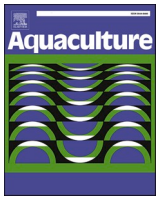
Aquaculture 613 (2026) 743388

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Mothers know best: Maternal signaling boosts larval resilience under ocean acidification conditions

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ARTICLE INFO

Keywords:
Ocean acidification
Shellfish aquaculture
Environmental priming
Lipidomics
Transcriptomics

ABSTRACT

Bivalve aquaculture is a growing sector worldwide, producing sustainable animal protein to meet growing demand from consumers. Yet, the industry remains vulnerable to environmental changes that can impact their product across life stages, especially at the larval stage. Parental priming, or the exposure of broodstock to adverse environmental conditions as they undergo gametogenesis, holds promise as a method to increase resilience in bivalve offspring. We exposed Manila clam (*Ruditapes philippinarum*) broodstock to low pH conditions (pH 7.4 for 78 days during gametogenesis). Larvae were produced from primed (low pH) and unprimed (ambient pH) broodstock and exposed to ambient or low pH conditions in a full factorial design. Larval phenotype in response to low pH was partially rescued by broodstock priming: larvae from low pH-exposed broodstock had better survival and growth than larvae from broodstock held under ambient conditions. Clam egg lipidomic and transcriptomic analyses were performed to determine the physiological differences associated with broodstock environmental conditions. Egg lipid abundance profiles were not significantly different between parental treatments. The egg transcriptome revealed 48 differentially expressed transcripts associated with parental environmental conditions. These genes are involved in important processes for early larval physiology, including metabolism, cell cycle, and transcriptional regulation. Broodstock clams were minimally impacted by their exposure to low pH for 78 days, however we show here that subtle maternal signals may contribute to the vastly improved larval performance observed under low pH conditions.

1. Introduction

In a rapidly changing climate, the aquaculture industry needs to quickly adopt mitigation measures to maintain and grow operations to serve an increasing world population. Ocean acidification (OA) - decreasing ocean pH due to increasing atmospheric CO_2 - has been impacting aquaculture of calcifying invertebrates for years (e.g., (Barton et al., 2015); (Cooley et al., 2017)) and remains a major challenge in the consistent production of oysters, clams, mussels, scallops, and other species. Adaptive strategies are desperately needed to support the maintenance and growth of the invertebrate aquaculture industry. Shellfish farming is one of the most sustainable protein production systems in the world (Hilborn et al., 2018) and contributes significantly to domestic aquaculture production, helping to offset the substantial U.

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https://doi.org/10.1016/j.aquaculture.2025.743388
Received 18 August 2025; Received in revised form 14 October 2025; Accepted 3 November 2025
Available online 4 November 2025

S. seafood trade deficit (Kite-Powell et al., 2013). Manila clams (*Ruditapes philippinarum*) are an iconic aquaculture species in Washington State (USA) and worldwide. They have a long history as part of "Pacific Northwest" culture and their farming generated $17 million in sales in Washington State in 2013 (Washington Sea Grant, 2015), yet the growth of this industry is slowed by the harmful impacts of an increasingly warm and CO_2-rich world. New approaches that provide resilience to worsening OA conditions are critical to the viability of the industry.

In invertebrate aquaculture, parental priming offers a promising means of producing stress-resilient larvae. Priming is an alternative strategy to genetic selection that promotes rapid acclimation of larvae via environmental exposures in the parent generation. Parental priming with positive carryover effects occurs when environmental drivers experienced by the parent generation result in a different and higher-fitness phenotype in their offspring. In this growing field of study, there have been reports of positive impacts of parental low pH priming on bivalve larvae and juveniles (Diaz et al., 2018; Gibbs et al., 2021a, 2021b; Parker et al., 2012, 2015; Spencer et al., 2020; Zhao et al., 2017, 2018, 2019, 2020), which can be interpreted as anticipatory maternal effects (AME). In the AME context, mothers adjust their offspring's phenotype to maximize the offspring's fitness in a challenging environment (J. Marshall and Uller, 2007). In some cases of parental low pH priming, there are examples of negative impacts (Griffith and Gobler, 2017; Venkataraman et al., 2019; Xu et al., 2024a; Xu et al., 2024b), or selfish maternal effects (SME), whereby maternal fitness is increased at the expense of offspring fitness. SME potentially gives the mother an opportunity to save reproductive energetic resources for a more optimal time/environment (J. Marshall and Uller, 2007). When priming has a positive effect, offspring tend to grow faster and larger in the condition similar to that experienced by their parents. Based on this growing evidence, it has been suggested that the aquaculture industry could harness the benefits of parental priming by deliberately exposing broodstocks to a short-term environmental driver in order to increase resilience of the offspring (Gavery and Roberts, 2017; Green et al., 2016). Phenotypes associated with AME in a successful priming scenario may include larger egg size, high spawning success rate, and robust larval and juvenile phenotypes in the same environment in which the parents were primed. Both AME and SME are likely achieved via environmentally sensitive molecular signals passed from parent to offspring. Two candidates are lipids – with roles as signaling molecules and the main nutritive resource for early developing larvae - and maternal RNA, which regulates early transcription, and thus physiology, of the developing zygote. Despite increasing evidence of beneficial phenotypes associated with parental priming in shellfish, the molecular mechanisms whereby parents confer these advantages to their offspring remain unknown. With a combined 'omics approach (lipidomics and transcriptomics) of clam eggs, we can make progress towards understanding the comprehensive mechanism of priming and how offspring outcomes are directly influenced by maternal signals.

Lipids represent the energetic resources necessary for embryonic development. We analyzed these molecules, which are transferred from parent to egg to offspring (Tadros and Lipshitz, 2009; Winata and Korzh, 2018) for insight into how environmental response can be transferred across generations. Lipids constitute a large fraction of egg biomass and fill diverse roles as metabolic substrates, membrane building blocks, or signaling molecules. For lipids to participate in a priming mechanism, the mother would have to provision different relative abundances of lipids to her eggs depending on environmental exposure. In bivalves, the full-body lipidome can be environmentally sensitive (Chan and Wang, 2018; Liu et al., 2022) and egg lipid content sometimes changes in response to OA (Gibbs et al., 2021a; Xu et al., 2024a). We hypothesized that a variety of lipids with different physiological roles would be implicated in the transgenerational inheritance of low pH response. In vertebrates and insects, different lipids play roles in the regulation of oocyte metabolism and maturation (Fruttero et al., 2017; Khan et al., 2021; Mostafa et al., 2022) and since molluscs have similar egg lipid constituents to vertebrates and insects (e.g., Imbs et al., 2021; Zagalsky et al., 1967), we expect lipids and their derivatives to play important regulatory roles in bivalve egg development as well.

Traditionally, investigations into parental effects have focused primarily on maternal metabolic resources deposited into eggs, however, molecules such as RNA and proteins may also underlie important signaling pathways that are mechanisms for inheriting environmental tolerance. Maternal mRNAs provide the template for gene and protein expression in early embryonic development before zygote genome activation. Maternal mRNA encodes embryonic chromatin, tubulin for mitotic spindles, and cell adhesion proteins, among many others (Dworkin and Dworkin-Rastl, 1990). Later hatching success is correlated with levels of specific maternal mRNAs and proteins in unfertilized eggs of the great scallop and Pacific oyster, respectively (Corporeau et al., 2012; Pauletto et al., 2017), reinforcing the importance of maternally provisioned genes and proteins in offspring outcomes. In round gobies, expression of maternal mRNA in early embryonic development is influenced by the maternally experienced environment (Adrian-Kalchhauser et al., 2018), suggesting an environmentally sensitive transference of gene expression patterns. In Eastern oyster gonad tissue, gene expression regulation is sensitive to parental OA exposure (Venkataraman et al., 2024). Maternal mRNAs are essential molecules that can contribute to determining offspring outcomes and are environmentally sensitive, making them likely players in the mechanism of broodstock priming.

Here, we examined the impacts of low-pH priming on Manila clam broodstock and its impacts on broodstock physiology and larval outcomes. Measures of broodstock and larval phenotype were selected that would reveal how low pH impacts both generations and the transmission of environmental information between generations. In broodstock, we assessed energy metabolism and reproductive conditioning. Larval clams were reared in both ambient and low pH conditions to determine the impacts of priming on survival, growth, and shell formation. Based on different larval phenotypes resulting from the two broodstock pH conditions, we investigated the impact of maternal effects via egg lipidomes and maternal RNA. This study contributes to the growing body of knowledge on the potential utility of environmental priming to improve environmental resilience in both commercial and restoration aquaculture.

2. Materials and methods

All code and files necessary to replicate the analyses described in the sections below are available on https://github.com/emmats/Clam-OA-priming.

2.1. Experimental set up

Adult Manila clams (n = 307, shell height 34.2 +/- 4.5 mm), were collected by hand using a raking fork in the low intertidal zone (between +1 to -1 m mean lower low water (MLLW)) of Liberty Bay in Puget Sound, Washington State (47°43'14.4"N 122°37'48.5"W) on January 18, 2023. Clams were immediately transferred to the Northwest Fisheries Science Center's Manchester Research Station (Port Orchard, WA, USA) and placed in a flow-through seawater system (Fig. S1).

Clams were acclimated for two weeks to common conditions (until January 30, 2023). Water conditions were ambient during the acclimation period and similar to the conditions found at the site during the time of collection (temperature = 10.0°C, salinity = 29.6 psu). In order to facilitate sex determination via gonadal biopsy later in the experiment, small holes (~4 mm) were drilled in the margin of the clam shell using a diamond bit for a Dremel handheld rotary tool.

The flow-through experimental system used seawater filtered to 5 μm pumped into four 200 L polyethylene header tanks, each equipped with independent temperature and pCO_2 control. Temperature was controlled within 1°C with a chiller capable of both heating and cooling.

Partial pressure of CO_2 (pCO_2) was controlled by direct injection of pure CO_2 through a venturi injector into each header tank. A pH probe (Durafet III) within each tank relayed to a Honeywell UDA analyzer/controller which controlled a solenoid valve on the CO_2 injection line to maintain the desired pCO_2/pH level. Seawater from each header tank was distributed by gravity to two replicate (100 L) tanks per treatment through a ball valve to control flow rate to (0.5 +/- 0.1 L/min). Forty adult clams were placed in each replicate tank by suspension in plastic mesh bags from the frame placed over the tank, with 10 clams per bag. There was no clam size difference between tanks.

