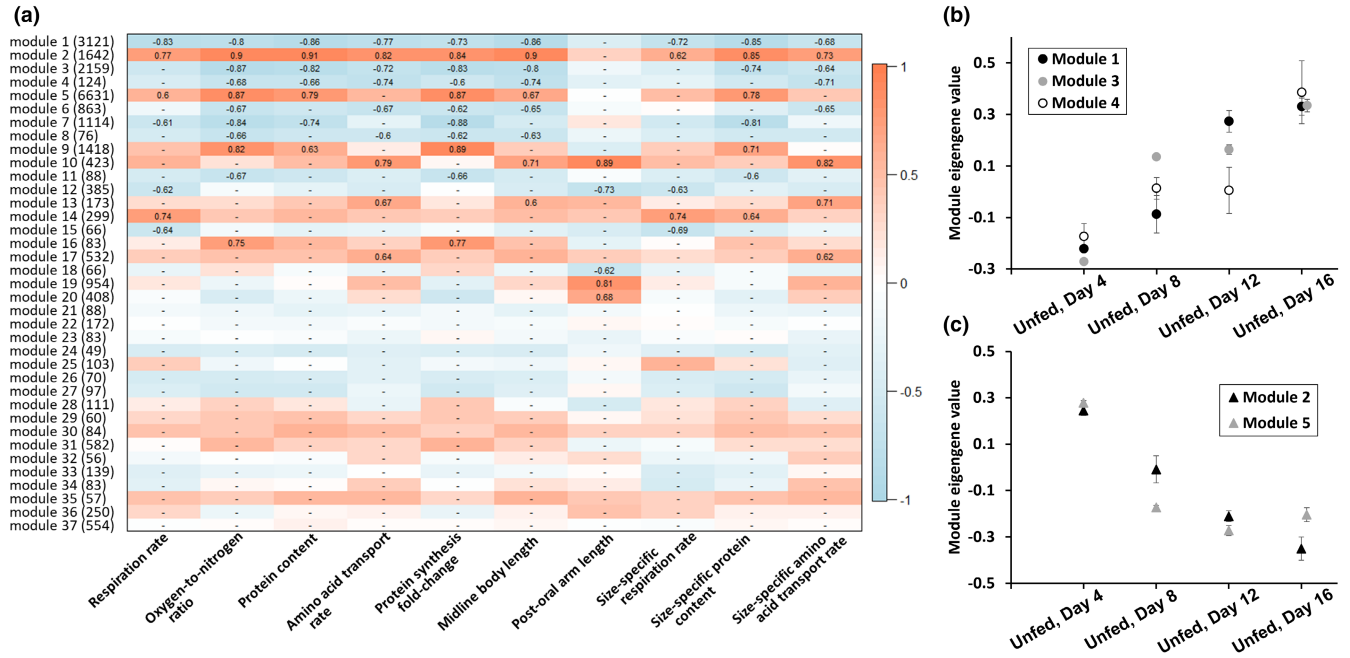

FIGURE 5 Weighted Gene Co-expression Network Analysis for unfed larvae of *Strongylocentrotus purpuratus* of different ages. (a) Module–trait relationships showed correlation of 37 modules with 10 traits. Each individual gene was assigned as a member of a specific, single module. Each row corresponded to a module and each column to an individual trait. Pearson's correlation coefficient is indicated in the bar on the right side of the blue-to-red heatmap (from −1 to +1). Listed in each cell of the heatmap are Pearson's correlation coefficients for high module–trait correlations ($p < .01$). Modules ranked 1–37 based on total number of traits correlated with a given module. The number of unique genes included in each module is listed in parenthesis. (b) Modules 1, 3 and 4. The genes in these modules (number of genes in each module given in panel a) had an average increased expression pattern with increasing time of food deprivation for unfed larvae (Data point labelled as 'Unfed, Day 4' refers to 4-day-old pre-feeding larvae). Error bars represent 1 SEM ($n = 16$ unfed larval samples analysed). The units on the y-axis are calculated from the first principal component of each module (Eigengene value). (c) Modules 2 and 5. The genes in these modules (number of genes in each module given in panel a) had an average decreased expression pattern with increasing time of food deprivation for unfed larvae (data point labelled as 'Unfed, Day 4' refers to 4-day-old pre-feeding larvae). Error bars represent 1 SEM ($n = 16$ unfed larval samples analysed). The units on the y-axis are calculated from the first principal component of each module (Eigengene value).

a significant relationship between midline body length and protein content ($p = .03$; $n = 10$), highlighting the importance of measuring mass (protein) and both morphological variables (post-oral arm and midline body lengths).

