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Narrowed gene functions and enhanced transposon activity are associated with high tolerance to ocean acidification in a juvenile subarctic crustacean

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Abstract

Ocean acidification (OA) threatens marine crustaceans, particularly those at high latitudes where conditions are rapidly changing. Red king crab (RKC) support important fisheries in Alaskan waters. RKC early life stages are sensitive to OA when exposure occurs as embryos and juveniles. However, in a supporting study, RKC were surprisingly tolerant of varying OA conditions (pH 7.5, 7.8, & 8.0) when reared long-term from larval hatching to the first crab stage (C1). Here, we examined gene expression in the C1 juveniles to characterize transcriptional activity of these OA-tolerant crabs. Expression of nearly half of all genes (44%) correlated with OA treatment, suggesting a strong molecular response to OA, contrary to the phenotypic results. Downregulated functions were numerous in response to OA, and included reduced energy production, biosynthesis, immune function, and notably lipid and carbohydrate metabolic processes, which suggest a shift in metabolic strategy to protein catabolism, possibly to reduce CO_2 production and facilitate acid/base regulation. Only a handful of functions were enriched in OA-induced genes, which were related to transcription regulation, control of growth and cell death, and signaling activity. Transposon activity was high in OA-reared crab, many of which were upregulated at consistent levels, suggesting that transposon mobilization may be a component of the RKC OA-response system. Genetic composition did not differ among OA treatments indicating that transcriptional differences in OA-reared crab were more likely attributed to phenotypic plasticity than selective mortality. Our results suggest that OA-reared RKC have a narrowed, possibly optimized, set of gene functions that enables OA-tolerance through the early juvenile stage. OA-exposure from hatch may benefit RKC and related species by "hardening" them against OA through physiological reprogramming. Future studies should test OA-hardened crabs in additional challenges, as metabolic and immune limitations may ultimately make them more vulnerable to infection or secondary stressors.

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Introduction

Global oceans are rapidly changing due to increased concentrations of atmospheric greenhouse gasses. In addition to warming, deoxygenation, and sea level rise, oceans are acidifying [1]. This is occurring due to the increased partial pressure of carbon dioxide (CO_2) in the ocean, which has absorbed ~30% of added atmospheric CO_2 emitted since the industrial revolution [2]. As a result, the concentration of aqueous CO_2, hydrogen ions, and bicarbonate ions are increasing, and ocean pH, carbonate ion concentrations, and calcium carbonate saturation states are decreasing. This shift is referred to as ocean acidification (OA) [3], and, in addition to the effects of lower pH, is particularly concerning for calcifying species including crustaceans, molluscs, and corals due to their reliance on calcium carbonate (aragonite, calcite) to form shells and exoskeletons [4, 5].

While OA is a global phenomenon, changes in high latitude coastal zones are likely to have outsized biological effects. Carbon dioxide is more soluble at high latitudes due to colder water, resulting in lower pH levels and carbonate saturation states [6, 7]. Increased freshwater inputs from sea ice melt and river runoff, and enhanced upwelling and respiration are likely to augment acidification in high latitude coastal zones [6]. Conditions in those regions are therefore more likely to become undersaturated with carbonate ions and reach severely low pH levels [7, 8]. The Bering Sea shelf, a coastal area off Alaska which supports highly productive commercial, subsistence, and sport fisheries [9], already experiences seasonal levels of aragonite saturation (Ω) below one, which is the biological threshold for calcium carbonate dissolution [10–13]. Model projections for the Bering Sea through the year 2100 predict longer seasonal periods of $\Omega<1$ (up to five and two months in the winter for aragonite and calcite, respectively) [14, 15, Darren Pilcher *pers. comm.*], and that pH will decrease 0.07–0.35 pH units in surface waters and 0.05–0.30 in bottom waters [14, 15]. These carbonate changes have significant socio-economic implications for Alaskan crustaceans, given their importance to fisheries as both prey species (krill, copepods) [16, 17] and fishery stocks (e.g. king, tanner, and snow crabs) [18, 19].

OA has broad but variable physiological effects on crustaceans [20]. Many are capable of maintaining hemolymph pH homeostasis by acid-base regulation [21, 22], and have exoskeletons composed of both calcium carbonate and chitin, which may protect against direct dissolution [23, 24]. Still, tissue and shell growth [25], molt cycle [26, 27], exoskeleton properties [28, 29], metabolic activity [30, 31], reproduction [22, 32], behavior [33, 34], and immune function [22, 35] can all be altered by OA, particularly at higher pCO_2 concentrations [36]. Effects are most acutely observed in early life stages (larvae, juveniles), and vary greatly by species, severity of OA, life stage, and duration of exposure [20, 28, 37, 38].

The diversity of responses in crustaceans, and all marine invertebrates for that matter, has highlighted the need for a more mechanistic understanding of how organisms function in OA conditions. Targeted gene expression analyses in green shore crab (*Carcinus maenas*) gill tissue reported that genes involved in acid-base regulation are affected by high pCO_2 [39, 40]. Transcriptome-wide expression analysis identified changes in genes involved in energy metabolism and apoptosis activity in shrimp (*Exopalaemon carinicauda*) [41], and immune functions, energy metabolism, and ion transport in the Chinese mitten crab (*Eriocheir sinensis*) [42, 43]. Metabolomic analyses in juvenile Dungeness (*Cancer magister*) and green shore (*Carcinus maenas*) crabs both found amino acid metabolism to respond to acidified conditions, perhaps due to increased buffering needs [44, 45]. As with biometric responses, molecular effects of OA can vary by life stage. Metabolite analyses of American lobster larvae (*Homarus americanus*) indicate that large metabolic shifts (fatty acids, amino acids, and citrate cycle) perhaps underlie their buffering capacity and tolerance of OA, whereas metabolic reprogramming is

not observed in the more sensitive juvenile stage [46]. Together, molecular assays to date reveal that OA induces changes in energy metabolism, acid-base regulation, immune function, and cellular stress-response processes, with the strongest responses perhaps reflective of physiological reprogramming. Changes are, however, somewhat unpredictable due to species-, stage-, and exposure-specific effects. For that reason, the most informative studies pair molecular assays with survival and growth data preferably from the same individuals.

Paralithodes camtschaticus, red king crab, is one of several crab species that are a highly valued fishery in Alaskan waters (17M pounds, or $90M USD, in annual commercial landings on average from 2000–2022, for all king crab species [47]) but that may be threatened by ocean acidification [18]. Early life stages, which are thought to be particularly vulnerable to OA, are present as brooded embryos year-round and from hatch in late spring through settlement in fall [48, 49] and may encounter low carbonate saturation states that already occur in bottom waters and during seasonal carbonate cycles [13]. Laboratory studies on embryos, early zoea, and juveniles indicate that OA negatively affects early red king crab life stages such that development is altered, and growth and survival typically decreases [50–52]. OA also interacts with warming by increasing intermolt duration and decreasing survival in juveniles [53]. One study to date has explored molecular changes in OA-exposed red king crab, and found no pronounced effects on the larval transcriptome (individuals were pooled) [54] which corresponded with decreased survival rates [52]; whereas in juveniles, expression of genes involved in cuticular processes were strongly affected by OA exposure [54] despite no changes in morphology (survival and growth were negatively affected) [51]. Interestingly, Long et al. [55] recently found that when red king crabs are exposed to OA during multiple stages of development—from hatch through the early juvenile stage—they are surprisingly tolerant of moderate (pH 7.8) and severe (pH 7.5) acidification, with no change in survival, growth, or development [42]. Therefore, when OA-exposure is prolonged and begins at hatch, red king crab may effectively acclimate without negative physiological impacts. The mechanisms that enable OA tolerance are of profound interest to those that manage and rely on the red king crab fishery.

In this study, we used functional genomics to examine the molecular response of OA-exposed red king crab. We leveraged juveniles from Long et al. [55] that were exposed to (and tolerated) three carbonate chemistry treatments from hatching to the first crab stage (C1), thus capturing transcriptional differences among crab that are reared in historically ambient conditions along the Bering Sea shelf (pH 8.0), and those acclimated to a moderately (pH 7.8) and severely (pH 7.5) acidified environments that are projected to occur in surface and bottom waters by the end of this century [15]. Using RNA-Seq, a high-throughput sequencing approach that measures gene-activity, our study provides a snap-shot of system-wide changes in energy allocation due to acidification exposure by identifying genes, their functions, and biological processes that differ in OA-reared crab [56]. Libraries were constructed from at least 13 individuals per treatment, rather than pools of individuals which can obscure genotype-dependent variation. Importantly, since the crab used in this experiment were quite tolerant of OA conditions [42], the molecular mechanisms and pathways described here may be potentially critical to survival in an acidified environment.

Methods

Ethics statement

Red king crabs are non-cephalopod invertebrates and research involving them is exempt from ethics approval. All research was conducted according to applicable national and international guidelines.

Animal collection

Ovigerous females for this study were collected in Bristol Bay in October 2016 in crab pots during the commercial fishery. Animals were transported to Kodiak in the live hold of a fishing vessel. Females were held communally in tanks at the NOAA Kodiak Laboratory in the Kodiak Fisheries Research Center supplied with flow through sand-filtered seawater pumped from Trident Basin, Kodiak, at local ambient temperature and salinity, and were fed to excess on chopped frozen fish and squid. Embryos were monitored and, as they approached hatching, 48 females were moved to individual 48L tubs with flow-through seawater. Tanks were monitored for larvae. Larvae for this experiment were collected during peak hatch from as many females as possible over a period of three days (April 10–12, 2017), pooled, and immediately moved to experimental tanks. Larvae used to stock experimental tanks were from the same 21 females on the first 2 days of larval stocking, and from 20 of those 21 on the third (one female completed hatching after the second day of stocking). Animals were collected under ADFG permit P-16-011 and subsequently held under P-17-023.