Following the acclimation period, two header tanks supplied with flow-through seawater obtained from Clam Bay, WA remained at ambient conditions (pH ~7.8), while pCO_2 was increased (pH decreased) in the other two header tanks for the low pH priming treatment (Fig. S2). In the two low-pH header tanks, CO_2 injection was used to lower pH to a target of 7.4, reducing the pH by 0.1 every 24 hours over the course of six days. The low pH treatment chosen for this study represents a pH value that has been observed in Puget Sound bottom waters (Feely et al., 2010). After six days, pH was held constant for the duration of the experiment, encompassing adult gametogenic conditioning (described in the next paragraph) and 14 days of larval rearing until May 3, 2023 (Fig. 1).

Once pH reached the target, the water temperature was increased in all tanks to stimulate gonadal maturation (Fig. S2). From a starting point of 9.9°C, temperature was raised 0.5°C every 24 hours over 20 days to reach a target of 20°C. Temperature was then held constant at 20°C for the duration of the broodstock conditioning (until April 18 and 19, 2023). Approximately one month prior to spawning, clams were redistributed across tanks within their respective treatments by sex to reduce the risk of premature spawning of females. Sex determination was performed via gonad biopsy using a hematocrit tube inserted into the small hole of the shell margin and visualizing the presence of sperm/eggs.

Broodstock were sub-sampled after the initial acclimation to the tanks for reproduction staging via histology and condition index (CI T_1, January 30, 2023). Subsequent condition index time points were March 1 (CI T_2, one month into conditioning) and April 3 (CI T_3, two months into conditioning). Gill tissue was sampled for ATPase activity on February 6 and March 31 (ATP T_1 and T_2, respectively). Time points are summarized in Fig. S2.

Throughout the experiment broodstock clams were fed a mixture of live microalgae (*Tisochrysis lutea*, *Pavlova lutheri*, *Tetraselmis sp.*, *Rhodomonas salina*, *Chaetoceros calcitrans*, *C. muelleri*, *Thalassiosira sp.*) grown on site. The specific combination of algae strains and concentrations varied daily and were supplied to a 200 L algae reservoir. Clams were fed *ad libitum* by distributing algae directly into the feed lines of each of the four headers using electronic metering pumps (EZB11D1-VC) that were randomly rotated daily to ensure feed was allocated equally across all treatments and replicate tanks.

2.2. Seawater chemistry

Water temperature and pH in header tanks was monitored continuously via the UDA system and using the APEX data logging system. Temperature and salinity were measured three times per week by handheld salinity probe (ThermoScientific Orion Star A322). At these same time points, pH was also measured spectrophotometrically. Water samples were collected in an Ocean Insight CV-Q-100 cuvette and warmed to 25°C for 30 min. Cuvettes were placed in the path of an Ocean Optics light source and measured using a Flame mini-spectrometer. Spectrograms were captured using Ocean Optics software before using m-cresol purple dye (Dickson et al., 2007).

Total alkalinity was measured February 7 and 17, March 1, 13, and 28, and April 10 from water samples (120 mL) collected from each tank and preserved using HgCl (50 µL). Alkalinity was not measured during the larval experiment and was estimated from the average total alkalinity measured on April 10 since salinity was similar from April 10 onwards. Total alkalinity determination was analyzed using a Mettler Toledo T5 Excellence Titrator with LabX software. Alkalinity data was forward- and back-filled for an equal number of days surrounding each alkalinity sampling time point. Based on these data, the seacarb package in R (Gattuso et al., 2021) was used to calculate pH, pCO_2, dissolved inorganic carbon (DIC) and aragonite saturation (Ω aragonite).

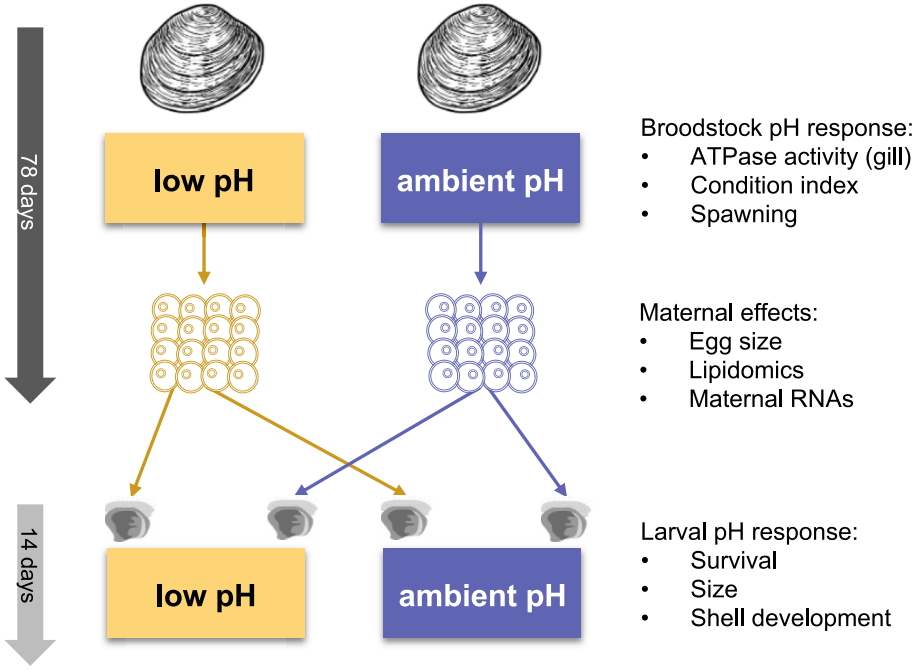

Fig. 1. Overview of the experimental design. Adult broodstock clams were exposed to low or ambient pH for 78 days, which included the main gametogenic period. Broodstock pH response was assessed and clams were spawned, with eggs collected to measure maternal effects. Larvae were reared in a full factorial design, with larvae resulting from each broodstock treatment raised at both ambient and low pH.

2.3. Broodstock

2.3.1. Reproductive maturation assessment

Histological samples were taken at the beginning of the experiment (January 30) to determine the initial reproductive stage. Individuals were sectioned through the center of the body in a plane perpendicular to the shell margin. Samples were preserved in 10% formalin in seawater for 24h followed by 70% ethanol for storage. Tissues were processed through a graded series of ethanol and xylene, embedded, sectioned, and slides were stained with hematoxylin and eosin. Clam reproductive maturity was staged based on Drummond et al. (2006).

2.3.2. Gill ATPase activity

Gill (ctenidia) sections were sampled post-acclimation and before pH treatment (January 31, ATP T_1, Fig. S2) and after two months' exposure to low pH conditions (March 31, T_2) to determine the impacts of low pH exposure on cellular energy production in the gill. This tissue and assay were chosen to understand potential impacts of pH treatment on adult physiology and energy balance. Approximately 50 mg of gill was preserved in ice cold SEI buffer (250 mM sucrose, 10 mM Na_2EDTA, and 50 mM imidazole; (Zaugg, 1982)) and stored at -80°C. The Na+/K+ ATPase activity, reported in units of μmol ADP x mg protein^{-1} x hr^{-1} of frozen sample, was measured following McCormick (1993) with modification from Bianchini and Wood (2003), as also described in George et al. (2023).

The ATPase activity data were checked for normality using the Shapiro-Wilk normality test. The data were not normally distributed and the bestNormalize package (Peterson, 2021) determined that a boxcox transformation was optimal. Boxcox (MASS package; Venables and Ripley, 2002) revealed that the data should be squareroot transformed, which resulted in normally distributed values (Shapiro-Wilk $p>0.05$). Levene's test revealed that variance across samples was equal with respect to time, sex, and treatment ($p>0.05$). The ATPase data were analyzed with two linear mixed effects (lme) models: 1) time only, 2) sex and treatment (including sex*treatment) using the nlme package (Pinheiro and Bates, 2000; Pinheiro et al., 2024). Tank was included as a random effect.

2.3.3. Condition index

Body Condition Index was determined at the beginning of the experiment (February 6, CI T_1, Fig. S2), after one month (March 3, CI T_2), and after two months (April 3, CI T_3) in the low and ambient pH conditions. For T_1, eight clams were selected using a random number generator. At T_2, three clams were randomly selected from each culture tank (but avoiding very small or very large clams) (total n=24). At T_3, two males and two females were randomly selected from each tank (total n=32). For each clam, the entire soft tissue and empty shell were dried separately at 60°C for 72 h and weighed. Body condition index was defined as the ratio of dry tissue weight to dry shell weight (CI = dry tissue mass (g)/dry shell mass (g) x 100).

As described in 2.2.2, a Shapiro-Wilk test and a Levene test were applied to determine the need for data transformation, which was not required. A one-way ANOVA was used to determine if the OA treatment had an impact on condition index at CI T_2 and T_3, also considering the interaction of time and treatment (p-value cut-off of 0.05).

2.3.4. Spawning and fertilization

Clams were spawned after 44 days at 20°C (Fig. S2). Spawning for low and ambient pH groups were carried out over two consecutive days (ambient pH clams were spawned on April 18 and low pH-primed on April 19, 2023) to minimize potential differences in water chemistry and reproductive development and to ensure adequate time for spawning and larval monitoring. All clams were spawned at ambient pH and larvae spent the first few hours of development at ambient pH. Fertilization and spawning occurred at ambient pH to avoid potential negative impacts of pH on fertilization rate so we could more clearly measure the effect of parental priming on larval outcomes. For each treatment group, males and females were separated and placed in black-bottomed spawning trays to help identify spawning individuals. The standard hatchery practice of thermal fluctuation was used to induce spawning (Helm et al., 2004). Specifically, for each spawning cycle, clams were air dried and subjected to physical disturbance (manual tray shaking) for approximately 15 seconds, followed by a high temp exposure (25°C water with continuous flow) for 30 min and finally a low temperature exposure (15°C with continuous flow) for 30 min. This cycle was repeated three times during which clams were monitored continuously. When a spawning individual was visually identified, it was moved to an isolated 1 L container at ambient temperature to collect gametes.

Duplicate egg samples (15 mL, ~50-200k eggs/sample) from individual females were spun gently, seawater was removed and eggs were snap frozen in liquid nitrogen for lipid (2.2.6) and transcriptome (2.2.7) analyses. Egg size was determined for a subset of females (section 2.2.5). The total number of spawning and non-spawning clams per sex per treatment was recorded to evaluate the effects of low pH priming on spawning activity using a chi-squared test with a significant cut-off of 0.05.