A total of 37 modules were identified, with 36 used for further analysis. Module 37 was not included in this analysis, as that module contained the 554 genes that could not be assigned to any network (The number of 37 modules identified in this study is not exceptionally high; values of 40–50 are reported in literature: Sun et al., 2017; Wang et al., 2019). Only two modules—module 1 (3121 genes) and module 2 (1642 genes)—were highly correlated with 9 of the 10 traits ($p < .01$ for all 9 traits; Figure 5a). This was expected since one of the 10 traits (post-oral arm length) did not significantly change with protein content. The other morphological trait, midline body length, was significant. Module 3 correlated with 7 of the 10 traits, and modules 4 and 5 showed a lower number of correlations with only 6 of the 10 traits. The remaining 31 modules all correlated with <6 traits and, hence, were not included in any further analysis. Module 1 contained 3121 genes that had a pattern of increased expression with increasing time of food deprivation for unfed larvae (Figure 5b). In contrast, the 1642 genes in module 2 had a decreased expression pattern (Figure 5c). These contrasting patterns in gene expression between module 1 and module 2 reflect an average up- and down-regulation of groups of genes in those respective modules. The increased or decreased expression patterns for modules 3, 4 and 5 are also shown in Figure 5b,c.

Further analysis identified possible gene expression signatures for resilience to food deprivation, with a focus on unfed larvae that sustained respiration, conserved lipid and protein reserves and maintained rates of protein synthesis and turnover (Figure 1). First, within modules 1 and 2, all core genes that had high module membership and gene significance were retained in the analysis (Table S2). This data filtering step resulted in a total of 94 genes (90 from module 1 and 4 from module 2) that displayed high correlation with complex traits. In the second filtering step, genes that showed up-regulation in unfed larvae were retained, based on differential expression analysis relative to newly formed 4-day-old pre-feeding larvae. This resulted in the reduction of the number of genes from 94 to 27. In the third filtering step, to identify potential biomarkers of physiological state in unfed larvae, the analysis identified 14 genes that were up-regulated in unfed larvae and whose pattern was not present in fed larvae. From genome annotation of *S. purpuratus* (Spur_5.0; Arshinoff et al., 2022), these 14 genes were categorized in several putative functional categories (Table 1).

TABLE 1 Categories of 14 biomarker candidate genes (gene ID listed) based on gene annotation of *Strongylocentrotus purpuratus*.

Gene ID	Category	Gene annotation	TPM, Day 4	Unfed, Day 8	Unfed, Day 12	Unfed, Day 16	Fed, Day 8	Fed, Day 12	Fed, Day 16
LOC575259	Membrane transporter	Sodium- and chloride-dependent glycine transporter 1	150.5	0.8	1.7	2.9	0.4	0.6	0.6
LOC115918921		Monocarboxylate transporter 7-like	0.4	1.4	3.4	6.6	0.1	0.0	0.0
LOC594364		Organic cation transporter protein-like	0.1	2.4	9.2	27.9	0.2	0.7	0.0
LOC574693	Heat shock protein	Heat shock 70 kDa protein IV	3.9	1.2	3.0	8.7	1.3	0.4	0.8
LOC115929375		Heat shock 70 kDa protein IV-like	3.3	1.1	2.4	6.8	1.3	0.4	0.8
LOC584383	Extracellular matrix protein	Fibrillin-2	1.6	1.0	1.6	2.2	0.9	1.1	1.0
LOC754732		Fibropellin-1	20.3	1.0	1.4	2.1	0.9	1.1	1.2
LOC589984		Neural cadherin	0.2	0.9	1.6	2.2	0.7	0.7	1.1
LOC105442843		Neural cadherin-like	0.3	0.9	1.6	2.3	0.6	0.8	1.1
LOC576716	Immune response	Scavenger receptor cysteine-rich type 1 protein M160	0.3	1.1	2.9	5.3	0.3	0.7	1.2
LOC115918657		C-C chemokine receptor type 8-like	2.0	1.1	1.6	2.1	0.6	0.9	0.8
SpAN		Protein SpAN	0.5	1.2	3.0	12.4	0.7	0.9	1.1
LOC100890724	Receptor	Dopamine receptor 2-like	0.8	0.9	2.3	2.2	0.9	0.8	0.6
LOC115924079	Gene regulation	Non-coding RNA	1.9	0.8	2.1	2.2	0.7	0.9	1.0

Note: Column labelled "TPM, Day 4" gives the transcripts per million (TPM) for each gene in 4-day-old pre-feeding larvae. For later larval stages (8-, 12- and 16-day-old unfed larvae and fed larvae, respectively), fold changes of gene expression were calculated relative to 4-day-old pre-feeding larvae (e.g. Unfed, Day 16 has a 2.9-fold increase in LOC575259 gene expression relative to 4-day-old pre-feeding larvae). Sequencing read counts for each of the 14 genes listed are given in Table S1.