Experimental design

Experiments were performed in 180L conical bottomed tanks. Each tank was randomly assigned to one of three pH treatments: 1) Local ambient (pH ~ 8.1), 2) pH 7.8, and 3) pH 7.5, with five replicate tanks per treatment. Tanks were stocked in a random order with larvae on the same day they hatched. Flowthrough water for this experiment was passed through a 5 μm filter and UV sterilized and flow into each tank was 2 L/min. The temperature and salinity of all tanks were allowed to vary with seasonal change of the intake water. The pH in each tank was adjusted via direct bubbling of CO_2 controlled by feedback from Honeywell controllers connected to an in-tank Durafet III pH probe. Discrete temperature and pH measurements were collected daily in each tank using a Durafet III pH probe calibrated with TRIS buffer [57]. Water samples were taken once a week from each tank beginning the second week of exposure, poisoned with mercuric chloride to saturation and analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA). DIC and TA were measured using a VINDTA 3C (Marianda, Kiel, Germany) and a 5012 Coulometer (UIC Inc.) according to DOE [58] using Certified Reference Material from the Dickson Laboratory (Scripps Institute, San Diego, CA, USA [59]). The seacarb package [60] in R (V3.6.1, Vienna, Austria) was used to calculate the other parameters of the carbonate system.

Larval rearing

Larvae were stocked at 50 larvae/L, or approximately 9,000 larvae per tank, and reared according to Swingle et al. [61], except that in this experiment we used ambient incoming seawater rather than elevated temperature to avoid potential interactive effects between pH and temperature. In brief, larvae were fed daily *ad libitum* on a diet of *Artemia sp*. enriched with DC DHA Selco (Inve Aquaculture) except during the non-feeding glaucothoe stage. Once the larvae molted to the glaucothoe stage, artificial seaweed was provided as a settling substrate. Glaucothoe continued to be monitored until they molted to the first crab stage. Survival, growth, and development metrics were captured at each of the four zoea, glaucothoe, and C1 stages (for details, see [55]).

RNA extraction & sequencing

For each OA treatment 13–15 juvenile crabs at the first juvenile instar (C1) stage were sampled for RNA-seq (2–4 crab from each replicate tank, Table 1), placing whole crabs in RNAlater per

Table 1. RNA-Seq sample size and replication by treatment after the removal of one outlier sample from the Ambient treatment. All libraries listed were used in all analyses (genetic, global patterns, gene co-expression, differential expression, and expression variation).

Treatment	Individuals / Libraries	Replicate Tanks	Replicates per Tank
Ambient (pH 8.0)	14	5	2 or 3
Moderate OA (pH 7.8)	13	4	3 or 4
Severe OA (pH 7.5)	15	5	3

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manufacturer's instructions (Thermofisher Scientific, Waltham, MA). To standardize among tanks/treatments and ensure that all crabs were at the same point in their molt cycle, sampling date varied by tank to target the C1-stage, and occurred five days after 100% of crabs in a tank reached the C1-stage. RNA isolation, library construction, and sequencing were performed by the University of Oregon. Briefly, RNAlater-preserved crabs were homogenized with silica beads using a Spex Geno/Grinder®, then RNA was isolated following TRIzol™ Reagent protocol for total RNA (Invitrogen Inc., Carlsbad, CA). The purity and quality of RNA were assessed with a NanoDrop™ Spectrophotometer (ThermoFisher Scientific) and a *Fragment Analyzer*™ (Agilent Technologies, Boulder, CO). Stranded mRNA-Seq libraries were constructed with the NuGen Universal Plus mRNA kit (Tecan Genomics, Inc, Männedorf, Switzerland). Libraries were prepared for sequencing using TruSeq RNA sample prep kits (Illumina). Paired-end sequencing was conducted on seven lanes of a Hi-Seq 4000 with 100-bp read length.

Bioinformatics

Raw sequence data from each of the seven lanes was demultiplexed, concatenated by library, then trimmed using Cutadapt v3.5 [62] to remove Illumina adapters, poly-A tails, flanking N bases, reads less than 50bp, and low-quality ends from reads using minimum quality scores of 20 and 15 for the 5' and 3' ends, respectively. Raw and trimmed data were inspected using FastQC [63] and MultiQC [64]. Reads were aligned to the draft Red king crab (*Paralithodes camtschaticus*) genome (Genbank accession GCA_018397895.1) [65, 66] using Bowtie2 v2.4.2 with the preset option—sensitive [67, 68]. The number of fragments aligning to gene coding regions of the *P. camtschaticus* genome was quantified using featureCounts v2.0.3 [69] with settings -p—countReadPairs to count paired-end fragments and -C and -B to exclude chimeras and singletons, respectively. Gene functions were identified by querying coding sequences of the *P. camtschaticus* genome, derived from gene annotations published along with the *P. camtschaticus* genome [66], against the Uniprot/Swissprot database [70] using blastx from blast v2.11.0 (e-value $< 1^{-10}$) [71].

Genetic analysis

As with many rearing experiments, the cumulative survival rate to the C1 juvenile stage was low, averaging 1.9% across all replicate tanks (for details see Long et al. [55]). While survival rates did not differ among treatments in this experiment [55], there could have been treatment-specific survival rates among the ~20 families, possibly resulting in genotype-specific expression patterns. We therefore assessed whether there were genetic differences among treatments. Single nucleotide polymorphisms (SNPs) were extracted from RNA-Seq reads to examine the genetic composition of sampled crabs. Variants were identified using the GATK toolkit [72]. Briefly, RNA-Seq reads were aligned to the draft red king crab genome [65]. The genome was first concatenated into 50 larger contigs, with 1000N separating each original contig, which was necessary to reduce the processing time in GATK. Alignment files were

deduplicated using MarkDuplicates, reads spanning splicing events and CigarN reads were split, variants were called using HaplotypeCaller, then joint-genotyped using GenotypeGVCFs. SNPs were filtered using VariantFiltration to hard-filter loci with any of the characteristics FS>60, QD<2, QUAL<30, SOR>3, DP<15, DP>150, or AF<0.30. SNPs were then pruned with `snpgdsLDpruning`from the R package *SNPRelate* v1.30.1 to remove those in linkage-disequilibrium and with >15% missing rate or <5% minor allele frequency. One sample from the ambient treatment was removed from the genetic analysis due to high missingness. From 227,781 candidate SNPs, 331 markers resulted from the above filtering and were retained for genetic analysis.

Differences in genetic composition among treatments was examined using multivariate analysis, estimates of diversity, and parentage analysis. Using *SNPRelate*, allele frequencies (major and minor) and per-SNP missing rate were calculated with *snpgdsSNPRateFreq()*, and PCA biplots of the first four principal components (PCs) were constructed with `snpgdsPCA()`. The first four PCs were selected as they explained over 25% of the total variance, and each additional PC explained less than 4% of the total variance. Pairwise Fst values [73] among treatments were calculated with `stamppFst()` from *STaMPP* v1.6.3 using 1000 bootstraps to generate 95% confidence intervals and *p*-values. Parentage analysis was performed using the 331 SNPs with *Colony* v2.0.6.6 for R [74], specifying polygamous males and females with three replicate medium-length runs using the full likelihood method with high precision. The parentage of samples with cluster probability < 0.5 were considered invalid (three samples from each treatment). The relationship between global expression patterns and genetic structure was assessed by regressing genotype PCA sample scores against the expression-derived PCA sample scores along the first two principal components, using OA treatment as a covariate.

Gene expression analysis

Analyses were performed in R v4.1.2 using RStudio interface v2021.09.1 [75, 76]. Unless otherwise specified, significance thresholds were alpha = 0.05 and representations of spread in data are 1 standard deviation.

RNA-Seq pre-processing for gene expression analysis. Gene counts were filtered to remove outlier samples and low-frequency genes. Outlier samples were identified using principal component analysis (PCA), which was performed on variance-stabilizing transformed counts of the top 500 genes using `vsd` and `plotPCA` from *DESeq2* v1.34.0 [77]. Genes with mean count <10 across all samples or those with counts <30 across at minimum 10% of the samples were discarded, and differences in the number of remaining fragments per sample among treatments was tested using ANOVA. This filtered gene count dataset was used in comparative gene expression analyses as raw counts, or transformed counts via variance-stabilization transformation in *DESeq2*.

Global patterns. Global gene expression differences among OA treatments were explored with PCA, using `prcomp` from the R package *vegan* v.2.5–7 on all gene counts that were transformed via variance-stabilization. Principal components that explained a significant amount of variance were identified using the scree test [78]. Global differences among treatments was assessed by permutational pairwise permANOVA with `pairwise.adonis` from the *pairwiseAdonis* package, which is a wrapper for `adonis`from the *vegan* package.

Gene co-expression network analysis. We identified groups of co-expressed genes (i.e. gene modules) with expression profiles that correlated with OA treatment using weighted gene co-expression network analysis (WGCNA v1.70–3 [79]). Briefly, a weighted gene network was constructed from transformed gene counts with a signed adjacency matrix using the soft thresholding power 15, and minimum module size 75. Modules were merged if their

eigengene expression correlated at $R > 0.75$, and those with eigengenes that correlated with pCO_2 concentration at alpha = 0.05 were determined to be associated with OA treatment. Modules with positive and negative correlations were designated as those with upregulated and downregulated expression profiles, respectively.

Differential gene expression analysis. Differentially expressed genes among pH treatments were identified using *DESeq2* with default settings [77, 80]. *DESeq2* uses raw count data to generate generalized linear models and internally corrects for library size, therefore counts were not transformed prior to differential expression analysis. No minimum log2 fold change (L2FC) was used to identify differentially expressed genes, but they were filtered for those with $|L2FC| > 0.5$ prior to functional analyses. In addition to examining the transcriptional responses of crabs to OA treatments, differentially expressed genes were used to characterize the gene modules with expression that correlated with pCO_2.

Variation in gene expression. Global differences in the variation of gene expression among OA treatments was assessed with a test of multivariate homogeneity of group dispersions. To do so, we used `betadisper` from the *vegan* package to calculate per-sample distances to group medians in multivariate space, then analysis of variance to compare per-sample distances among treatments for all genes, and for differentially expressed genes. Pairwise comparisons and associated permuted *p*-values identified which treatments differed. We then examined gene-wise variation in expression by treatment for genes that were upregulated, downregulated, or not differentially expressed for each treatment. The within-treatment coefficient of variation (CV = SD/mean) was calculated for each gene, providing a method of comparing variation in gene expression relative to the mean for each treatment [81]. CV was summarized by calculating the mean CV of genes that were upregulated, downregulated, and were not differentially expressed for each treatment.

Functional analyses. *Enrichment analyses*. Gene sets of interest, outlined below, were characterized by Gene Ontology (GO) enrichment analyses. For all gene sets, genes were filtered for those that mapped to the Uniprot/Swissprot database [70], and enriched GO terms were identified by entering UniprotID's into the Gene-Enrichment and Functional Annotation Tool from DAVID v2021 [82] to identify enriched biological processes, which were defined as those with modified Fisher Exact p-values (EASE Scores) <0.05. For all enrichment analyses the background list of genes included all examined genes that mapped to the Uniprot/Swissprot database (n = 32,435).