Five brood-groups were created per pH treatment. Each brood-group included eggs from five females and sperm from four males. Eggs were counted for each female in triplicate and 200,000 eggs from each female were combined (a total of 1 million eggs/brood-group) and mixed briefly before being distributed equally across four 1L containers (~250,000 eggs/container). Sperm from an individual male was added to each container, targeting 5-10 sperm/egg ratio to minimize sperm competition. Approximately 10 minutes after the addition of sperm, the four egg containers were combined and excess sperm was removed by rinsing eggs onto a 20 μm stainless steel mesh filter (Hogentogler). The fertilized eggs were placed into a clean 1L container containing 1 μm filtered seawater at 22°C. This process was repeated for each of the five brood-groups. Approximately four hours post fertilization (hpf), embryos from each brood-group were split equally into ambient and low pH treatments (approximately 500,000 embryos per group and treatment) by reducing the volume via gentle siphoning through at 20 μm screen to 250 mL and splitting the volume evenly into two clean 1 L containers to which either ambient or low pH water was added to a total of 800 mL. These containers were placed in sealed chambers with minimal headspace to minimize gas exchange and placed in a 22°C water bath. Broodstock mortality was tracked over the course of the experiment. A chi-squared test was used to evaluate the effect of low pH priming on broodstock mortality with a significant cut-off p-value of 0.05.

2.3.5. Egg size

Eggs from a subset of female clams (n=13 ambient pH and n=9 low pH) were imaged immediately for egg size. Images were taken using Amscope camera (model: MU1000) on Nikon Eclipse TS100 at 10X magnification. Measured eggs per female ranged from 10-50 eggs for a total of 579 eggs analyzed. Egg size was analyzed using ImageJ software to determine egg area. Scaling images were used for each clam to set the scale of 400-500 μm based on the distance measured in pixels. All images were processed by use of the *Thresholding Tool* as well as the Binary Tools *Fill Holes* and *Watershed.* Individual egg total area was analyzed by use of the *Analyze Particles Command.*

The impact of broodstock pH treatment on egg size (area) was assessed with a lme following Leinbach et al. (2021), with broodstock and larvae treatment as a fixed effect (including broodstock*larval treatment) and clam ID as a random effect. This analysis was performed on individual egg measurements (n=579) and on egg area averaged for each female (n=22 clams) with a p-value cut-off of 0.05 for significance.

2.3.6. Egg lipidomics

Egg lipidomic analysis was performed on frozen egg samples from 30 female clams (n=15 from each pH treatment). This sample size should

be large enough to detect biologically meaningful changes in the egg lipidome (e.g., Xu et al., 2024a). Clam egg samples were thawed at 4°C for 30 min, purified DI water (100 µL) was added and samples were homogenized in a Bullet Blender (Next Advance) for 10 min chilled with dry ice to 4°C. Each sample (100 µl) was transferred to 2 mL Eppendorf PCR-clean vial. A spiking solution containing 69 isotope-labeled lipid internal standards (25 µl), water (75 µl), methyl-tert-butyl ether (MTBE, 575 µl) and methanol (160 µl) was added to each sample, and the solution was vortexed for 10 seconds. Samples were shaken at 21°C/500 rpm for 30 min, followed by an addition of water (200 µl) and centrifuged at 14000 rpm for 3 min at 21°C. The hydrophobic lipid molecules were present in both resulting layers. The upper layer was collected into a new disposable glass culture tube (14x100 mm).

Lipids from the bottom layer were extracted by the addition of MTBE (300 µl), methanol (100 µl) and water (100 µl). Samples were shaken for 5 min at 21°C/ 500 rpm, and centrifuged at 14000 rpm for 3 min at 21°C. The upper layer from this second extraction was collected and combined with the previously collected upper layer from the first extraction in the glass tubes. Combined samples were dried at 30°C for 30 min in a Biotage N_2 drier and reconstituted in 10 mM ammonium acetate in 50/50 (v/v) methanol/methylene chloride and transferred to auto-sampler glass vials for mass spectrometry lipidomics analysis.

Lipid species were measured using a Sciex Lipidyzer system as described in Su et al. (2021). The Sciex Lipidyzer mass spectrometry platform consisted of Shimadzu Nexera X2 LC-30AD pumps, a Shimadzu Nexera X2 SIL-30AC autosampler, and a Sciex QTRAP 5500 mass spectrometer equipped with ESI source coupled with SelexION for differential mobility spectrometry (DMS). 1-propanol was used as the chemical modifier for the DMS. Samples were introduced to the mass spectrometer by flow injection analysis at 8 µl/min. Targeted lipidomics using multiple reaction monitoring (MRM) and positive/negative polarity switching electro-spray ionization was used to analyze 1,011 lipid species across 20 lipid classes and sub-classes and 69 isotope-labeled lipid internal standards. Each sample was injected twice, first with the DMS enabled to collect PC, PE, LPC, LPE, PG, PI, PS and SM (see Table S1 for lipid abbreviation definitions), and then with the DMS disabled to collect CE, CER d18:0, CER d18:1, DAG, FFA, HexCER, LacCER, LPG, LPI, LPS, PA and TAG. SM, DAG, CE, CER d18:0, CER d18:1, HexCER, LacCER, LPG, LPI, LPS, PA, LPE, LPC and TAG were ionized in positive and PE, PG, PI, PS, PC, and FFA in negative ionization modes. Data were acquired using Analyst 1.6.3 and processed with an open source Shotgun Lipidomics Assistant

v4.1.2 software. For quality control, an in-house pooled human serum sample was run at the beginning and at the end of the batch. The median percent difference between beginning and ending QCs for lipid class was 1.1% (Fig. S3). The final data contained absolute concentrations of each lipid species in micromolar calculated by comparing peak intensities for measured lipids to the internal standard peak intensities. Blanks were subtracted from experimental samples on a feature-wise basis; blank-corrected abundances <0 were replaced with 0.

Egg lipidome data was tested for physiologically relevant lipid structural trends that might not be detectable by comparing abundances of individual lipid species or groups. We calculated lipidome-wide and class-specific means for hydrocarbon chain unsaturation and length. These were compared between ambient- and low-pH groups using Welch 2-sample *t*-tests with Holm familywise error rate correction. We also visualized the hydrocarbon chain distributions, total lipidome, and phospholipidome using seelipids (https://github.com/octopode/seelipids). Multivariate statistical methods were used to perform sensitive, physiology-agnostic tests for treatment-induced changes in lipid composition. For these analyses, the lipidomics data were grouped in three different ways: by lipid species, by lipid class, and by hydrocarbon chain (the latter two being subsets of the first). If a lipid type had >80% missing values across all samples it was removed from further analysis. perMANOVA using adonis2 in the vegan package (Oksanen et al., 2024) was applied to each of the three groupings of the lipidomics data to determine whether pH treatment had an effect on the lipid composition of clam eggs. An Euclidean distance matrix was applied and missing values were removed from the perMANOVA analysis. Differential abundance of individual lipid species, classes, and hydrocarbon chains was determined with limma (Ritchie et al., 2015). Data were median-normalized and log-transformed before analysis with limma to achieve a normal distribution. The adjusted *p*-value (using the "fdr" adjustment) cut-off for significantly different abundance was 0.05.

2.3.7. Egg transcriptomics

RNA-Seq analysis was performed on frozen egg samples from the same 30 female clams (15 from each pH treatment) analyzed for lipidomics. RNA isolation, library preparation and sequencing were performed by Azenta Life Sciences (Waltham, MA). Briefly, RNA was extracted using the Qiagen RNeasy Plus universal mini kit, using the manufacturer's protocol and RNA concentration and quality were assessed using the Qubit RNA Assay and TapeStation, respectively. RNA-Seq libraries (n=30) were prepared using the NEBNext Ultra II RNA library prep kit and pooled prior to sequencing using the Illumina NovaSeq platform (PE 150bp).

Sequencing reads were quality and adapter trimmed using Cutadapt (v4.2) with the following parameters (*-a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA; -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT; -q 15,10; -m 40; –trim-n*). Trimmed reads were aligned to the *Ruditapes philippinarum* reference genome (GCF_026571515.1) using the RefSeq gene annotations (GCF_026571515.1_ASM2657151v2_genomic.gtf) in STAR (v2.5.3a) with default parameters.

To classify reads as belonging to the mitochondrial or nuclear genome and identify the proportion of reads mapping to mitochondria per sample, the total number of reads mapping to mitochondrial loci (i.e. loci on contig NC_031332.1; including 13 protein-coding genes 2 rRNA (16S and 12S), and 24 tRNAs) were divided by the total number of reads per sample.

A Student's T-test was used to determine if the proportion of reads mapping to mitochondrial loci were different between egg transcriptomes corresponding to the different parental rearing environments (*p*-value cut-off of 0.05 for significance). Proportion of reads mapping to a given RNA 'Type' (e.g. protein-coding, rRNA, lncRNA, etc.) according to the RefSeq annotation file (https://www.ncbi.nlm.nih.gov/datasets/gene/GCF_026571515.1/) were similarly calculated per sample. Egg transcriptome gene counts from STAR were pre-filtered and used to identify differentially expressed genes. To exclude the potential for bias from genes with low read counts (Love et al., 2014), genes were only considered for differential expression analysis if they met the following requirement: at least half (n=8) of the samples in at least one corresponding parental condition (low pH and ambient) must have a read count equal to or exceeding the median read count (27) for all genes. Genes that passed filtering were analyzed for differential expression between conditions using DESeq2 (v1.38.3) with Benjamini and Hochberg *p*-adj < 0.05 for significance (Love et al., 2014). Differentially expressed genes (DEGs) were visualized using a heatmap (pheatmap v1.0.12; Kolde, 2019), with log2 fold-change (LFC), an effect size estimate, as cell input values.

Functional enrichment analysis was performed to identify biological processes significantly overrepresented in DEGs associated with pH treatment using DAVID (Huang et al., 2009a, 2009b). UniProt Accession IDs were obtained for Manila clam genes via BLAST (Altschul et al., 1990) (<1e-5 e-value cutoff) and the DEG annotations were used as input for DAVID as the gene list and with annotations for all genes passing the filter for expression as the background list. Significantly enriched pathways were defined within DAVID using a Fisher's Exact test with a Benjamini-adjusted *p*-value < 0.05 (Benjamini and Hochberg, 1995).

2.4. Larvae

2.4.1. Larval rearing and sampling

Twenty-four hours post-fertilization, a water change was performed by removing water via gentle siphoning through a 20 μm filter and refilling with the appropriate treatment water. At this time algal feed (*Tisochrysis luteas*) was added at a concentration of 20,000 cells/mL.