3.6 | Biomarkers

An important characteristic of unfed larvae observed in this study was their ability to sustain metabolism in the absence of algal food and subsequently recover once food became available (Figures 1a,b and 2a,b). This physiological state was achieved without the need to fully deplete endogenous lipid and protein stores, since 85% of the respiratory costs of unfed larvae were not supported by utilization of endogenous energy reserves (see, Section 3.2). This energy gap would need to be supplied by an exogenous source of energy (non-algal). Of the 14 biomarker genes identified in unfed larvae, the neutral amino acid transporter gene LOC575259 was the most highly expressed (Table 1 and Table S1). This gene in larvae of *S. purpuratus* is physiologically homologous to other amino acid transporter genes in this species (Meyer & Manahan, 2009), as well as in other species (Bröer & Gether, 2012; Pramod et al., 2013).

A series of biomarker validations for putative amino acid transporter gene LOC575259 (herein, AAT) was applied to assess the expression of AAT in unfed larvae (4-, 8-, 12- and 16-day-old). Analysis by gene expression from transcriptomes showed a pattern of increased (scaled to transcripts per million, TPM) expression of AAT, with 16-day-old unfed larvae expressing AAT at 417 TPM—2.8-fold higher than the 150 TPM in 4-day-old pre-feeding larvae (Figure 6a). The TPM for AAT was 5.6-fold higher in unfed larvae compared to fed larvae (417 ± 46 in 16-day-old unfed larvae; cf. 75 ± 2 in 16-day-old fed larvae: Figure 6a). A qPCR validation revealed near-identical results to transcriptomic expression of AAT (Figure 6b). A validation of observed increase of AAT was repeated with a time-course series for another cohort of unfed larvae. Again, AAT showed an increasing pattern of expression in unfed larvae in the absence of algal food, compared to 4-day-old pre-feeding larvae (Figure 6c). Finally, a qPCR protocol was developed and applied to measure AAT expression in a single larva (replicated on a series of single larva samples: Figure 6d). A 4-day-old pre-feeding larva had lower amounts of AAT transcripts, as indicated by a higher cycle threshold (C_t) value of 32.5 ± 1.3. The C_t value for older unfed larvae decreased from 32.5 ± 1.3 to statistically lower values of 25.1 ± 0.5 for 8-day-old larvae (Welch's *t*-test, $p = .01$) and 23.6 ± 0.6 for 12-day-old larvae (Welch's *t*-test, $p = .005$). The C_t values were not significant between 8- and 12-day-old unfed larvae (Welch's *t*-test, $p = .12$). These data support a conclusion that an unfed larva, deprived of algal food for longer periods, had a significantly higher number of transcript copies for AAT than a newly formed 4-day-old larva.

4 | DISCUSSION

The central issue addressed in this study is to understand the mechanisms by which larval forms cope with particulate food deprivation. Traditional analyses of this phenomenon have tended to focus on the role of maternally endowed energy reserves to extend larval lifespan (Byrne & Sewell, 2019; Emlet, 1987; Grote et al., 2011; Helm et al., 1973; Marshall et al., 2008; Moran et al., 2013; Vance, 1973). Yet, in this study, we showed that the utilization of remaining maternal reserves in newly formed 4-day-old larvae only accounted for 15% of the energy needed for the long-term survival (up to 20 days) of larval forms in the absence of algal food. Notably, there was a lipid and protein depletion threshold of 23%, beyond which there was no further utilization of energy reserves (Figure 1b,f). This energy gap of 85% to sustain respiration, conserve biomass and maintain the essential process of protein turnover in unfed larvae was not accounted for by feeding on algae (absent by experimental design), nor by feeding on bacteria (Figure 3). Here, we provide quantifiable evidence by flow cytometry that sea urchin larvae do not feed on bacteria in seawater which are routinely present in non-axenic standard larval culturing protocols. This finding is consistent with biomechanical observations that cilia-based feeding behaviour in echinoid larvae does not capture small, bacteria-sized particles (Strathmann et al., 1972). Also, direct counting by microscopy revealed that bivalve larvae do not feed on bacteria (Manahan & Richardson, 1983). Further evidence for a lack of bacterivory to supply the energy gap in sea urchin larvae was obtained from the following analysis.