Co-expressed genes. To characterize functions that respond to pCO_2 in a dose-dependent manner, enrichment analyses were performed on co-expressed gene modules (from WGCNA analysis) for which eigengenes correlated with pCO_2 concentration, filtered to retain genes that either correlated individually with pCO_2 (Gene Significance p-value < 0.05) or were differentially expressed.

Differentially expressed genes. To determine the functions of differentially expressed genes two enrichment analyses were performed for each of the three pairwise treatment contrasts: (1) genes that were upregulated ($L2FC > 0.5$) and (2) genes that were downregulated ($L2FC < -0.5$) in response to the more severe OA treatment.

Low-variance differentially upregulated genes. To identify processes that are likely critical to the function of OA-reared crab, enrichment analysis was performed on a subset of differentially expressed genes that were upregulated in OA compared to ambient: those that had very consistent expression levels (genes with within-treatment CV $< 3\%$, referred to as low-variance genes).

Transposable element composition. Given that not all transposable elements are assigned to transposition-related GO terms and may be overlooked by traditional enrichment analyses, gene sets were also interrogated for transposable element composition. The proportion of

genes that were transposable elements was estimated for each gene set (co-expressed gene modules, differentially up/down-regulated genes in each pairwise contrast, and low-variance genes) by searching within the protein names of annotated genes for the words "transposon", "transposable", "LINE" (representing long interspersed nuclear elements), "retrotransposable element", "transposable element", "mobile element jockey" (a LINE), and "pol polyprotein". Pol polyprotein was included as it is core to the replication of retrotransposons, but may also be associated with retroviral activity [83]. These terms were determined to represent the majority of TEs by manual review of annotated genes. Beta-regression and a likelihood ratio test assessed whether the TE proportions differed for gene sets that were up-regulated and down-regulated in OA treatments.

Code and analysis files used in this study are available in the accompanying repository https://github.com/laurahspencer/red-king_RNASeq-2022 (https://doi.org/10.5281/zenodo.10547911).

Results

Experimental design

Experimental pHs were well controlled to within 0.01 pH units in experimental treatments (Table 2). Water temperatures increased from about 5˚C at the beginning of the experiment to about 10˚C by the end and averaged about 7.2˚C throughout (Table 2). Incorporating this seasonal shift in temperature provided ecologically relevant variability in conditions, including slight shifts in pCO_2 and saturation state conditions over time [84, 85] (S1 Fig).

Survival, growth, and development

Survival, growth, and development results were reported in Long et al. [55]. Briefly, survival, developmental time, calcification, and mass did not differ among treatments at any stage, nor did survival or developmental time cumulatively from hatch to the C1 juvenile stage differ.

Genetic relatedness analysis

Principal component analysis (PCA) constructed from SNPs (n = 331) indicated genetic homogeneity among treatments (Fig 1, S2 Fig). Individuals loosely aggregated into two or three clusters, primarily along PC1 and PC2 which explained 8.7% and 7.3% of variation, respectively, but no treatments were overrepresented in any of the clusters. Pairwise F_{ST} values

Table 2. Water parameters during the experiment. Temperature and pH were measured daily; salinity, dissolved inorganic carbon (DIC) and alkalinity were measured weekly beginning on the second week of the exposure period; all other parameters were calculated. Values are mean ± standard deviation, calculated across replicate tanks per treatment (N = 430 for pH and temperature, and N = 53 for other parameters). See S1 Fig for water parameter time-series and S1 Table for per-tank means.

Treatment	Ambient	Moderate OA	Severe OA
Temperature (C)	7.24 ± 1.40	7.25 ± 1.40	7.23 ± 1.44
Salinity	31.267 ± 0.142	31.277 ± 0.149	31.288 ± 0.163
pH_T	8.05 ± 0.03	7.79 ± 0.05	7.50 ± 0.06
pCO_2 (μatm)	370.74 ± 26.92	703.89 ± 90.45	1414.71 ± 287.82
HCO_3^- (mmol/kg)	1.89 ± 0.08	1.96 ± 0.05	2.00 ± 0.04
CO_3^{-2} (mmol/kg)	0.11 ± 0.01	0.06 ± 0.01	0.03 ± 0.01
DIC (mmol/kg)	2.01 ± 0.08	2.06 ± 0.05	2.10 ± 0.05
Alkalinity (mmol/kg)	2.16 ± 0.08	2.12 ± 0.05	2.09 ± 0.06
$\Omega_{Aragonite}$	1.66 ± 0.09	0.96 ± 0.13	0.52 ± 0.19
$\Omega_{Calcite}$	2.65 ± 0.15	1.53 ± 0.21	0.83 ± 0.31

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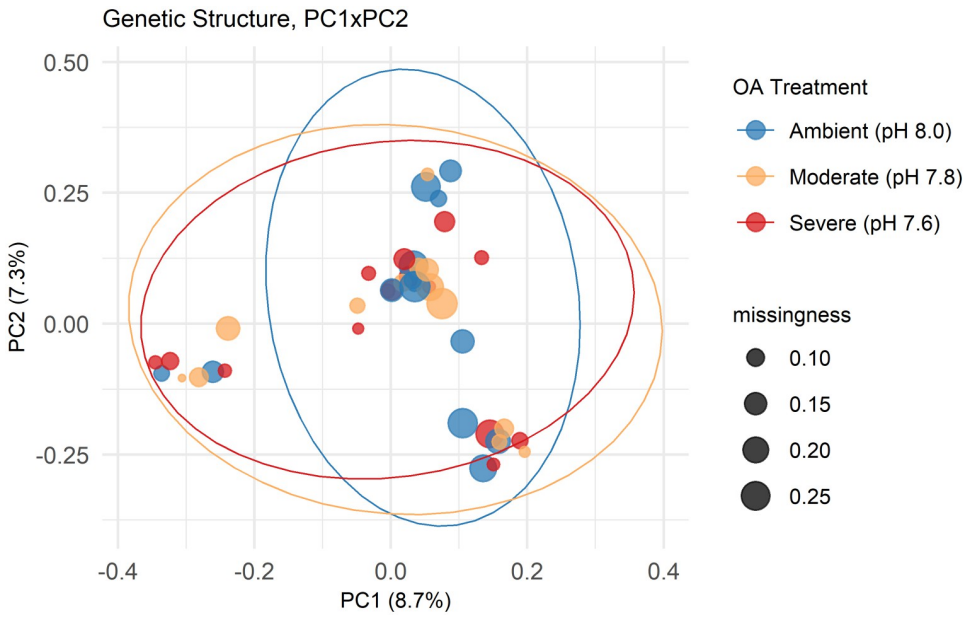

Fig 1. PCA biplot of the first two principal components, constructed from RNA-Seq derived SNPs (n = 331). Points represent individual crabs that are color-coded by OA treatment, which do not indicate clustering by treatment, and sizes represent the percent of SNPs that are missing in each individual. The PCA and other genetic analyses (parentage, pairwise F_{ST}) does not indicate that genetic composition of individuals surviving the three-month exposure differed among treatments.

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among samples from the three treatments did not differ from zero for any contrast (Table 3). Parentage analysis using Colony estimated the same number of mothers (n = 9) and fathers (n = 10) represented by surviving offspring in each treatment. No correlation was found among SNP-derived PCA scores and gene expression-derived PCA scores along the first two principal components (S3 Fig).

RNA-Seq pre-processing for gene expression analysis

A total of ~2.65B paired-end reads (henceforth "fragments") remained after discarding ~4.55M (0.17%) during initial quality-filtering, with a per-sample mean of 61.7M +/- 10.7M fragments. Across all samples ~2.14B reads were aligned to the *P. camtschaticus* draft genome [65, 66], for a total alignment rate of 80.47%. The average per-sample alignment rate was 80.43%±2.15%, which included 43.19%±1.50% and 24.52%±0.89% of concordantly mapped fragments that mapped uniquely and multiple times, respectively. The multi-mapped fragments were assigned by Bowtie2 to the "best" location, and therefore counted once in the downstream analysis. Of the ~2.14B aligned fragments, ~1.27B were assigned to gene-coding regions of the *P. camtschaticus* draft genome (59.6%). The remainder were not included in the downstream analyses as they mapped to non-coding regions (22.3%), or were assigned

Table 3. Pairwise F_{ST} values.

Treatment 1	*Treatment 2*	F_{ST}	*CI Lower Bound*	*CI Upper Bound*	*p-value*
Ambient	Severe	-0.0015	-0.0107	0.0073	0.67
Ambient	Moderate	-0.0123	-0.0207	-0.0035	1.00
Moderate	Severe	-0.0033	-0.0125	0.0061	0.78

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ambiguously (6.7%), as singletons (8.3%) or chimeras (3.1%). Initial examination of the gene counts using PCA identified one sample from the ambient pH treatment as an outlier, which was removed from the dataset and resulted in 14, 13, and 15 samples for the ambient, moderate, and severe OA treatments, respectively (Table 1). In total, we detected all 162,611 gene features that are in the draft *P. camtschaticus* genome [66], but after removing low frequency genes (totaling 0.75% of fragments), 74,778 genes remained for analysis, 32,435 of which mapped to genes in the Uniprot/Swissprot database. The high number of genes to which reads mapped reflects the large *P. camtschaticus* draft genome, which includes a high degree of repeat elements characteristic of crustacean genomes [65]. A one-way ANOVA indicated that the number of fragments retained for analyses did not differ among OA treatments ($F_{(2,39)}$ = 0.22, *p*-value = 0.80), ranging from 15.4M to 38.2M per sample and averaged 29.4M±5.1M, mapped to on average 75K±80 genes.

Global expression patterns

Global expression profiles of red king crabs reared in ambient conditions differed from those reared in either moderate or severe OA. Pairwise permANOVA tests detected significant differences in multivariate space among ambient conditions and OA treatments (moderate OA: F(1) = 1.84, *p*-adj = 0.042; severe OA: F(1) = 2.70, *p*-adj = $3.0e^{-3}$), but not between the two OA treatments (F(1) = 1.20, *p*-adj = 0.50), which is evident from the biplot of the first two principal components (PC1 & PC2) (Fig 2). Ambient-reared crabs were separated from those reared in OA treatments along PC1 (19.0%) and PC2 (8.4%), which combined explained 27.4% of variation in global expression. While the scree test indicated that PC3 and PC4 also explained a significant amount of variation (7.0% and 6.2%, respectively), there was no separation among treatments along those axes (S4 Fig).