At 48 hpf, larvae were robust enough for handling (D-hinge) and were split into additional replicates. Approximately 4,000 larvae, estimated from a known concentration of larvae/mL, from each of the containers (each container representing a single brood-group at ambient or low pH) were added to triplicate 48 μm mesh bottom silos (2" diameter PVC, approximately 10" in length, volume ~700 mL). Sixty silos were placed into triplicate sealed chambers with either ambient or low pH seawater (n=15 per parent-larval treatment combination). Temperature was maintained by placing the chambers in a circulating water bath set at 22°C. Larvae were fed algae (*Tisochrysis lutea*) at a concentration of 40,000 cells/mL/day. Water was changed daily.

Larvae were counted at days 7 and 14 post-fertilization (dpf) to estimate relative survival. Larvae were collected on 2, 7 and 14 dpf for image analysis of size and shell development.

2.4.2. Morphometrics

Images of larvae were taken using an Amscope camera on an inverted microscope (Nikon Eclipse TS100) at (10X) magnification. Live samples of larvae at 2, 7 and 14 dpf were taken from each spawning group at each time point and 10-15 images captured from each replicate within one hour of removing samples from the container. All images from each time point were given unidentifiable file names and randomly sorted for measuring shell growth and number of abnormalities. For each image, using ImageJ, a 5x6 grid was overlaid and a random list of numbers between 1 and 30 used to choose the order of grid sections to measure. Going through the order of grid sections, all shells with any portion in the section were analyzed until at least five shells had been analyzed from the image. For each larva, the length from the hinge to the margin was measured and the normality of the shell was determined as a binary assessment of normal or abnormal. Examples of shell abnormalities included large indentations or bulges in the margin, unequal size of upper and lower valves, and a concave hinge (Fig. S4). Image analysis proceeded until 20 larvae had been analyzed from each silo replicate.

The impact of time, broodstock priming, and larval pH treatment on larval shell size was assessed with a lme. Time, parent treatment, and larval treatment (including broodstock*larval treatment) were all fixed factors and brood group was set as a random factor. Larval shell abnormality was analyzed with a generalized linear model (glm) following (Timmins-Schiffman et al., 2013) with the same effects as described for larval shell size and using a binomial distribution, with a p-value cut-off of 0.05 for significance.

2.4.3. Survival

The proportion of larvae surviving to 7 dpf was calculated by dividing the larval counts on 7 dpf by the total larvae moved into the silos at 2 dpf (48 hours post-fertilization). Similarly, at 14 dpf the relative survival of larvae was measured by dividing the larvae counted at 14 dpf by the larvae counted at 7 dpf.

3. Results

3.1. Seawater chemistry

Distinct pH treatments were maintained for both broodstock priming and larval rearing (Tables S2 and S3, Figs. S5 and S6). Across all broodstock tanks, pCO_2 in the ambient pH treatment averaged 826 μatm and the low pH tanks averaged 2353 μatm, corresponding to average pH of 7.8 and 7.4, respectively. For the ambient pH larval tanks, average pCO_2 was 672 μatm (pH 7.8) and for the low pH tanks pCO_2 was 1942 μatm (pH 7.4). Aragonite saturation state was <0.75 for most of the experiment for the low pH larval treatment tanks and >1.5 for ambient pH (Tables S2 and S3, Figs. S5 and S6). Total alkalinity (A_T) was consistent across all treatments, but shifted between March 13th to March 28th from approximately 2400 μmol/kg to 2080 μmol/kg (Table S4). There was no change in salinity during the duration of the experiment (Figs. S5 and S6). A different type of bottle for A_T sample storage was used starting March 28th and is likely the source of the shift since it was consistent across every sample taken.

3.2. Broodstock

3.2.1. Reproductive maturation

Histology samples were taken from clams just after collection from the natural environment. All clams were found to be in a stage of quiescence and graded at either 0 or 1 out of 5 (Drummond et al., 2006). In most cases, no oogonia or spermatogonia were visible and so sex could not be determined. Visible oocytes were detected through biopsy after 21 days of maturation at 20°C, and all clams were then sampled and sorted by sex. In total, 45 males and 87 females were in the low pH group and 50 males and 83 females were in the ambient pH group. Broodstock mortality was relatively low over the course of the experiment (n= 11 low pH, n=4 ambient) with a majority of mortalities occurring after the maximum conditioning temperature was reached. There was no significant difference in mortality between pH treatments ($X2$=3.3457, p = 0.07).

3.2.2. Gill ATPase

ATPase activity in gill sections did not differ by any single factor (time, pH treatment, sex), nor was it impacted by the interaction between sex and treatment (p>0.05) (Fig. 2A).

3.2.3. Body condition index

All condition index data was determined to be normally distributed based on the Shapiro-Wilk test and homoscedastic between treatments based on Levene's Test, justifying the use of a one-way ANOVA test to determine statistical significance of differences between treatment groups at the endpoint, between treatments and baseline measurements at sampling point 1, and between males and females. There was no significant difference in body condition between pH treatment, sex or between sampling timepoints (p>0.05) (Fig. 2B).

3.2.4. Spawning

In the low pH group, 30 males spawned out of 33 (91%) and 47 females spawned out of 76 (62%). In the ambient pH group, 27 males spawned out of 42 (64%) and 29 females spawned out of 74 (39%) (Fig. 2C). Based on a chi-squared test, spawning rate was significantly higher in the low pH group versus the ambient pH group in both males ($X2$ = 5.7959, p = 0.016) and females ($X2$ = 6.8177, p = 0.0090).

3.2.5. Egg size

Egg area was measured from digital images taken directly after spawning, with the mean of all measurements from one individual used as a replicate. The area for eggs from low pH females was 3669 μm^2 +/- 134 (mean ± SD) and from ambient pH females was 3720 μm^2 +/- 128 (mean ± SD) (Fig. 2D). Egg area was not significantly impacted by broodstock pH treatment (p > 0.05), when assessed for individual eggs, nor for averaged area for each female.

3.2.6. Egg lipidomics

The total number of different lipid species detected was 1241 (Skyline document and raw files are available on PanoramaWeb; https://panoramaweb.org/wAKAD2.url; Table S5) (MacLean et al., 2010; Sharma et al., 2014, 2018). Physiologically relevant lipid structural parameters were remarkably stable (Fig. 3): neither mean chain length nor unsaturation changed significantly with parental low-pH exposure

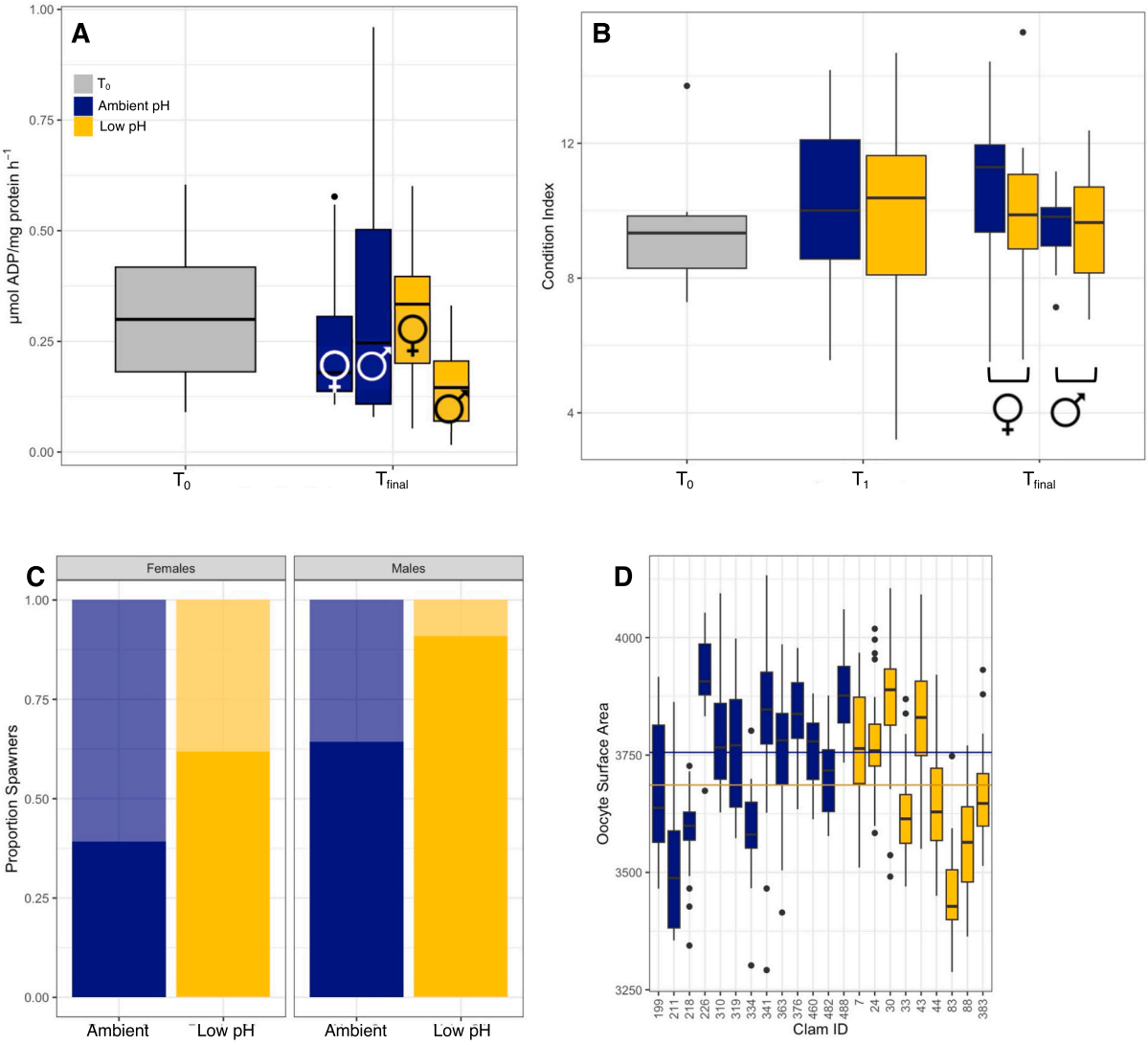

Fig. 2. A) ATPase activity by treatment and clam sex. B) Condition index by treatment and clam sex. C) Proportion spawners by treatment and clam sex. The darker bars represent the clams that spawned. D) Oocyte surface area for each clam, by treatment; horizontal lines represent the mean oocyte surface area for all oocytes measured in each treatment. Broodstock low pH treatment results are represented in yellow and ambient pH results are in blue. Results from before pH treatments began are represented in gray.

in the total lipidome, the phospholipidome, or five major lipid classes ($p_{adj} > 0.35$). In the parallel analyses of individual lipid species, classes, and hydrocarbon chains some entries were removed due to >80% missingness, encompassing 170 lipid species, two lipid classes, and 59 hydrocarbon chains. There was no significant difference in abundances of individual lipid species (Fig. 4A), classes or chains (Fig. 4B) between parent pH treatments, as determined using limma. In the absence of any significant changes in lipid species abundance, we here summarize the egg lipid composition as an average across both treatments. Triacylglycerides were the dominant class of lipids (68.8 mol % on average), with substantial concentrations of phosphatidylcholine (PC; 13.9%), phosphatidylethanolamine (PE; 8.2%), phosphatidylinositol (PI; 3.3%), and cholesteryl ester (CE; 3.2%) (Fig. 3). Each of the other 15 classes of lipids detected fell below 0.01 mole fraction of the egg lipid content.