The protein content of planktonic marine bacteria was estimated to be 24×10^{-15} g cell^{-1}, equivalent to 63% of dry weight (Simon & Azam, 1989; Zubkov et al., 1999). To provide a maximum possible estimate of the energy available in a single bacterial cell, it was assumed that the remaining 37% of dry weight was lipids (i.e. 14×10^{-15} g lipids cell^{-1}). Converting these biochemical contents into energy equivalents, the corresponding values were 5.8×10^{-10} J for protein (i.e. 2.4×10^{-14} g × 24.0 μJ ng^{-1}) and 5.5×10^{-10} J for lipids (i.e. 1.4×10^{-14} g × 39.5 μJ ng^{-1}). Combined, this equates to an energy content of 1.1×10^{-9} J per bacterial cell. Based on this value, the number of bacterial cells required to supply the energy gap of 552 μJ per larva over a 16-day period (see, Section 3.2) was 1307 cells per hour (assuming 100% efficiency of mass-energy conversion) [552 μJ ÷ (16 × 24) h ÷ 1.1×10^{-9} J cell^{-1}]. From these best-case scenario estimates of the energy content of a marine bacterial cell, over a 3-h experiment duration (Figure 3c), there would have to have been a decrease of 196,050 bacterial cells mL^{-1} (1307 cells h^{-1} × 50 larvae mL^{-1} × 3 h). As shown in Figure 3d, even a small 5% decrease (an average of ~83,900 bacteria per mL) in bacterial numbers could be accurately measured using the BD Accuri C6 Plus Flow Cytometer method. Hence, a 13% decrease would have been easily detectable, but was not observed (196,050 ÷ 1,525,699; starting bacterial number estimated from the intercept of regression in Figure 3c). Collectively, the data shown in Figure 3a,c support the conclusion that—while pluteus larvae of *S. purpuratus* can actively feed on microalgae (*R. lens*)—larvae of this species are not capable of feeding on bacteria.

Regarding other potential sources of exogenous energy, the possibility that larvae utilize dissolved organic matter as an energy source is highlighted in the current study by the up-regulation in expression of an amino acid transporter gene in unfed larvae (Figure 6). For context, the possibility that soft-bodied marine invertebrates can gain energy by transporting dissolved organic matter directly from seawater has been investigated for over a century (reviewed