Global gene expression was less variable among crabs reared in OA treatments compared to those reared in ambient conditions. Using a test of multivariate homogeneity of group dispersions, we found that variation in global gene expression differed by treatment

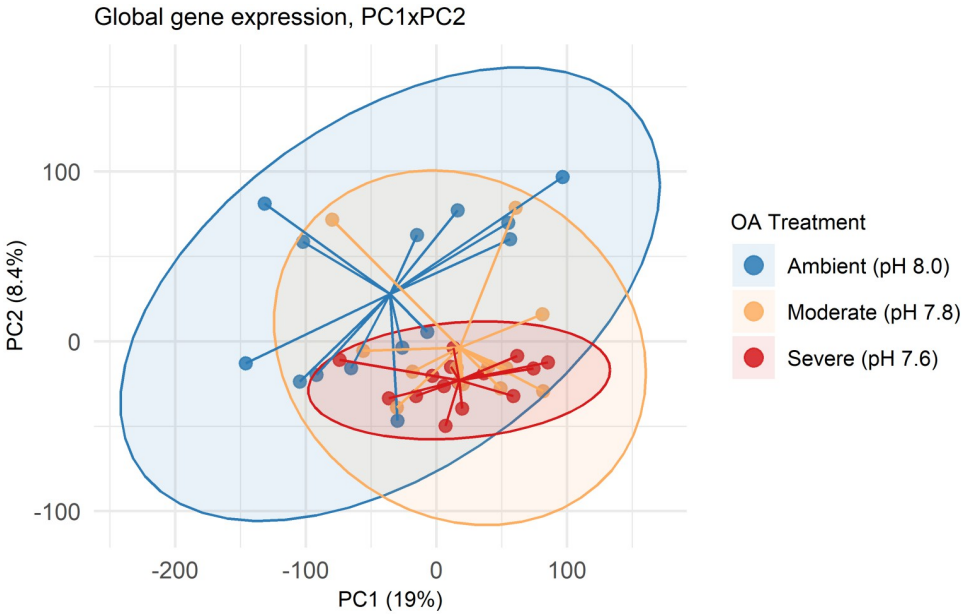

Fig 2. PCA biplot of first the two principal components, constructed from all expressed genes.

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(F(2,39) = 4.9, p = 0.012). Pairwise comparisons indicated that variation differed between ambient and severe OA (p-value$_{permuted}$ = 8.3e^{-3}), but did not differ between ambient and moderate OA (p-value$_{permuted}$ = 0.32) or moderate and severe OA (p-value$_{permuted}$ = 0.25).

Co-expression network analysis

We performed a weighted gene co-expression network analysis (WGCNA) to identify groups of genes that were co-expressed (i.e. gene modules), and for which expression correlated with pCO_2 concentration. The 74,778 examined genes were assigned to 41 modules, 14 of which had eigengenes that correlated significantly with pCO_2 (S2 Table). For eight of the modules, which contained in total 22,537 genes, the eigengenes correlated negatively with pCO_2 indicating that expression decreased as OA treatment became more severe. Six modules, containing 19,248 genes in total, correlated positively with pCO_2, indicating higher expression in OA treatments.

Differential gene expression

Analysis in DESeq2 identified 6,806 genes that were differentially expressed among treatments (9.1% of all examined genes). The number of differentially expressed genes increased with OA severity: 1,459 genes differed between ambient and moderate OA (526 upregulated in moderate OA, 933 downregulated, Figs 3 & 4A), and 6,257 genes differed between ambient and severe OA (2,350 upregulated in severe OA, 3,907 downregulated, Figs 3 & 4B). Only 47 genes differed between moderate and severe OA treatments (22 upregulated in severe OA, 25 downregulated, Figs 3 & 4C), indicating that the two OA treatments induced a similar transcriptional response (S3 Table). There was high overlap between the differentially expressed genes and the co-expression network analysis (WGCNA), with 92.8% of DEG's assigned to one of the 14 pCO_2-correlated co-expression modules.

Variation in gene expression

Within-treatment mean coefficient of variation (CV), which was calculated separately from genes that were upregulated, downregulated, or not differentially expressed, ranged from

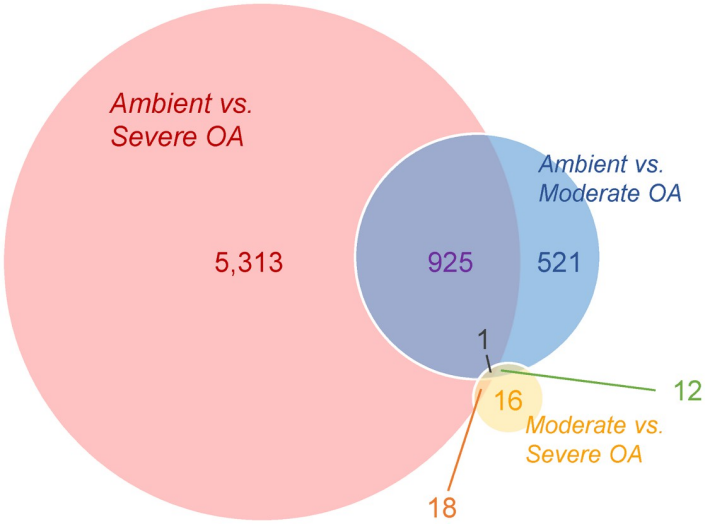

Fig 3. Venn diagram showing the number of differentially expressed genes among each pairwise OA treatment contrast, and the number that are shared among contrasts.

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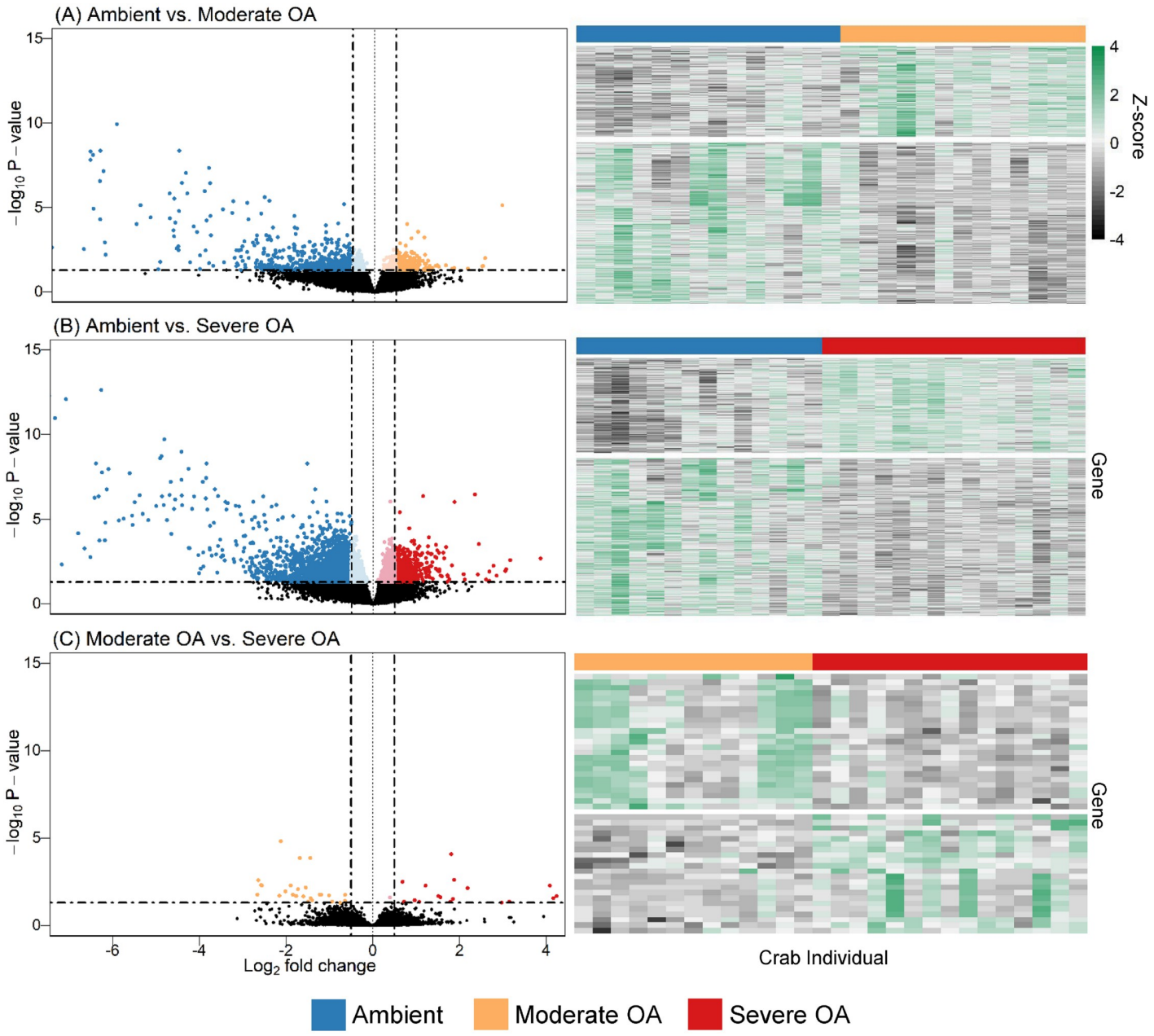

Fig 4. Pairwise differential expression among OA treatments for (A) ambient (pH 8.0) vs. moderate OA (pH 7.8), (B) ambient vs. severe OA (pH 7.5), and (C) moderate OA vs. severe OA. Volcano plots (left panels) show expression of all genes. Each point represents a unique gene, with non-black points residing above the dotted line representing differentially expressed genes. -Log10 p-value is along the y-axes, with higher numbers indicating higher significance, and $\log_2$ fold change is along the x-axes, with higher absolute values indicating larger differences among treatments. Differentially expressed genes (p-adj < 0.05) are color-coded to indicate those that are expressed at higher levels in ambient treatment (blue), moderate OA (orange), or severe OA (red). Points with darker shades indicate those that have | Log2FC| >0.5, which were used in functional analyses. Heatmaps (right panels) show expression of differentially expressed genes only (rows = genes) at per-sample resolution (columns = samples), with the green-black gradient indicating the z-score of expression values standardized across samples for each gene, where green and black indicate higher and lower expression, respectively.