3.2.7. *Egg transcriptomics*

In total, approximately 156 GB of sequencing data (NCBI SRA: PRJNA1289209) were generated across the 30 egg samples (n=15 per treatment). The alignment rate to the Manila clam genome ranged from 71% to 80%, with an average alignment rate of 75%. To evaluate the Manila clam egg transcriptome broadly, we first evaluated the proportion of transcripts derived from the mitochondrial or nuclear genome. While approximately half of the reads from the eggs mapped to loci on the mitochondrial genome, we found that a smaller proportion of reads mapped to mitochondrial loci in eggs from low pH exposed broodstock (Student's T, p=0.03). A majority of the reads mapping to mitochondrial features corresponded to the 16s rRNA locus (Table S6). More broadly, protein coding transcripts make up the largest proportion of transcripts in Manila clam eggs, followed by rRNA with other non-coding RNA types representing a small proportion of transcripts (Fig. S7).

Of the 12,124 genes evaluated for differential expression analysis 48 were differentially expressed (0.40 % of total genes), with most (n=34) exhibiting lower abundance in eggs from low pH broodstock (Fig. 5; Table S7). A majority of differentially expressed genes (32) were annotated according to RefSeq (Table S7). GO terms corresponding to the 48 DEGs included cytoskeletal terms (e.g., actin filament reorganization), membrane transport (e.g., endocytosis), cell division, chromatin organization, signal transduction, and others (see Table S7 for a complete list of GO terms). No functionally enriched Gene Ontology

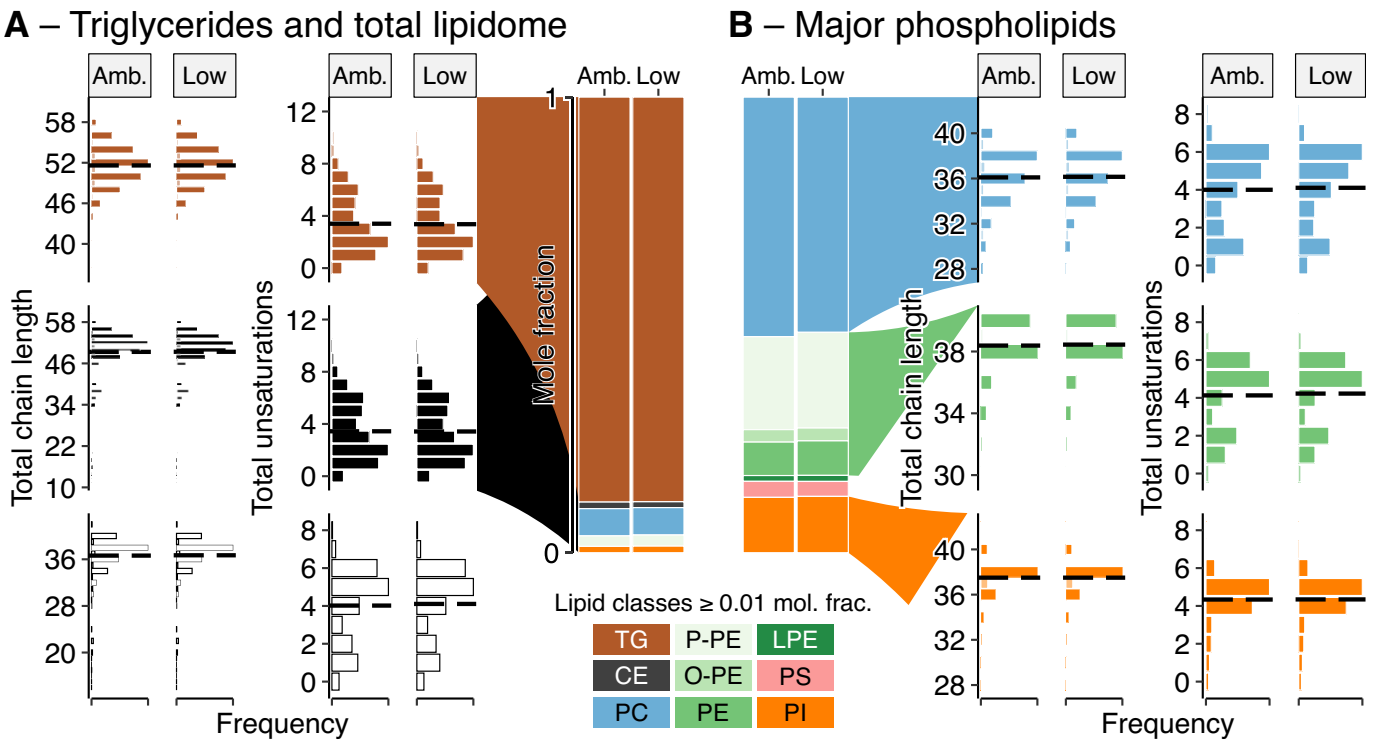

Fig. 3. Egg lipidomes are remarkably unperturbed by maternal low pH exposure. (A) Vertical bar plots show the mean total lipid class composition of eggs from ambient and low pH-reared clams. The majority class was triacylglyceride (TG) storage lipids. Horizontal bar plots show chain length and unsaturation (double bond) distributions for TG (brown bars), all lipids (black bars), and total phospholipids (white bars). Horizontal dashed lines in these plots indicate mean chain length and unsaturation, none of which differed significantly between maternal exposures. (B) Vertical bar plots show the composition of major (>0.01 mole fraction) phospholipid classes. Horizontal bar plots are the same as those in (A), but for PCs, PEs (ester and ether), and PI.

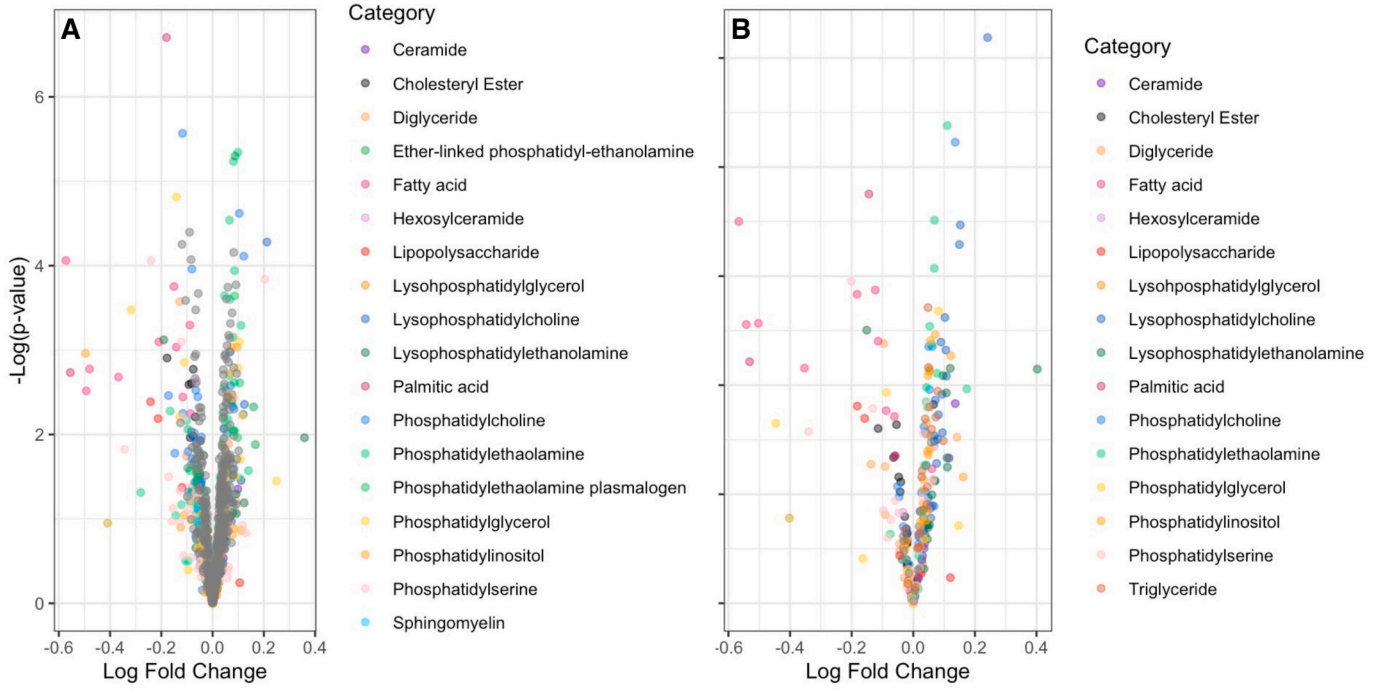

Fig. 4. A) Limma results for lipid species colored by broader lipid category; none are significantly different. B) Limma results for hydrocarbon chains alone; none are significantly different.