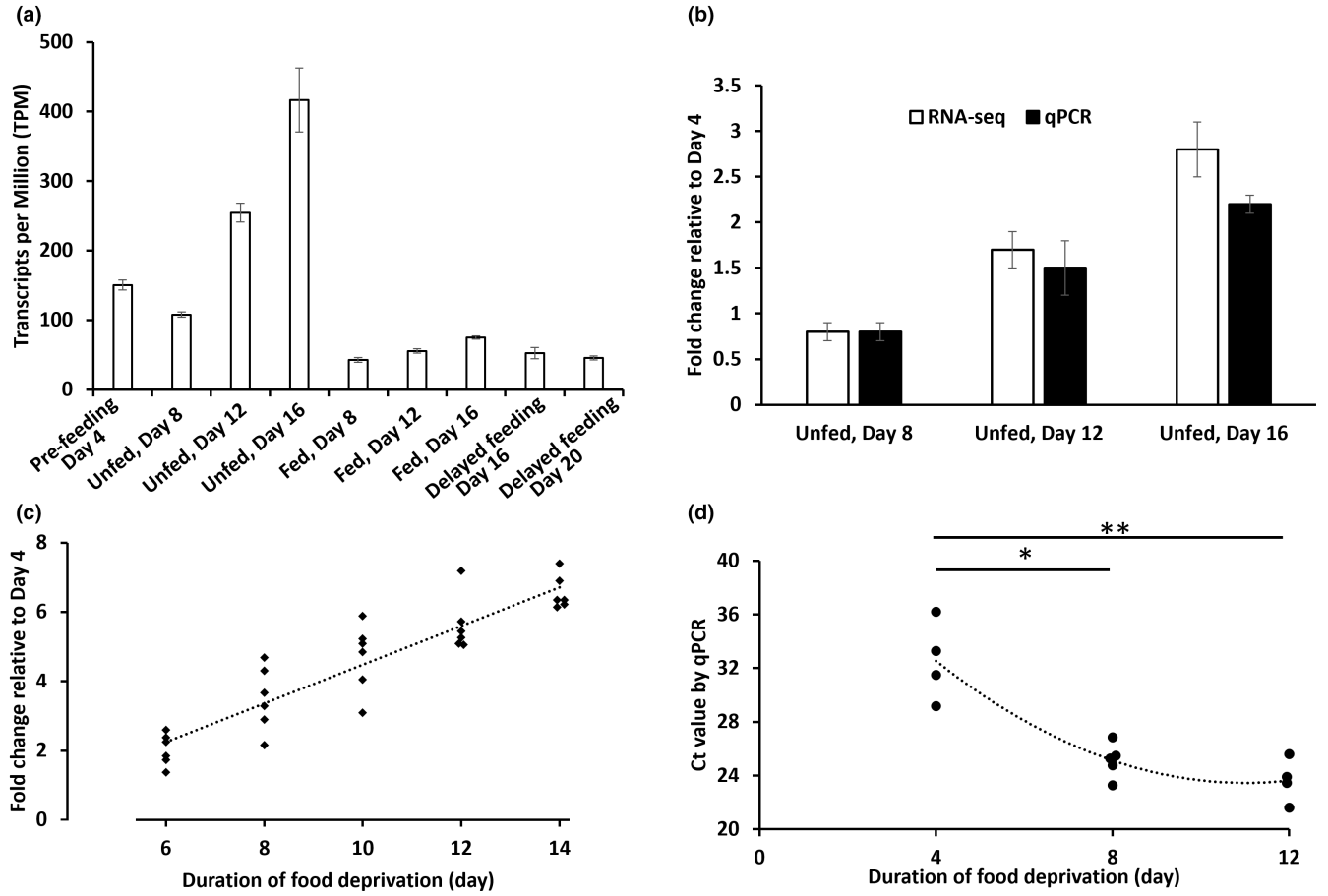

FIGURE 6 Expression of putative amino acid transporter gene LOC575259 (AAT) in larvae of *Strongylocentrotus purpuratus* of different ages and feeding treatments. For visual clarity, certain data points are graphically offset on the *x*-axis. (a) AAT gene expression analysed from nucleotide sequencing (RNA-seq), scaled to transcripts per million (TPM), in larvae from different feeding treatments. Error bars represent 1 SEM ($n = 3$–7 per larval sample). (b) Nucleotide sequencing by RNA-seq (open bars) and qPCR validation (solid bars) for AAT gene expression, illustrated as fold change in unfed larvae relative to 4-day-old pre-feeding larvae. Error bars represent 1 SEM ($n = 3$ for each age and each quantification method). (c) An independent (i.e. another cohort of unfed larvae) qPCR validation of AAT gene expression in unfed larvae, illustrated as fold change relative to 4-day-old pre-feeding larvae. Each data point represents an assay on different samples of larvae ($n = 6$ for each age sampled). (d) qPCR measurement of AAT gene expression in single unfed larvae. Each data point represents a qPCR assay on a single larva. Starting point was 4-day-old pre-feeding larva. Quantification of gene expression measured as cycle threshold value (C_t), with low value indicating high gene expression. **p*-value <.05; ***p*-value <.01.

by Manahan, 1990). Specifically for developing sea urchins, we calculate that an environmentally realistic concentration of dissolved organic matter (amino acids) could fully support the metabolic requirements of an unfed larva of *S. purpuratus* (see calculations in Appendix S1, Part-B).

Insights gained from observations of whole-organism growth—linked with biochemical and physiological analyses—were valuable for understanding the physiological bases of resilience to food deprivation (Figures 1 and 2). Such analyses, however, are limited in their applicability to the ecology of small larval forms in nature since, for the latter, it would not be appropriate to pool field-collected larvae for a biochemical analysis given the likelihood of high variance in individual larvae. Molecular biological analyses have been widely applied to understand whole-organism responses to environmental stress (Bedulina et al., 2013; Devens et al., 2020; Gleason & Burton, 2015; Heckmann et al., 2008; Lockwood & Somero, 2011). Here, we implemented an integrative approach to identify specific nutrition-responsive gene biomarkers that were linked to measurements spanning different biological hierarchies (biochemical and physiological processes, and organismal growth: Figure 5). The result was the identification of 14 potential candidates from a transcriptome of over 20,000 genes in the sea urchin *S. purpuratus* (Table 1 and Table S1). Of these 14 genes, the putative amino acid transporter gene LOC575259 was the most abundant (TPM, Table 1). This gene was successfully applied in independent verification assays (different larval cohort) for resilience to food deprivation in a single larva (Figure 6d).