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Table 4. Coefficient of variation mean ± SD for genes that were upregulated or downregulated relative to other treatments, or not differentially expressed (Non-DEG). To focus on differences among OA and ambient treatments, the 16 genes that were only differentially expressed among moderate and severe OA were not included in these calculations.

	Ambient	Moderate OA	Severe OA
Up-regulated	11.0%±10.3%	6.8%±4.7%	4.0%±4.2%
Down-regulated	5.5%±4.1%	11.2%±9.5%	8.5%±7.2%
Non-DEG	8.3%±5.6%	8.0%±5.3%	7.2%±5.2%

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4.0%-11.2% (Table 4). Genes upregulated in the severe OA treatment relative to ambient had the lowest mean CV (Table 4).

Functional analyses

Enrichment analysis of co-expressed genes that correlate with pCO_2. Genes that decreased significantly with pCO_2 (eight modules) were enriched for 193 biological process GO terms (65 at FDR<10%), which were predominantly related to protein production, energy production, metabolic activity, tissue development, muscle activity, cell cycle, immune function, chaperone mediated protein folding, and telomere maintenance (Fig 5, S4 Table). Genes that increased significantly with pCO_2 (six modules) were enriched for 48 processes (10 at FDR<10%), focused on transcription regulation and signaling (Fig 5, S4 Table). We also found that transposition and the related process DNA integration were enriched in both downregulated and upregulated modules (5 modules each).

Enrichment analysis of differentially expressed genes. Enrichment analysis revealed 26 and 104 biological process GO terms that were enriched in genes that were differentially upregulated and downregulated in an OA treatment compared to ambient, respectively (S5 Table). In genes upregulated in moderate-OA compared to ambient reared crab, 11 enriched processes were involved in negatively regulating (i.e. decreasing) cell growth and proliferation, transcription regulation, chaperone-mediated protein folding, chromatin organization, neurotransmitter exocytosis and signal transduction, and DNA integration via transposons (Fig 6). In severe-OA reared crab, 15 enriched processes in upregulated genes were involved in transposable element activity, regulation of transcription, nervous system development, signal transduction, and tissue development (Fig 6).

A variety of biological processes were downregulated in OA-reared crab compared to ambient-reared crab (Fig 7). Of the 23 processes that were enriched in genes down-regulated in moderate-OA reared crab, many were involved in the metabolism of various compounds (e.g. carbohydrates, lipids, fatty acids, estrogen), energy production (e.g. tricarboxylic acid cycle), and ceramide activity (biosynthesis, translocation, and metabolism), but also were involved in protein folding and N-linked glycosylation, toxin transport, and telomerase activity (see S5 Table for full list). There were 81 enriched processes in genes down-regulated in severe-OA reared crab. Many processes that were enriched in moderate-OA reared crab were also enriched in genes downregulated in severe-OA reared crab (Fig 7). Enriched processes that were uniquely downregulated in severe-OA reared crab included translation (the most significantly enriched down regulated process, p-value = $8.6e^{-14}$), and those related to DNA replication, microtubules, immune function, and aerobic respiration (Fig 7, see S5 Table for full list).

Five biological processes were enriched in genes that were differentially expressed among crab reared in moderate vs. severe OA. Two processes involved in molecular chaperone activity via the heat shock complex were enriched in genes with increased expression in the

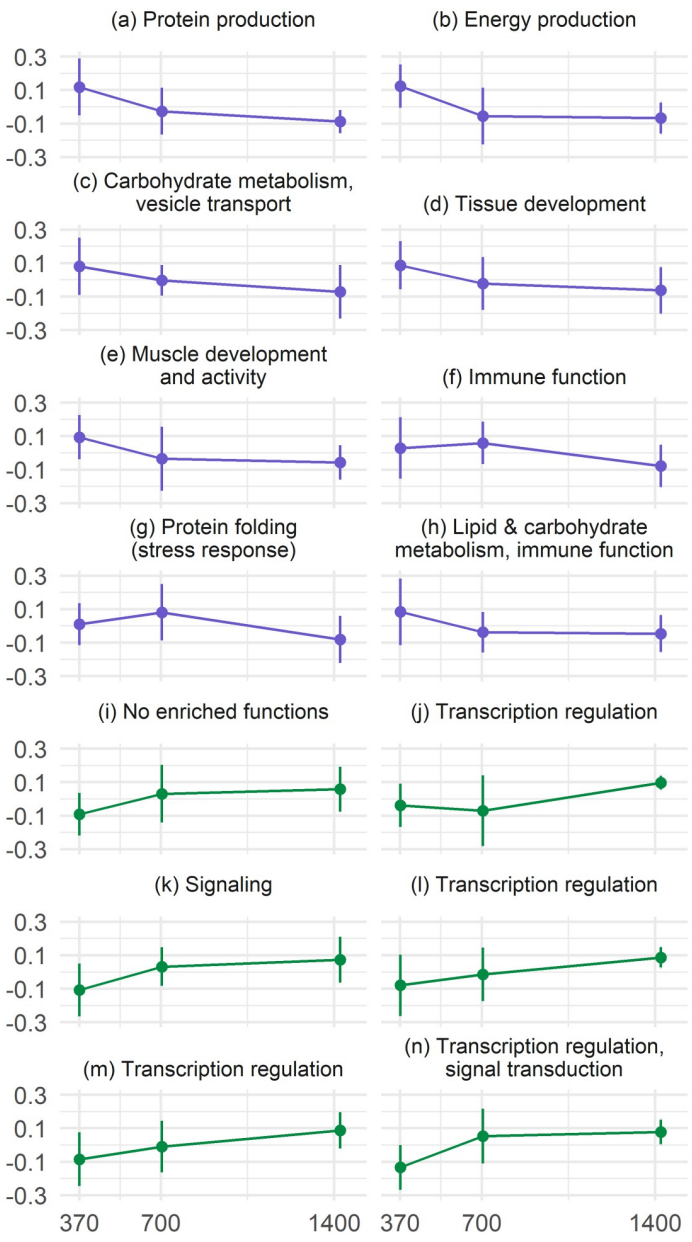

Fig 5. Expression profiles of co-expressed gene modules that correlated with pCO_2 treatment negatively (purple, a-h) and positively (green, i-n), indicating those genes that decreased and increased with OA severity, respectively, where 370 μatm is ambient (pH 8.0), 700 μatm is moderate OA (pH 7.8), and 1400 μatm is severe OA (pH 7.5). Points indicate the mean eigengenes (i.e. first principal component) for each treatment within each module, with standard deviation among individuals within treatments indicated by error bars. Figure titles indicate the predominant biological functions that were enriched in each module. Detailed enrichment analysis results, number of genes in each module, and correlation statistics are S4 Table. Transposition was omitted from figure titles as it was enriched in many modules.

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moderate OA-reared crab, and three processes involved in transposition were enriched in genes with increased expression in the severe OA-reared crab (S5 Table).

Enrichment analysis of genes potentially critical in an OA environment. Of the 526 and 2,350 genes that were upregulated in moderate and severe OA relative to ambient

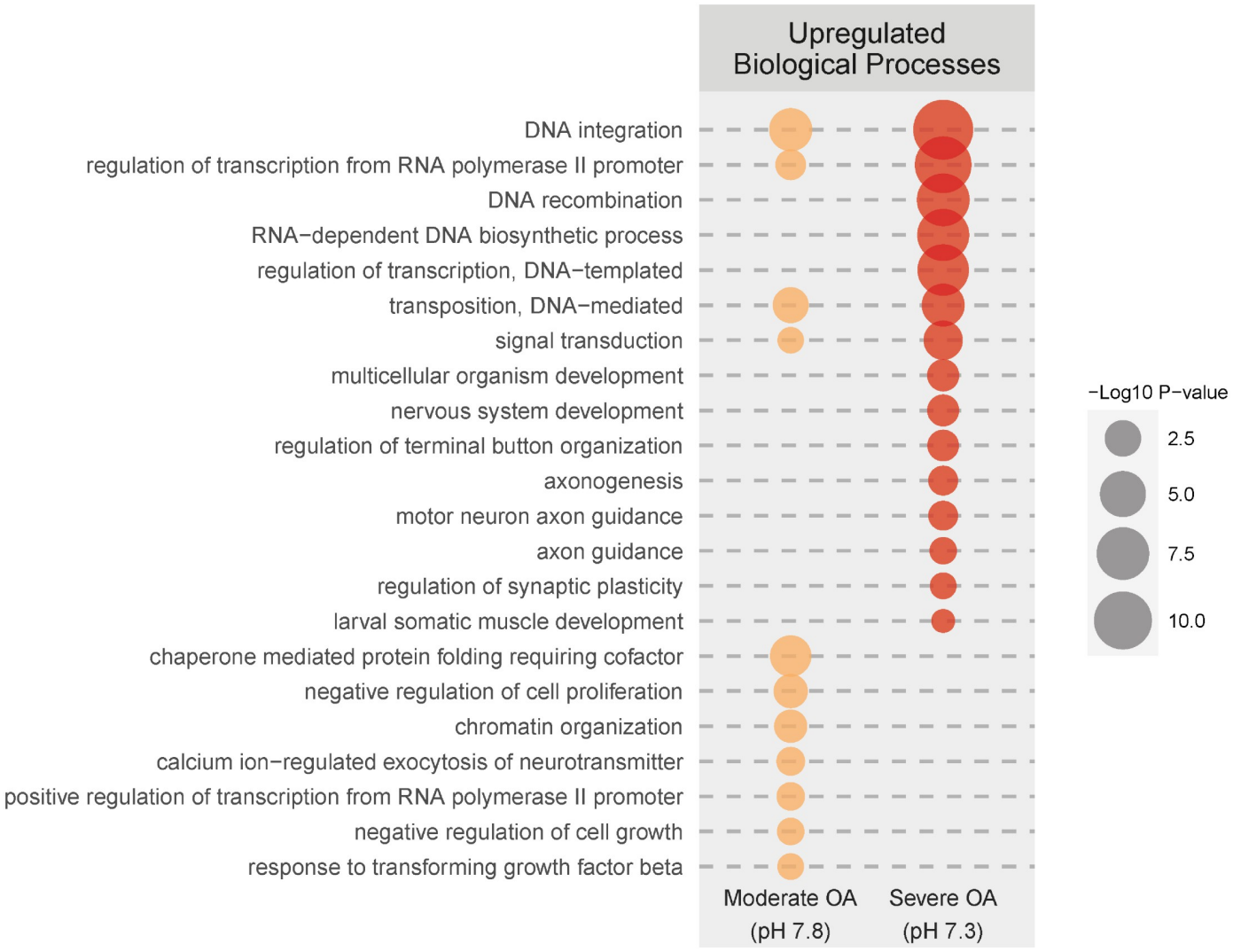

Fig 6. Enriched Gene Ontology biological processes in genes that were upregulated in moderate OA (pH 7.8, left column) and severe OA (pH 7.3, right column) compared to ambient conditions (pH 8.0). Point size indicates the -Log10 transformed *p*-value (all *p*-values < 0.05), such that larger points are more significantly enriched.