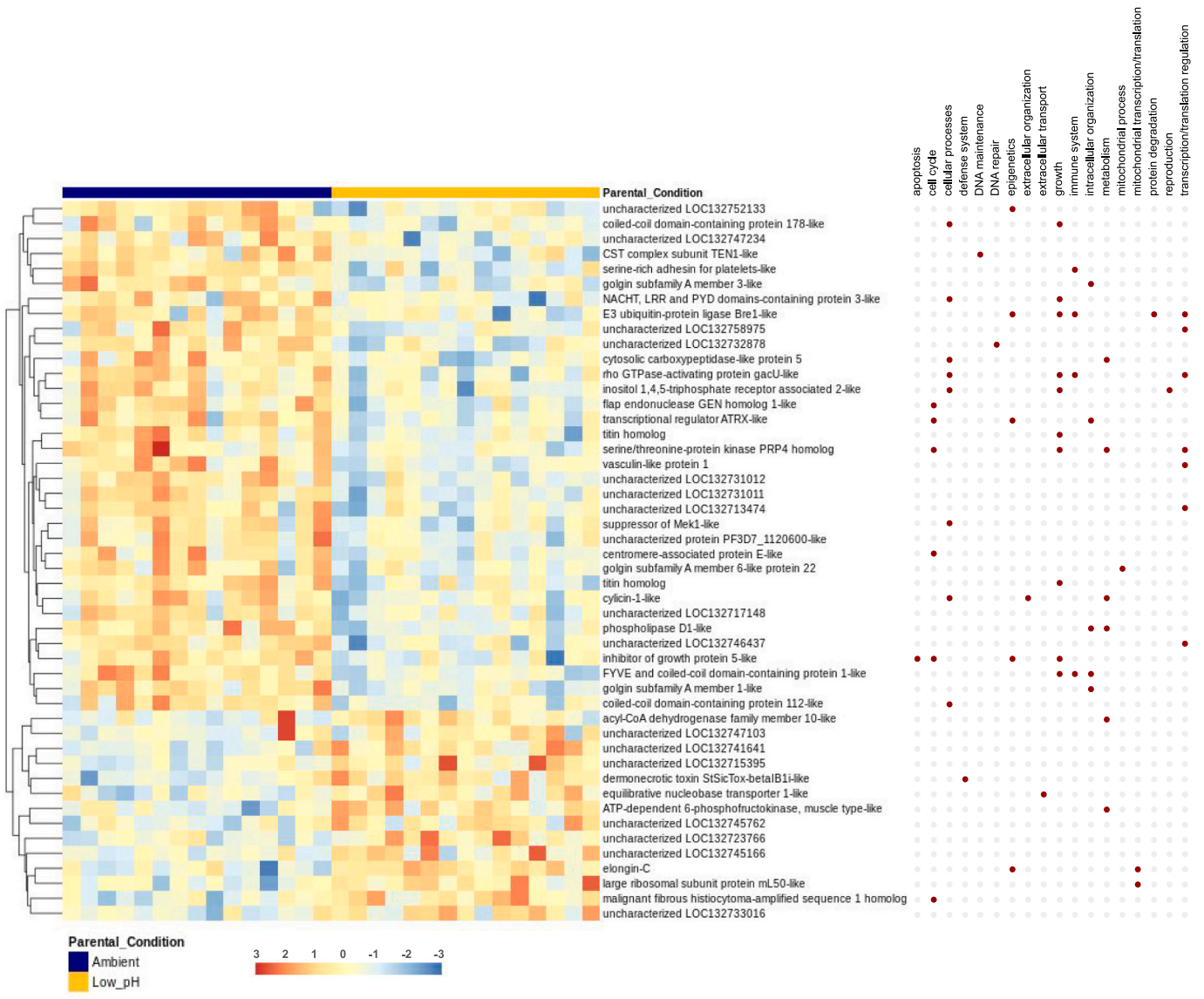

Fig. 5. Differentially expressed genes in eggs from low pH versus ambient parental conditions clustered by expression levels. Heatmap grouping along the top of the columns indicates broodstock treatments - low pH (yellow) and ambient pH (blue). Coloration of cells based on log2 fold change, where red = increased log2 fold change (increased abundance) and blue = decreased log2 fold change (decreased abundance). Dot matrix indicates functional processes associated with each differentially expressed genes (red=associated process).

biological processes were identified.

3.3. Larval development

Larval size was significantly impacted by time, parental pH treatment and larval pH rearing conditions, and the interaction between broodstock and larval pH treatment ($p < 0.05$). The larvae reared at ambient pH that had low pH parents had the largest shell length across all days (Fig. 6A-C). Ambient pH larvae from ambient pH broodstock were the second largest. Larvae reared in low pH conditions were the smallest, with the entire cohort of low pH larvae from the ambient pH broodstock group dying before 14 dpf (Fig. 6D-F).

Larval survival was not significantly impacted by broodstock pH treatment (p=0.18), nor by larval pH treatment (p=0.052) at 7 dpf (Fig. 6G). By 14 dpf, there was a significant effect of broodstock pH treatment on larval survival (p=0.016), but no significant effect was found for larval pH treatment or the interaction between broodstock and larval pH treatment (p>0.05) (Fig. 6H).

More larvae reared at low pH had shell abnormalities (Fig. 6I; Fig. S4). Time (p<2e-16), broodstock pH (p=0.00025), larval pH (p=0.00021), and the interaction between broodstock and larval pH treatment (p=2.86e-9) all had significant impacts on larval shell abnormalities.

4. Discussion

A changing ocean environment, including decreasing pH, will continue to influence the viability of the shellfish aquaculture industry. Leveraging environmental memory, particularly transgenerational acclimatization, is one important way to mitigate the impacts of ocean acidification on shellfish production by increasing offspring resilience. We have demonstrated positive effects of broodstock priming to promote transgenerational acclimatization in Manila clams and the underlying mechanisms have been explored via transcriptomic and lipidomic analyses of eggs from broodstock held under different pH regimes.

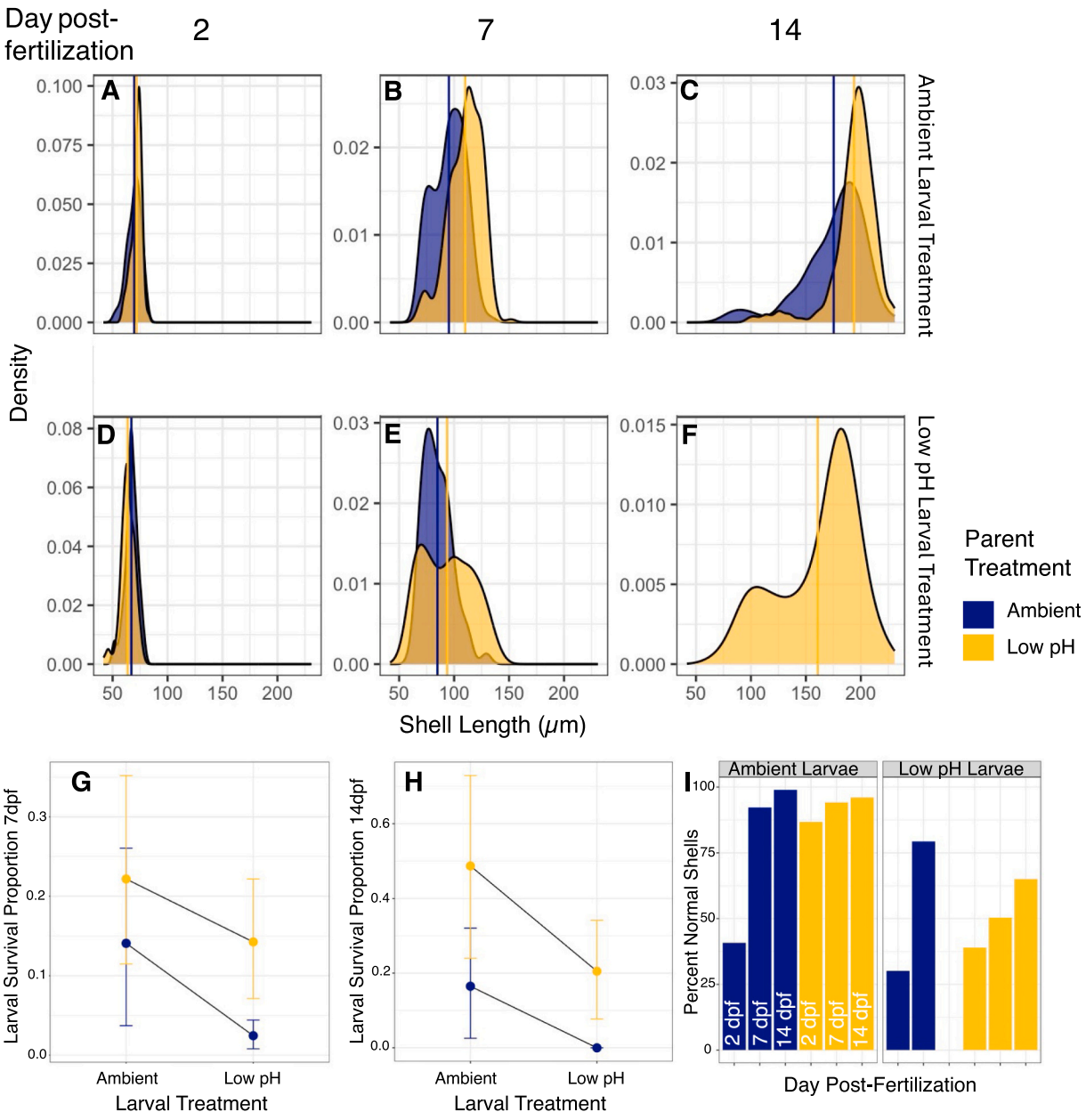

Fig. 6. Larval shell length, survival and normality. Panels A-C show the distribution of larval shell length for larvae reared in ambient pH conditions for days 2, 7 and 14 post-fertilization with shell lengths for larvae reared in low pH conditions depicted in panels D-F. If the larvae's parents were conditioned at ambient pH, the distribution is in blue; larvae from low pH broodstock are in yellow. Mean shell length for each group is marked with a vertical line. G and H show the reaction norms for larval survival at 7 and 14 days post-fertilization in larval ambient and low pH conditions, respectively. Panel I shows the percent normal shells for larvae reared in ambient conditions (left panel) versus low pH conditions (right panel) for days 2, 7 and 14 post-fertilization.

4.1. Adult broodstock clams were minimally impacted by low pH

The Manila clam broodstock in this study showed no signs of physiological stress in response to low pH conditions. An important aspect of parental priming in aquaculture is to have sufficient perturbation to elicit a carryover response in the larvae, but not to negatively impact the physiology of the broodstock, criteria which were met in this experiment. In fact, a higher proportion of low pH-primed broodstock spawned. Further, the two pH conditions were distinctly different (pH 7.8 and 7.4) and aragonite saturation state was consistently less than 1, which makes calcification energetically unfavorable, yet clams demonstrated no difference in cellular energy production in their gill tissue as determined by ATPase activity, nor in egg size. Low pH impacts on gametogenic adults are not consistent across invertebrate species and the realized low pH phenotype is likely determined by species-specific adaptations and environmental history. Across bivalves, some species, like the clams in this study, demonstrate little or no effect of low pH on adult gametogenic maturation (Diaz et al., 2018; Gibbs et al., 2021a), while others express detrimental effects of low pH on energy metabolism and/or gametogenesis (Neylan et al., 2024; Zhao et al., 2019). Even within a species, local adaptation likely plays a role in environmental response. Manila clam adults in the Washington population may be adapted to low pH conditions, in contrast to clams from the South China Sea that showed signs of pH stress across physiological metrics after low pH exposure (Jiang et al., 2023; X. Xu et al., 2016). Reproduction is an energy-intensive process (Ginther et al., 2024) and the impact of low pH stress on the process of gametogenesis depends on how much energy is diverted to respond to the stress resulting in variation in physiological

response to low pH based on evolutionary and ecological history.