The analyses by gene ontology (Figure 4) and gene networks (Figure 5) do not imply that the 14 candidate genes identified (Table 1) have been characterized functionally, although for amino acid transporter genes, the use of heterologous expression and reverse genetics has verified physiological function for specific amino acid

transporter genes in developmental stages of *S. purpuratus* (Meyer & Manahan, 2009). Rather, the goal of the current study was to identify biomarkers of relevance for ecological and physiological analyses of resilience to food deprivation in single larvae. An outcome from the biological filtering steps used to link gene network analysis with biochemical and physiological processes in unfed larvae was that the identification of three candidate genes in the category of membrane transporter (Table 1) supports the bioenergetic analysis of a need to acquire dissolved organic matter from the environment. In addition, given the observation of the maintenance of protein turnover in unfed larvae (Figure 1d), the identification of candidate genes that link to heat shock proteins was notable in the context of needing to maintain correct protein structure under food deprivation (chaperone function). Candidate genes identified in the category of extracellular matrix proteins would also assist in the integrity of cell–cell connectivity and communication under nutritional deprivation. Regarding morphological responses to food deprivation (Figure 2a,b), a dopamine receptor candidate gene identified in the current study is consistent with the findings of Adams et al. (2011) and other reports of increases in post-oral arm length in sea urchin larvae (Boidron-Metairon, 1988; Fenaux et al., 1994). These prior findings were interpreted to be an adaptive adjustment of feeding capacity, although Nilsson and Pernet (2022) reported that morphological response of developmental stages of echinoids can depend on the culturing conditions used to rear larvae.

The identification in the current study of specific nutrition-responsive genes (Table 1 and Figure 6) will assist with resolving decades-long debates (Boidron-Metairon, 1988; Fenaux et al., 1994; Nilsson & Pernet, 2022) regarding appropriate measurements to apply to understand the physiological state of larvae under various environmental conditions. In that regard, the nutrition-responsive gene expression for the putative amino acid transporter (LOC575259) showed high correlations with a range of traits in unfed larvae. When food limitation was experimentally continued, this gene showed enhanced expression in individual unfed larvae (Figure 6). The expression pattern of this gene was verified in different larval cohorts, suggesting that it is a robust biomarker that could be used to determine the nutritional status of single larvae (Figure 6). As illustrated in this study, confidence in the selection of a biomarker is enhanced when there is an experimentally based linking of biochemical and physiological phenotypic filters for interpterion of gene expression patterns.

In conclusion, the resilience of the larval form studied to survive an extended period in the absence of algal food is not based on the utilization of endogenous energy reserves, nor alternative sources of exogenous particulate food (feeding on bacteria). Instead, the energy gap could be supplied by direct acquisition of dissolved organic matter from seawater. This conclusion is supported by the new findings in the current study of the up-regulation of amino acid transporter gene expression in response to particulate food deprivation. Combined, these integrative approaches provide meaningful biological signals that reveal new insights to address long-standing challenges of identifying specific biomarkers from thousands of candidate genes (Figure 4) by linking gene expression with measured suites of physiological processes (Figure 5). The resulting outcome is an increased understanding of the mechanisms of organismal resilience and the promise of new approaches to analyse developmental stages in natural ecological settings undergoing environmental change.

AUTHOR CONTRIBUTIONS

NL, AWG and DTM conceived and designed the experiments. NL and AWG performed the experiments and collected data. NL, AWG and DTM analysed the data. NL, AWG and DTM wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The raw Illumina DNA sequencing reads were deposited at the U.S. National Center for Biotechnology Information (NCBI) BioProject PRJNA947343 under the Sequence Read Archive (SRA) accession numbers SRR23934862–SRR23934898.

BENEFITS SHARING STATEMENT

The work provides the benefits of public access to new RNA-seq data sets obtained for larvae of the sea urchin *Strongylocentrotus purpuratus* under different environmental conditions. The methodology used in this study provides an integrative approach spanning different biological hierarchies for effective identification of gene expression signatures for mechanisms of resilience in marine larval forms to variable food environments. It also addresses a priority concern regarding the development of predictive, single-individual indexes to assess the physiological and nutritional states of larvae in natural ecological settings.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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