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treatment, respectively, 66 (13%) and 1,319 (56%) were expressed at consistent levels across individuals within treatments (CV < 3%, hereafter referred to as low-variance genes). These upregulated low-variance genes are of interest as they may provide critical functions in the OA environment. Low-variance genes upregulated in severe OA treatment were enriched for biological processes involved in transcription regulation, DNA integration (transposon activity), nervous system processes (neurogenesis, signal transduction, calcium ion-regulated exocytosis of neurotransmitter), developmental processes (multicellular organism development, keratinocyte differentiation, glycosaminoglycan biosynthesis), regulation of cardiac muscle cell contraction, and potassium ion transport (S6 Table). Low-variance genes upregulated in moderate OA treatment were enriched for chromatin organization and transposition (S6 Table). In contrast, low-variance genes that were upregulated in ambient treatment relative to either OA treatment were primarily enriched for processes involved in protein biosynthesis, glycolysis, and ion transport (including hydrogen ion transport) (S6 Table).

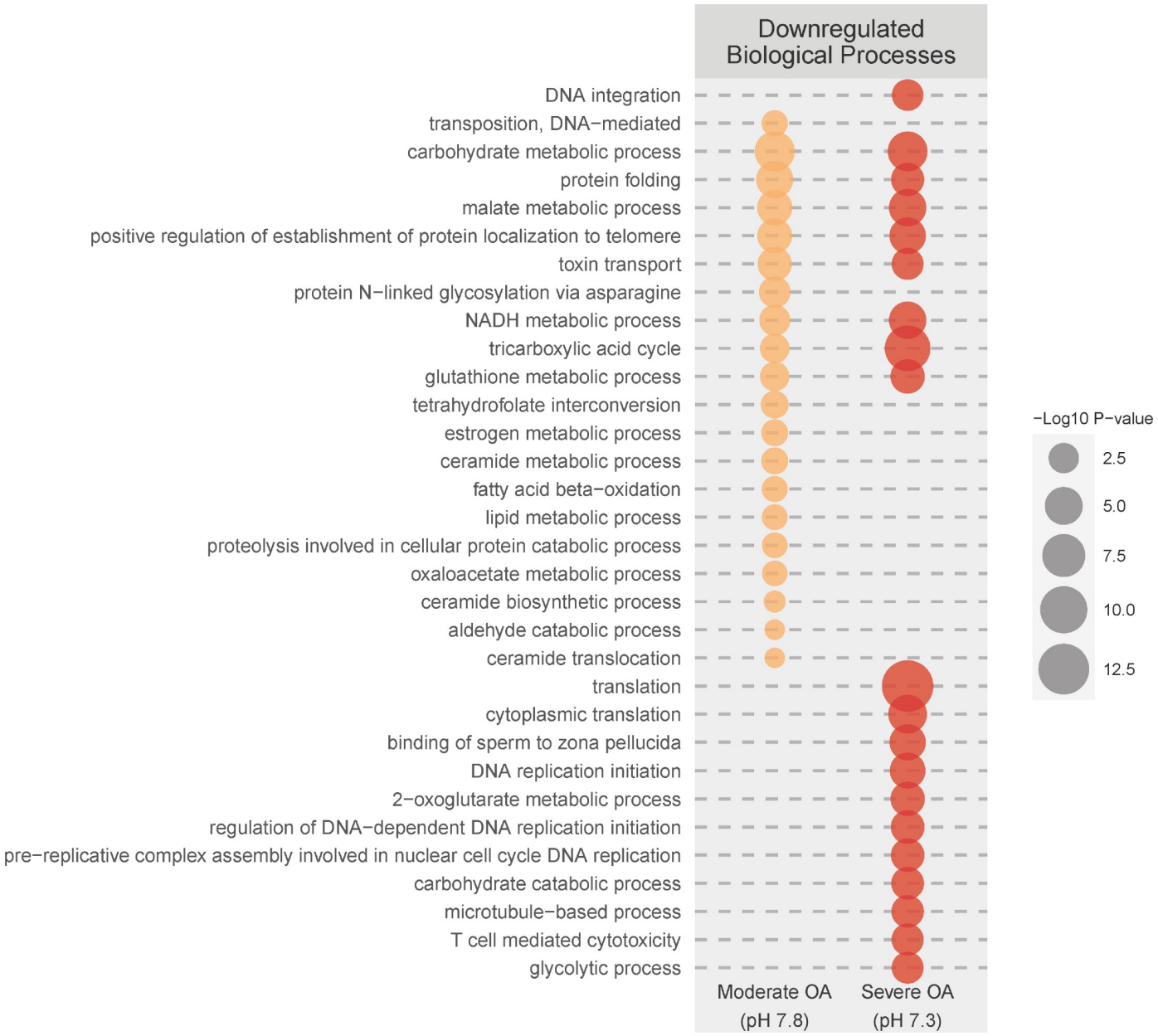

Fig 7. Enriched Gene Ontology biological processes in genes that were downregulated in moderate OA (pH 7.8, left column) and severe OA (pH 7.3, right column) compared to ambient conditions (pH 8.0). Point size indicates the -Log10 transformed *p*-value (all *p*-values < 0.05), such that larger points are more significantly enriched. Only the top 20 processes by *p*-value per OA treatment were included in this figure; see S5 Table for full list.

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Transposable element composition. A large portion of the red king crab transcriptome mapped to transposable elements (20,860 TEs), comprising 28% of the 74,778 analyzed genes, and 64% of the 32,435 annotated genes. These TEs mapped to 67 distinct Uniprot Species IDs, all of which were retroelements (S7 Table). Transposable element activity was high in all treatments, but activity increased with OA severity. Of the annotated genes that were upregulated in severe OA compared to ambient and moderate OA treatments, 65% and 86% mapped to TE's, respectively, while 51% of genes upregulated in moderate OA compared to ambient were

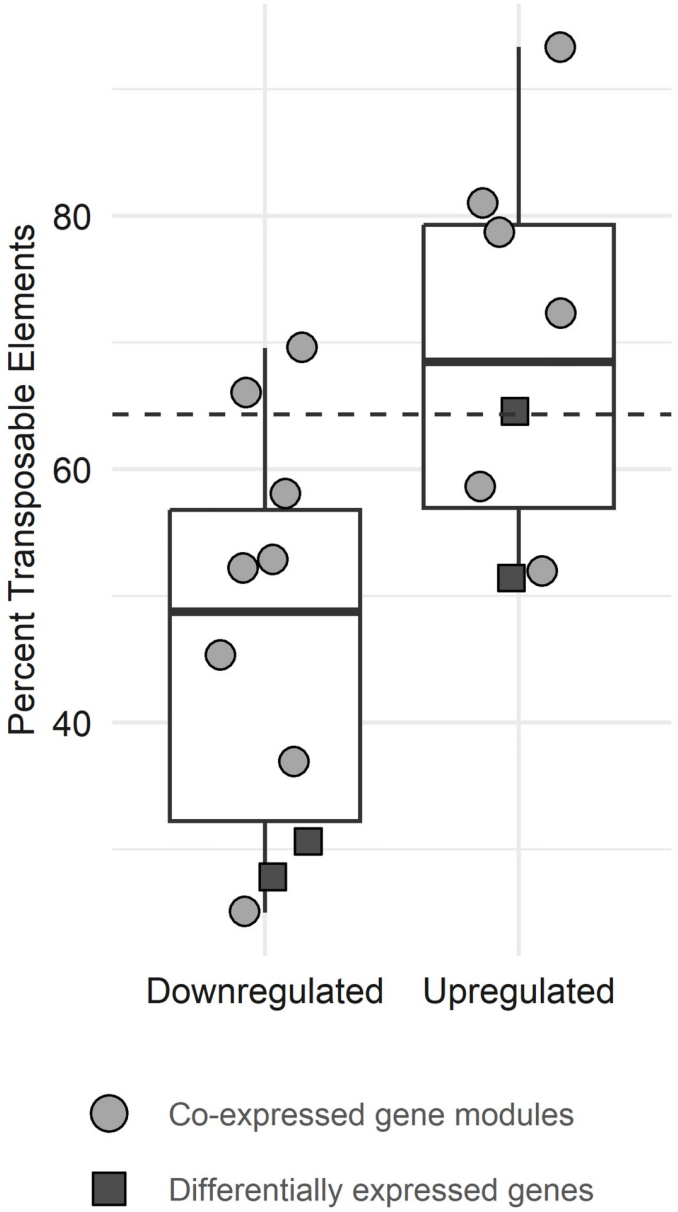

Fig 8. The percent of genes in each co-expressed gene module and differentially expressed gene set (DEGs) that were transposable elements (TEs), categorized by whether genes were downregulated or upregulated in OA relative to ambient treatment, which shows the high percentage of TEs in OA-upregulated genes. The proportion of all genes examined that were TEs (64%) is indicated by the dotted line. TEs were identified by searching within the protein names for the words transposon, transposable, LINE, retrotransposable element, transposable element, mobile element jockey, and pol polyprotein. Lines in the middle of each boxplot indicate the median percent TEs in downregulated and upregulated gene sets.

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TE's (Fig 8, S8 Table). In contrast, 31% and 28% of genes that were more abundant in ambient treatment compared to moderate and severe OA mapped to TEs (Fig 8, S8 Table). A large percentage of upregulated low-variance genes were also transposable elements, comprising 50% and 60% of those genes in moderate and severe OA-treated crab, respectively. In contrast, 16% of the low-variance upregulated genes in ambient-pH-reared crab were transposable elements

(S8 Table). The TE composition of co-expressed gene modules was similar- on average the percent of genes that increased and decreased with pCO_2 was 73% and 51%, respectively (Fig 8). A likelihood ratio test examined the proportions of gene sets that were TEs, and found higher TE proportions in upregulated gene sets compared to downregulated gene sets (χ^2 (2, N = 20) = 10.7, p = 0.001, Fig 8).