As mentioned, low pH increased the proportion of male and female spawners. The trend of a higher proportion of spawners was also observed in Asian date mussels, although other physiological reactions to low pH diverged from the Manila clams in this study (Zhao et al., 2019). A review of impacts of low pH on marine invertebrate reproductive processes reveals that clams in this study diverged in some aspects of their low pH response in comparison to other molluscs (Padilla-Gamiño et al., 2022). In most other molluscs studied, low pH impacted males and females differently and led to a decreased number of spawners (Padilla-Gamiño et al., 2022), neither of which were observed in this study. Egg quality appears to be robust to low pH across bivalves (Padilla-Gamiño et al., 2022), a trend that was also observed in this study as egg diameter and lipidomes did not differ by pH treatment. The impact of low pH on the mechanisms underlying these important reproductive phenotypes is understudied, hampering our understanding of how low pH affects the fundamentals of reproduction in marine invertebrates (Padilla-Gamiño et al., 2022). No measurements or observations were taken of sperm so we do not know how low pH may have affected sperm motility and other functions. Generally, sperm motility and function are inhibited under low pH conditions across invertebrates (Eads et al., 2016; Esposito et al., 2020; Shi et al., 2017; Vihtakari et al., 2013), but since fertilization for these clams occurred in ambient pH water, it is very likely that the sperm were not affected by parental low pH exposure.

Exposure to an environment that is within the clam's established acclimatory ability may be the key to successful transgenerational priming. The clam broodstock were likely experimentally exposed to low pH conditions which are not dissimilar to their habitat, due to their adult infaunal life history and origin from a low pH estuary (Feely et al., 2010), resulting in relatively unimpacted adult physiology and reproduction. There is evidence in other invertebrates that extreme environmental conditions for adults yields larvae that are not well acclimatized to the same environmental stressors (Griffith and Gobler, 2017; Neylan et al., 2024; Parker et al., 2012; Xu et al., 2024a; Xu et al., 2024b), or larvae that have variability in resulting phenotypes (Gibbs et al., 2021a). In some species, a longer period of adult acclimation may result in no observable effect in larvae (Suckling et al., 2015), or an extended length of parent exposure to low pH may worsen the impact of low pH on larvae (Neylan et al., 2024). The balance between negative and positive transgenerational effects likely lies in the level of timing, physiological state, and degree of environmental perturbation in parents, and how this translates into offspring provisioning.

4.2. Parental priming improved larval performance at low pH

The highest performing larvae in terms of survival and growth were those reared in ambient pH water (pH 7.8) whose parents were low pH-primed. Of the larvae whose parents were low pH-primed, those reared at low pH were smaller and had more shell abnormalities than larvae reared at ambient pH (Fig. S8). Bivalve larvae are more sensitive to low pH than adult life stages (Kroeker et al., 2010) and it is well established that larval calcification and shell size are impacted by pH (e.g., Frieder et al., 2017; Gibbs et al., 2021a; Thomsen et al., 2017; Timmins-Schiffman et al., 2013). The difference in shell size between ambient and low pH-reared larvae appears to increase through 14 days of development, as previously observed in geoduck clam (Timmins-Schiffman et al., 2020). Despite their smaller size, the low pH larvae from low pH-primed parents survived through day 14 post-fertilization, while low pH larvae from ambient pH parents did not. The larvae without abnormalities at low pH show clear growth through the 14 days the cohort was followed (Fig. S8). Low pH exposure of invertebrate parents has varying impacts on larvae, ranging from positive effects on larval and juvenile survival (Diaz et al., 2018; Gibbs et al., 2021a, 2021b; Palombo et al., 2023; Parker et al., 2015; Spencer et al., 2020; Thomsen et al., 2017) to negative (Griffith and Gobler, 2017; Neylan et al., 2024; Venkataraman et al., 2019; Xu et al., 2024a; Xu et al., 2024b). Based on these previous results, since the Manila clam parents were physiologically robust to low pH and early development of larvae benefitted from parent low pH priming, it is likely that the larvae from low pH-primed broodstock would continue to mature and settle at low pH. The long-term benefits of low pH-priming are less clear. In some species, the beneficial impacts of low pH-priming may not extend past the F1 generation (Thomsen et al., 2017), while in others, transgenerational low pH exposure continues to realize positive growth and survival results in subsequent generations (Parker et al., 2015). Parent genetics and physiology also play a role in the transgenerational impacts of low pH (Goncalves et al., 2016; Thomsen et al., 2017). In an aquaculture setting, the focus is on growth and survival of the F1 generation, which is clearly improved by priming in this study and in others.

The Manila clams in this study demonstrate anticipatory maternal effects (AME) (J. Marshall and Uller, 2007), or the adjustment of offspring phenotype to maximize offspring fitness in an environment. When environmental conditions do not result in physiological stress, there is physiological and energetic flexibility to produce high quality offspring that will thrive via AME. Molecular signals from the mother via the egg, such as mRNA or lipids, can mediate broodstock priming AME.

4.3. Exploring mechanisms underlying positive effect of OA priming

There is increasing evidence across bivalve species that parents dictate larval outcomes by transfer of environmental memory based on their experienced environment. In cases where parental environmental exposure results in positive outcomes in the next generation and improves offspring resilience in adverse conditions, elucidating these molecular mechanisms will benefit commercial and restoration aquaculture. Unlocking how and under what circumstances broodstock priming is realized will have wide-reaching impacts on the industry. To begin to investigate this we provide insight into shifts in the egg lipidome and transcriptome of Manila clam broodstock that will form the foundation for a more in-depth understanding of broodstock priming.

4.3.1. Lipidomic analysis of eggs

The Manila clam egg lipidome in this study resembled the lipidomes previously reported in clams and other bivalves (Gibbs et al., 2021b; Xu et al., 2024a). Most of the lipids detected were triacylglycerides (TGs), an important storage molecule in marine invertebrate eggs and a source of energy for developing pelagic larvae (e.g., Frieder et al., 2017; Laudicella et al., 2023; Lee, 1974; Wehrtmann and Graeve, 1998). In marine invertebrates, many pelagic larvae depend upon maternally provisioned lipids to successfully complete the larval stage, mature, and settle. Of the hydrocarbon chains in clam eggs, most had even chain lengths, with C16 and C18 being the most dominant, as has been observed in other lipid chain analyses of marine invertebrate eggs (Benkendorff et al., 2005; Laudicella et al., 2023; Lee, 1974; Wehrtmann and Graeve, 1998) (Fig. S9).

There was no significant impact of low pH-priming on egg lipids in this study. When an egg producer is under physiological stress, fewer lipids may be transferred to eggs since more energy is being spent on acclimatization and survival. Low pH can have this impact in bivalves, resulting in lower lipid content in the eggs of low pH-exposed parents. Total lipid content was not measured in this study, but egg size is generally considered to be a proxy for maternal investment in eggs and is likely strongly correlated with egg lipid content. In Manila clams collected in China, two months of low pH conditioning during a similar reproductive period to this study resulted in decreased TG in eggs (Xu et al., 2024b) and detrimental impacts in the larvae (Xu et al., 2024a). In contrast, total lipids and TGs were higher in eggs of low pH-conditioned Sydney rock oysters, but unimpacted in Pacific oysters (Gibbs et al., 2021a, 2021b). The impact on the egg lipidome in the Chinese clams was reflected in the larval lipidome: larvae from low pH-exposed parents had lower TG abundance and rapidly used these limited resources when

exposed to low pH themselves (Xu et al., 2024b). In other studies, low pH influences on bivalve eggs did not translate to offspring (Gibbs et al., 2021b; Zhao et al., 2020), once again suggesting evolutionary and acclimatory differences in transgenerational inheritance. The differing results between our study and Xu et al. (2024b) may be due to the fact that clams in the South China Sea do not regularly experience pH as low as that experienced by clams in Puget Sound (Feely et al., 2010; Zhai et al., 2014).

The clam egg lipidomes measured in this study were notably unimpacted by prolonged maternal exposure to low pH. In Manila clams from the South China Sea, hundreds of lipids significantly changed abundance in eggs from clams conditioned to low pH (Xu et al., 2024b); but, as stated above, those clams may not have been adapted or acclimatized to low pH conditions. Lipids play essential roles in oocyte and embryonic development (e.g., Uzbekova et al., 2025), although most of these research developments are from vertebrates. In mammals, there are delicate balances among fatty acid quantities and ratios that influence reproductive success and maturation (McKeegan and Sturmey, 2011). The amount of docosahexaenoic acid (DHA) in red drum eggs determines larval growth, survival and swimming speed, with results suggesting that DHA plays an irreversible role in establishing metabolic capacity in larvae (Perez and Fuiman, 2015). If lipids do play a role in the transgenerational plasticity that we observed in the clam larvae from low pH-primed parents, then it must involve exotic lipid species or extremely subtle quantitative adjustments not detectable by our analysis. However, it is clear that maternal lipid provisioning of eggs was not impacted by prolonged low pH exposure, laying a foundation for later larval developmental success.

4.3.2. *Maternal RNAs in low pH-primed eggs*

To evaluate the Manila clam egg transcriptome broadly, we first evaluated the proportion of transcripts derived from the mitochondrial or nuclear genomes. Transcripts derived from the mitochondrial genome, which includes 13 protein-coding genes, the 12S and 16S rRNA and various tRNAs, were abundant with approximately half of the reads mapping to mitochondrial loci. This is similar to mitochondrial transcript abundances reported in eggs of other species including sea urchin (Cabrera et al., 1983), frogs (Dworkin et al., 1981) and fish (Kleppe et al., 2012; Ma et al., 2019). The largest proportion of mitochondrial reads mapped to the 16S ribosomal rRNA. The RNA-Seq libraries employed Poly(A) retention, which aims to reduce the proportion of reads mapping to rRNA, yet we found over 70% of the reads mapping to mitochondrial transcripts are associated with the 16S rRNA locus. Comparatively, less than 5% poly(A) selected reads mapped to rRNA in trout eggs (Ma et al., 2019). This could be the result of high AT content found in many molluscan mitochondrial genomes rendering the rRNA “sticky” and challenging to purify using poly(A) enrichment (Adema, 2021). Interestingly, we found that low pH-priming had an effect on the proportion of mitochondrial transcripts. Specifically, low pH-primed eggs had a smaller proportion of reads mapping to mitochondrial loci. Regulatory control of maternal RNA processing is complex (Winata and Korzh, 2018) and it is unclear how the relative proportion of reads mapping to mitochondrial transcripts would impact the clam embryos. Complementary proteomic and mitochondrial DNA analyses could help to shed light on whether these observations are due to translational control or mitochondrial copy number variation and should be considered in future work.

To elucidate the mechanisms underpinning the improved growth and survivorship observed in larvae from primed broodstock, a differential expression analysis was performed on maternal transcripts. Similar to the clam egg lipidome, we detected a relatively small shift in gene expression between primed and ambient clams. Forty-eight genes were differentially expressed between the two broodstock pH groups, only 0.4% of the entire sequenced egg transcriptome. These genes are from functional categories such as metabolism, cell cycle and transcriptional regulation. Even though the number of DEGs is small, these are all processes that are important for early embryonic development and their effect size on embryonic and larval physiology may be relatively large and play an important role in zygotic genome activation. Other studies have varying results in the number of DEGs that may explain differences in offspring performance, with 1904 genes correlated to D-larval hatching rate in scallops (Pauletto et al., 2017) to 66 DEGs explaining larval survivorship in zebrafish eggs (Cheung et al., 2019). Subtle changes in gene expression can have significant impacts on physiology if they have regulatory functions (e.g., Adrian-Kalchhauser et al., 2018 and references therein) and we propose genes among the 48 DEGs detected here are instrumental in altering larval phenotype.

The cellular functions of metabolism, cell cycle, transcription and translation are essential for progression of embryonic/larval development and may be sensitive to low pH exposure (Gurr et al., 2022; Harry and Zakas, 2024; Kim et al., 2020; Waite and Sorte, 2025). The changes in gene expression in these functional categories suggest that low pH-exposed Manila clam mothers alter the maternal mRNA in key pathways that allow for successful development in an environment that would otherwise be damaging to calcifying larvae. These changes may be an example of adaptive plasticity (Gotthard and Nylin, 1995) that allows for AME and better larval fitness. Low pH impacts organismal energy balance and changes in metabolic biomarkers are a common part of the low pH response in larval life stages (e.g., Kelly et al., 2016; Strader et al., 2020; Stumpp et al., 2011). These differentially expressed maternal mRNAs may have primed larval metabolic pathways such that they were better prepared for the metabolic shift necessary to thrive in low pH conditions. Under low pH metabolic stress, larvae may more quickly use their maternal lipid reserves (Xu et al., 2024b), but we saw no evidence of depleted lipids over the 14 days of larval rearing. We did not observe a change in egg surface area or lipidome between broodstock groups, suggesting that the total amount of lipids provisioned between conditioning treatments were the same. Since the larvae grew and survived well through day 14 post-fertilization, it is likely that these maternally-derived transcripts successfully primed the larval metabolic processes and physiology. Similarly, increased abundance of cell cycle genes and proteins in eggs are associated with better larval growth and development in other bivalves (Corporeau et al., 2012; Pauletto et al., 2017). Zygotes and larvae grow rapidly, mitotically producing new cells that differentiate into specialized tissues and structures essential for growth and survival. The improved growth in larvae from low pH-primed parents may have been supported by changes in expression of cell cycle genes. Underlying all of these molecular changes would be regulation of transcription and translation, producing the genes and proteins necessary to drive forward cellular and organismal growth and physiological processes. These regulatory pathways are environmentally sensitive in gonad/gametes and are likely instrumental in transgenerational plasticity. In Eastern oyster (*Crassostrea virginica*) gonad tissue, low pH conditioning changed the epigenetic profile of genes in a way that impacted the overall "transcriptional noise", although few DEGs were detected (Venkataraman et al., 2024). Gene expression in offspring of low pH-primed Sydney rock oysters had a different transcriptional response to low pH than larvae from non-exposed parents (Goncalves et al., 2016), suggesting different baseline physiology inherited from primed parents. In round gobies, expression levels of maternal mRNA involved in transcriptional regulation in early embryos were highly influenced by maternal environmental experience (Adrian-Kalchhauser et al., 2018). The authors suggest that this key regulatory change is likely highly influential for early embryonic development. Further work on embryonic and larval gene expression leading up to and past the zygotic genome activation would clarify the role of these environmentally sensitive maternal RNAs in determining later larval phenotype. It is worth noting, that there may be other contributing factors to the phenotypes observed that should be considered moving forward, namely investigating paternal contributions to offspring as the literature strongly suggests sex-specific responses to low pH exposure (Kelly et al., 2016; Lymbery et al., 2019; Padilla-Gamiño et al., 2022; Venkataraman

et al., 2024; Vihtakari et al., 2016).

4.3.3. Molecular signals underlie parental priming effects

Manila clam parents, and other marine bivalves, transfer a wide range of molecules to their eggs and offspring and it is likely that some combination of these transferred molecules underlie observed transgenerational plasticity due to broodstock priming. In this study's results, we do not have a clear understanding of how mRNA or lipids play a role in this maternal transfer of environmental memory, but the observed subtle differences lay the groundwork for further exploration into mechanisms underlying broodstock priming. Given the variability in transgenerational response to low pH across studies of marine bivalves, it may be possible that specific mechanisms of transfer vary by species. Molecular mechanisms likely include some combination of epigenetic factors, mRNA, proteins and lipids. In bivalves, DNA methylation patterns are sensitive to low pH (Lim et al., 2021; Putnam et al., 2022; Venkataraman et al., 2022, 2024) and environmentally sensitive epigenetic marks can be heritable (X. Wang et al., 2023). In Eastern oyster gonad tissue, there were few changes in gene expression between low pH-exposed and control adults, similar to the current study; however, there were broadscale shifts in gonad DNA methylation and transcriptional variation (Venkataraman et al., 2024), suggesting that the mechanism of maternal effects may be highly influenced at the epigenetic level. Inheritance of maternal proteins and mRNA can determine quality of bivalve offspring (Corporeau et al., 2012; Pauletto et al., 2017), and it is likely that some species/populations of bivalves see changes in oocyte gene and protein expression under low pH conditions, although this is understudied. Egg lipidome shifts in response to low pH can be inherited by the next generation of larvae, likely impacting larval performance (Xu et al., 2024b). Bivalve gonad microbiomes are sensitive to low pH and can be inherited by offspring (Scanes et al., 2023; Unzueta-Martínez et al., 2022). Microbiomes and their hosts interact biochemically, exchange metabolites that can alter host and microbe physiology. Synthesis of these different streams of evidence for understanding the mechanisms of broodstock priming that improves resilience can help us identify species and populations that can thrive in low pH conditions and that can be potentially leveraged to introduce greater resilience into wide-ranging aquaculture programs.

5. Conclusions

The clear phenotypic difference in larvae from broodstock held at low pH points to an environmental signal that is transferred from parent to offspring that underlies a physiological change. Female invertebrates provision their eggs with many molecular resources - lipids, maternal RNAs, proteins, immune defense molecules, etc. (Chille et al., 2021; Corporeau et al., 2012; Lee, 1974; Pauletto et al., 2017; L. Wang et al., 2015; Xu et al., 2024b) - and the inheritance of a phenotype is likely complex, involving multiple molecular pathways. In this study, some combination of these inherited molecules leads to a robust phenotypic response to low pH in larvae when Manila clam broodstock are exposed to low pH. These results are promising for the local aquaculture industry and hatcheries that have struggled with low pH-related production roadblocks. Bivalve hatcheries in the Northeast Pacific are already broadly implementing ocean acidification mitigation measures and there is cautious interest in broodstock priming as an additional tool (Lewis-Smith et al., 2025). Notably, growers reported that they would be more likely to implement broodstock priming if there was documented evidence of its benefits (Lewis-Smith et al., 2025). If growers have the capability to lower, control, and monitor $pCO2$/pH during broodstock conditioning, they would be able to implement the type of parental priming described here. We would caution growers to ensure that their bivalves are from a species and population that would benefit from low pH priming as not all studies in this domain have yielded positive impacts on offspring (see Section 4.2). Our results contribute to the growing body of research on the mechanistic understanding of parental priming and positive carryover effects.

Data and code repository and DOI

NCBI (transcriptomics): NCBI SRA : PRJNA1289209
Panorama (lipidomics): https://panoramaweb.org/wAKAD2.url
reviewer351@proteinms.net
https://github.com/emmats/Clam-OA-priming.

CRediT authorship contribution statement

Emma Timmins-Schiffman: Writing – original draft, Visualization, Formal analysis, Data curation. **Larken Root:** Writing – original draft, Investigation, Formal analysis. **Ryan Crim:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Mollie A. Middleton:** Writing – review & editing, Investigation, Formal analysis. **Megan M. Ewing:** Writing – original draft, Visualization, Formal analysis. **Jacob Winnikoff:** Writing – review & editing, Visualization, Formal analysis. **Gracelyn Ham:** Writing – review & editing, Investigation. **Giles Goetz:** Writing – review & editing, Formal analysis. **Steven Roberts:** Writing – original draft, Funding acquisition, Conceptualization. **Mackenzie R. Gavery:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding Sources

This work was supported in part by the National Oceanic and Atmospheric Administration's Ocean Acidification Program (grant #NRDD21408) to MG; a grant from Washington Sea Grant, University of Washington, pursuant to National Oceanic and Atmospheric Administration Award No. NA14OAR4170078 to SBR; and funding from Washington Ocean Acidification Center provided salary support for ETS. The views expressed herein are those of the author(s) and do not necessarily reflect the views of NOAA or any of its sub-agencies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to acknowledge and thank the Puget Sound Restoration Fund hatchery staff for providing animal husbandry support, Alexa Romersa for guidance on Manila clam spawning protocols, Mike Riffle for statistical support and Aster Earls for generating the map of the experimental area. Laura Zelter and Brian Pratt assisted with creating the portal for sharing the lipidomics data. Danijel Djukovic performed the lipidomics mass spectrometry.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.743388.

Data availability

All data are available in public repositories (e.g. NCBI, GitHub, etc)

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