Discussion

Calcifying marine species living at high latitudes may be particularly vulnerable to the effects of OA due to more extreme changes projected to occur in those regions [1, 15]. Crustaceans in some high latitude regions are likely already experiencing acidified conditions seasonally; at present the Bering Sea shelf drops to seasonal lows of around pH 7.5 [13] and mean bottom pH is projected to drop by a further 0.3 units by 2100 [15]. Red king crab, which is one of several valuable commercial fisheries in Alaskan waters, are sensitive to changes in ocean chemistry at the juvenile stage, resulting in high mortality and decreased growth [51, 86]. However, our supporting study [55] found that red king crab are surprisingly resilient to OA when exposure begins early in life—at hatch—with no impact to survival, growth, or development through the early juvenile (C1) stage. Here, we explore aspects of red king crab molecular physiology that are altered by long term exposure to acidification, and which may be critical to their survival, to understand their adaptive potential and improve population predictions. We provide the first study to describe the molecular signatures of juvenile red king crab that were reared from the larval stage in acidified conditions.

Expression of nearly half of all genes (44%) correlated significantly with pCO_2, suggesting a strong molecular response to OA conditions, contrary to the phenotypic results. There was a general reduction in transcriptional activity and inter-individual variability in OA-reared crab, suggesting that OA reduces the breadth of physiological functions compared to crabs reared in ambient seawater. Functional analysis of downregulated genes indicates that energy production is depressed in OA conditions, which is likely associated with decreases in biosynthesis, the immune system, and myriad metabolic processes. Downregulated lipid and carbohydrate metabolic processes suggest a shift in metabolic strategy to protein catabolism, possibly to reduce CO_2 production and facilitate acid/base regulation. The limited energy is shunted towards transcriptional regulation mechanisms, signaling systems, and control of growth. Transposable elements (TEs) in OA-reared crab were highly active, particularly in the most severe OA treatment, and were expressed at consistent levels, suggesting that TEs play a role in the OA-response. In the remainder of this section we describe processes that are suppressed and triggered by OA exposure during development in red king crab, and expand on effects to TE activity. Finally, we discuss differences in gene expression variation observed among treatments, and consider whether there were significant genetic differences among treatment groups that may have contributed to the observed gene expression profiles.

Processes that are suppressed in OA-reared crab

OA resulted in a widespread downregulation of metabolic processes involved in multiple respiratory pathways, metabolism of a variety of compounds, and protein synthesis machinery (Fig 7). Reduced metabolic activity may be one way that red king crabs mitigate acidosis [87, 88]. Carbon dioxide is produced during respiration, therefore a decrease in metabolic activity reduces internal CO_2 production. Decreased mitochondrial activity can also reduce oxidative cellular stress by reducing mitochondrial production of reactive oxygen species, which might otherwise increase due to environmental stressors [89–91].

Another outcome of changes in metabolic activity could be a shift in metabolic strategy away from carbohydrate and lipid substrates and towards proteins to improve acid/base regulation [92, 93]. Protein catabolism is less energy efficient, but instead of CO_2 it produces ammonia and bicarbonate, which can be used to buffer intracellular pH [94]. While we did detect downregulation of carbohydrate & lipid metabolism that suggests a metabolic shift (Fig 7), and increased transmembrane signaling, we did not detect increased ion regulation processes in response to OA. It is possible that ion transport was indeed higher, but was uncoupled from mRNA levels in our fully OA-acclimated crab [95]. Pairing gene expression with more direct physiological assays (e.g. excreted O:N ratio, in vivo Na+,K+-ATPase activity) would improve interpretation of how red king crab metabolic and ion regulation strategies respond to OA.

Metabolic changes are quite common in response to OA [96, 97]. OA can cause metabolic depression (and more generally, reduction in gene activity) in crustaceans and other marine invertebrates [98–102]. In the present study, metabolic processes were downregulated in both the moderate and severe OA treatments, which likely reflects the sensitivity of red king crab to changes in ocean chemistry. Given these findings, we might expect metabolic rate to decrease in response to OA in red king crab. In previous respiration trials [50] there was no change to metabolic rate measured by oxygen consumption in juvenile red king crab exposed to the same OA conditions after three weeks. The decreased metabolic gene activity in the present study could reflect an acclimatory response that is only induce by long-term and/or multi-stage exposure—in our case three months from hatching—which may influence the physiology of crabs in a way that short term- or single life stage- exposure does not [97]. Decreased expression of metabolic functions may also reflect an interactive effect between OA treatment and seasonal temperature increase, which was not a factor in Long et al. [50] (they held crab at 5˚C). Future studies should pair gene expression analysis with more direct measurements of metabolic rate (and other traditional physiological measurements) at varying temperatures to untangle the effects of OA and temperature on red king crab metabolic functioning.

OA affected the red king crab immune system. Genes involved in neuroinflammation (microglial cell activation), viral response, and the innate immune system decreased with pCO_2, although effects were less pronounced compared to metabolic changes. OA can negatively affect the immune system in crustaceans [22, 35, 103–105] and other marine invertebrates [106–108]. The mechanisms by which OA alters immune function are not fully understood, and could relate to energy constraints and malfunction of immune-related enzymes and signaling/recognition pathways (among other mechanisms) [106, 108]. We find evidence that immunosuppression may in part be due to activation of the stress response [109]. Genes coding for the octopamine receptors and a receptor for tyramine, the direct precursor of octopamine, were upregulated in OA. Octopamine is the invertebrate orthologue to norepinephrine, and is one of the key neurotransmitters that regulates the acute stress-response [110]. Interestingly, molecular chaperones (HSP70 and DNAJA1), which respond to a variety of stressors by refolding or facilitating the destruction of damaged proteins [111, 112], increased in moderate OA but decreased substantially in severe OA, which suggests that those processes become ineffective as OA severity worsens. Further, it suggests that at moderate OA levels increased oxidative stress damages proteins, or that altered intracellular or extracellular pH affects folding patterns of proteins. While these changes did not ultimately result in mortality in the present study, our findings indicate that red king crab may be more vulnerable to co-occurring secondary stressors, such as thermal stress [53, 54], due to immunosuppression and changes in cellular stress-response mechanisms. Additional multi-stressor studies are needed that expose red king crab to a range of pH levels alongside other stressors, particularly pathogen challenges.

Transposable elements are highly active in OA-reared crab

Many of the genes upregulated under OA conditions were transposable elements (TEs, or transposons). While TEs were present in all gene sets, including those that were more active in ambient conditions, they comprised a much larger percentage of genes upregulated in OA-reared crab (Fig 8, S8 Table). TEs, or "jumping genes", are DNA elements that move to new locations in the genome when activated, resulting in insertional mutations [113]. Retrotransposons, the class of TEs detected in our study, are mobilized by a copy-and-paste mechanism where the DNA sequence is transcribed and an RNA intermediary is then reverse-transcribed into a cDNA copy before being integrated into the genome [114]. Increased transposable element activity in response to environmental stress is well documented in a variety of eukaryotes [115]. There have been, however, only a few studies to detect stress-activated TEs in marine crustaceans [116], and to our knowledge this is the first to do so in response to OA. While TEs comprise large portions of the genomes of arthropods [117], including crustaceans [65, 118], previous transcriptional characterization of stress-responses in crustaceans may have overlooked TE's due to the common practice of masking repeat elements from genomes during analysis [119]. Recent reports from a wide range of other taxa have also implicated TEs in the OA-response, including anemones [120], clams [121], and diatoms [122]. This breadth of taxa, which now includes crustaceans, suggests that TEs are a common response to acidification exposure.

Why TEs can become activated under stress is still under debate, as is whether they are detrimental or beneficial to an organism's survival [114]. TEs may become more active because the mechanisms that suppress them are no longer functioning, which could negatively affect fitness due to pathological mutations. Alternatively, stress-activated TE's could act as regulatory elements and/or increase diversity of proteins available to the organism or its descendants, which may cause beneficial phenotypic variation. TEs may also be a component of the antiviral defense system, as they can stimulate the antiviral inflammatory response [123, 124]. Deciphering why TE were more active in OA-reared crab is beyond the scope of this study. However, given that red king crab early life stages tolerated OA in this study, and that many genes that were upregulated at consistent levels among OA-exposed individuals were TEs, TE mobilization may be an effective component of the red king crab stress-response system, rather than simply the result of genomic instability. TEs are also strong facilitators of adaptive evolution [125, 126], thus OA-induced TE activity may benefit red king crab at an evolutionary scale, given that it could provide a mechanism for rapidly increasing genetic diversity [115, 127]. It would be informative to examine whether the stress-induced mobilization of TE's increases mutation rates by re-integration into the germline, resulting in heritable changes to the genome, which can occur in other species [128]. If so, this could provide a mechanism by which OA exposure increases phenotypic diversity across generations.

Other processes that are more active in OA-reared crab

OA triggered a heavy investment in transcriptional regulation at multiple levels of transcriptional control. DNA methylation and histone demethylase activity was upregulated in OA-reared crab, which are epigenetic mechanisms that control transcription through changes to chromatin structure and DNA accessibility [129]. A large percentage of upregulated genes (18%) were associated with the regulation of transcription from RNA polymerase II promoters. Increased expression was also detected in genes that code for post-translational gene control, such as Exportin-5, which is involved in mRNA silencing by microRNAs [130]. Increased transcription regulation activity in OA-reared crab explains the widespread changes in gene expression (44% of all genes correlated with pCO_2). Given that OA resulted in widespread

downregulation of a variety of biological functions (Fig 7, S4 & S5 Tables), one purpose of the transcriptional regulatory response of OA-reared crab may be to shut down (or dampen) less critical processes. Additionally, since physiological metrics were unaffected by OA [55], changes in transcription regulation may reflect physiological reprogramming that optimizes gene activity for the OA-environment, for instance by shifting metabolic pathways to improve acid/base regulation, and decreasing activity of less-critical processes to conserve energy. In addition to the broad transcriptional effects, these regulatory elements may be responsible for the unleashing of transposable element activity in response to OA [115].

Cell signaling and nervous system development was substantially more active in OA-reared crab. Many upregulated genes were associated with signal transduction, and interacted with or spanned cell membranes (e.g. Teneurin-m, Semaphorin-1A, Ankyrin-2). Increased expression of these genes suggest that OA increases the need to transfer information between the external and internal environments, particularly across the plasma membrane, to regulate activity inside the cell. Investment in signaling molecules may also reflect negative effects of OA on transmembrane signaling, perhaps by damaging the membrane or cytoskeleton (e.g. due to oxidative stress [91]), or by decreasing signaling molecule binding affinity [131, 132]. Investment in the nervous system may be necessary to fortify systems that monitor conditions in the environment. A gene coding for the protein "pinocchio", which was upregulated in severe OA, is expressed in the antenna of some arthropods (e.g. fruit fly, [133]), and acts as a chemosensory receptor [133, 134]. OA-associated carapace dissolution around neuritic canals is correlated with damaged setae in larval Dungeness crab or their underdevelopment [135]. Setae are important sensory structures which are innervated with chemo- and mechano-receptors [136]. Further, OA can alter the response of crabs to chemosensory cues suggesting a reduction in their ability to detect such cues [137]. The heavy investment in neurogenesis found here may be one way that OA-reared crab counteract the negative effects of OA to external sensory structures.

Genes involved in negative regulation of growth and cell proliferation were also upregulated in OA conditions (Fig 4A). Differentially upregulated genes included negative growth regulators Menin, Brain tumor protein, and Forkhead box protein O (FOXO). FOXO is a transcription factor that specifically inhibits growth in response to cellular stressors, including oxidative stress and nutrient deprivation [138], and may therefore be one regulatory mechanism connecting oxidative stress with OA exposure [5] and decreased growth rate previously seen in juvenile red king crab and related species [25, 50, 51]. Interestingly, our supporting study did not find any effects of OA on growth measured throughout the larval stages and into the early juvenile stage. Had treatments continued it is possible that growth through the juvenile stage could have been impacted. Alternatively, negative growth regulators could reflect increased cellular response to DNA damage caused by OA. For example, FOXO negatively regulates growth by promoting cell cycle arrest, DNA repair and detoxification, and apoptosis [139], which may be needed in OA conditions due to damage from oxidative stress [5]. While this experiment's three-month exposure is long relative to much of the other research, it would be informative for future studies to extend OA treatments through the full juvenile stage, and where possible sexual maturity, to fully capture effects of OA on growth across life stages.

A previous study reported that OA-exposed juvenile red king crab predominantly upregulated genes related to the cuticle [54], which we did not see. On the contrary, five genes coding for cuticle proteins (CP1158 & CPAM119) were downregulated in severe OA-reared crab (but cuticle processes were not enriched). The Stillman et al. [54] experimental design was similar to the present study in many ways (similar laboratory & OA conditions), but the treatments were shorter in duration (three weeks) and occurred only during the juvenile stage. The transcriptional response of juvenile red king crab may therefore depend on whether they are OA-

naive, or if they were previously exposed to OA conditions as larvae. Exposure to OA during early life stages may be particularly impactful, as it can alter an organism's physiological trajectory [140, 141]. For instance, exposure to OA during oogenesis and embryogenesis positively impacts larval performance in snow crab [142], and negatively affects larval size, morphology, mineral content, and metabolic rate in Tanner crab [143]. Another possible factor influencing the transcriptional response to OA is when red king crab are exposed relative to their molt cycle, which greatly influences gene expression [144, 145]. We sampled juveniles early in the intermolt stage and, because of the design, know that all crabs were sampled at the same stage in the molt cycle (at ~5 days post molt). The crabs sampled by Stillman et al. [54] were not standardized by where they were in the molt cycle, thus possibly capturing some effects of OA on crabs in the early pre-molt stage when the new exoskeleton is being synthesized. A time-series analysis of the transcriptome in varying OA environments, particularly across multiple stages of the molt-cycle, would provide a more integrated view of the crustacean OA response.

OA decreases gene expression variability, which is not explained by genetic differences

The variability in gene expression was much lower among individuals reared in OA treatments than those reared in ambient pH. This is evident in the PCA constructed from genome-wide expression data (Fig 1), which reveals tighter clustering as OA treatment severity increases. Genes upregulated in OA-reared crabs were also expressed at more consistent levels, particularly in the severe OA-reared crab (Fig 2, Table 4), suggesting the need for highly controlled levels of transcripts that perform critical functions. This tight transcriptional control may reduce the scope for potential responses to other stressors (e.g. warming, pathogens), which could explain synergistic effects of OA and warming on survival reported for red king crab [53]. Given the possibility for genotype-specific gene expression influencing our transcriptional results, we investigated whether genetic composition differed among OA treatments (i.e. did larvae from only a few families survive in OA treatments). We found no evidence of genetic differences among treatments—samples did not cluster by treatment in the genetic PCA (Fig 1, S2 Fig), and no families were over- or under-represented in any treatment. The constricted gene expression pattern observed in juveniles reared in OA treatments therefore is not likely attributed to genotype-specific expression, but is a plastic response to OA conditions that is consistent across many families. This may reflect a lack of standing genetic variability that is needed to fuel rapid adaptation to OA [146], as transcriptional variability and other measures of phenotypic plasticity in response to OA may be associated with the tolerance of a species or population [147], or their potential for adaptive selection [148]. It must be noted that our genetic analysis is limited to variants within transcribed genes, and therefore may not fully capture variation across the genome. As the OA literature continues to mature, closer attention should be paid to transcriptional variability among OA-exposed organisms, and how it relates to the species' tolerance.

Conclusion

Red king crab juveniles are quite sensitive to short-term OA exposure, even when compared to closely related species [50, 86]. When reared in OA from hatching through the early juvenile stage, however, typical response metrics (growth, development, survival) are unaffected [55], suggesting a shift in molecular mechanisms that enable OA tolerance. Our findings reveal that red king crab reared in OA conditions have a narrowed, possibly optimized, set of gene functions that may reflect physiological reprogramming for the OA environment [149]. Still unknown is whether OA tolerance in OA-reared crab persists through all juvenile stages, as

metabolic limitations and depressed immune function, revealed here by gene expression analysis, may ultimately make them more vulnerable to infection or secondary stressors and limit growth. Only a handful of processes are upregulated in OA, indicating a critical need for a more active and developed nervous system, and tight regulation of transcription and control of growth. Given the universal and invariable upregulation of transposable elements in OA-exposed crab, TE activity may serve as an effective OA response by producing novel or cryptic transcripts, but these effects may only be fully realized in future generations. Multi-stressor studies are needed, with a focus on the immunological effects of OA, the role (and vulnerability) of the nervous system in an acidified world, and the potential role of TE's in fueling adaptation.

Supporting information

S1 Fig. Water parameters during the experiment. Temperature and pH were measured daily; salinity, dissolved inorganic carbon (DIC) and alkalinity were measured weekly, beginning Week 2; all other parameters were calculated. Lines are mean values across tanks for each treatment.
(TIF)

S2 Fig. PCA biplot of principal components 3 and 4, constructed from RNA-Seq derived SNPs (n = 331). Points represent individual crabs that are color-coded by OA treatment, which do not indicate clustering by treatment, and sizes represent the percent of SNPs that are missing in each individual.
(TIF)

S3 Fig. Correlation plots among SNP-derived PCA scores and gene expression-derived PCA scores along the first two principal components, with Pearson correlation coefficients and p-values calculated for each treatment.
(TIF)

S4 Fig. PCA biplot of principal components 3 and 4 constructed from all expressed genes.
(TIF)

S5 Fig. Network of biological processes (Gene Ontology terms, "GO") that were enriched in genes differentially expressed in moderate acidification (pH 7.8) compared to control conditions (pH 8.0), where green and red indicate processes enriched in upregulated and downregulated genes, respectively. The network was constructed using the GO hierarchical structure.
(PDF)

S6 Fig. Network of biological processes (Gene Ontology terms, "GO") that were enriched in genes differentially expressed in severe acidification (pH 7.5) compared to control conditions (pH 8.0), where green and red indicate processes enriched in upregulated and downregulated genes, respectively. The network was constructed using the GO hierarchical structure.
(PDF)

S7 Fig. Network of biological processes (Gene Ontology terms, "GO") that were enriched in genes differentially expressed in severe acidification (pH 7.5) compared to moderate acidification (pH 7.8), where green and red indicate processes enriched in genes upregulated and downregulated in the severe treatment, respectively. The network was constructed

using the GO hierarchical structure.
(PDF)

S1 Table. Mean water parameters for each tank. Also available on GitHub: Supplemental-Table-1_Water-parameters-by-tank.csv.
(XLSX)

S2 Table. Statistics for modules with eigengenes that correlated significantly with pCO_2 concentration. Modules in purple (↓) and green (↑) indicate modules for which gene expression decreased and increased with pCO_2, respectively. The percent of genes that were transposable elements are also included for each module. Modules are randomly assigned color names, and the letters correspond to Fig 5 panels in the main text.
(XLSX)

S3 Table. Number of differentially expressed genes and enriched GO terms by pairwise treatment contrast. Colors and arrows indicate the number of DEGs and GO terms that were upregulated (↑) and downregulated (↓) in the more severe OA treatment, where ambient = pH 8.0, moderate = pH 7.8, and severe = pH 7.5.
(XLSX)

S4 Table. Enriched Gene Ontology terms in gene modules associated with pCO2 treatment, which were identified using WGCNA. Also available on GitHub: Supplemental-Table-3_WGCNA-Enrichment-Results.csv.
(XLSX)

S5 Table. Enriched Gene Ontology terms in differentially expressed genes, which were identified using WGCNA. Also available on GitHub: Supplemental-Table-4_DEG-Enrichment-Results.csv.
(XLSX)

S6 Table. Enriched Gene Ontology terms in upregulated low-variance genes, which were identified using WGCNA. Also available on GitHub: Supplemental-Table-5_Upregulated-Low-CV-Enrichment-Results.csv.
(XLSX)

S7 Table. A list of the 68 unique transposable elements detected in gene expression data. See separate file or Supplemental-Table-6_Transposons.csv.
(XLSX)

S8 Table. Percent of genes differentially expressed among pairwise contrasts that map to transposable elements. Colors indicate genes that were more active in ambient (blue), moderate OA (orange), and severe OA (red) treatments in each pairwise comparison. Values below the dotted line indicate the % of upregulated genes with low within-treatment variation ($CV < 3\%$) that were TEs.
(XLSX)

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