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The Divergent Responses of Salinity Generalists to Hyposaline Stress Provide Insights Into the Colonisation of Freshwaters by Diatoms

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ABSTRACT

Environmental transitions, such as the salinity divide separating marine and fresh waters, shape biodiversity over both shallow and deep timescales, opening up new niches and creating opportunities for accelerated speciation and adaptive radiation. Understanding the genetics of environmental adaptation is central to understanding how organisms colonise and subsequently diversify in new habitats. We used time-resolved transcriptomics to contrast the hyposalinity stress responses of two diatoms. *Skeletonema marinoi* has deep marine ancestry but has recently invaded brackish waters. *Cyclotella cryptica* has deep freshwater ancestry and can withstand a much broader salinity range. *Skeletonema marinoi* is less adept at mitigating even mild salinity stress compared to *Cyclotella cryptica*, which has distinct mechanisms for rapid mitigation of hyposaline stress and long-term growth in low salinity. We show that the cellular mechanisms underlying low salinity tolerance, which has allowed diversification across freshwater habitats worldwide, includes elements that are both conserved and variable across the diatom lineage. The balance between ancestral and lineage-specific environmental responses in phytoplankton have shaped marine–freshwater transitions on evolutionary timescales and, on contemporary timescales, will affect which lineages survive and adapt to changing ocean conditions.

1 | Introduction

Environmental transitions are often landmark events in evolution (Carroll 2001; Donoghue et al. 2021; Grosberg, Vermeij, and Wainwright 2012). On shallow timescales, the colonisation of new environments can trigger rapid adaptive evolution, opening up new niches and creating the conditions for ecological speciation (Schluter 2000). Played out over macroevolutionary timescales, these processes can lead to adaptive radiations (Stroud and Losos 2016) or increases in the rate of speciation (Pinseel et al. 2020; Wiens 2015). Phenotypic plasticity can play a key role in the colonisation of new habitats and, once there, directional selection can tailor plastic phenotypes to the new environment, allowing populations

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to become permanently established (Ghalambor et al. 2007). Historical patterns of habitat shifts can be reconstructed through phylogenetics, but a full understanding of how environmental barriers are crossed requires direct observations from genetics or controlled experiments (Downey et al. 2023; Jones et al. 2012). Pushed to their physiological limits, generalists are natural candidates for experimentation to determine how species successfully colonise, become established, and eventually diversify within new habitats.

Diatoms are microalgae found throughout marine and freshwaters, where they play keystone roles in food webs and nutrient cycles. Diatoms are ancestrally marine, but as a result of numerous transitions across the salinity divide, freshwater species outnumber marine ones (Nakov, Beaulieu, and Alverson 2019; Roberts et al. 2023). In addition to marine and freshwater specialists, many lineages include 'euryhaline' generalists that survive a broad salinity range. Selection should favour this type of plasticity in species that experience environmental fluctuations (Bradshaw 1965; Ghalambor et al. 2007; Via et al. 1995), such as the rapid salinity changes that occur in coastal and estuarine biomes (Gibson, Barnes, and Atkinson 2002). The ability of populations to survive abrupt environmental change rests on their ability to survive the initial cellular stress. Certain stress responses are widely conserved across species (Lindquist and Craig 1988; Rhee, Kim, and Lee 2007; Scandalios 2002), but lineage-specific regulation of stress responses have also been identified (Brion et al. 2016) and are important because species able to mount more robust stress responses may be more likely to successfully colonise new environments. Through controlled RNA-seq experiments built upon decades of laboratory studies (Liu and Hellebust 1976; Paasche 1975; Schobert 1974), the physiological responses by diatoms to low salinity are coming into focus (Downey et al. 2023; Kamakura, Bilcke, and Sato 2024; Nakov et al. 2020; Pinseel et al. 2022), but comparative studies are necessary to show how some lineages have established a foothold in inland waters while others have not.

To better understand mechanisms of marine–freshwater transitions, we used experimental RNA-seq to characterise the short-term, minutes-to-hours, response to hyposaline stress in the diatom, *Skeletonema marinoi*. We compared this with published data on the acclimated state of *S. marinoi* exposed to weeks of hyposaline conditions to develop a more complete temporal model of salinity acclimation (Pinseel et al. 2022). We then compared the response of *S. marinoi* to that of another diatom, *Cyclotella cryptica*. The two species last shared a common (marine) ancestor roughly 90 million years ago (Figure 1). *Skeletonema marinoi* has deep marine ancestry with a recently evolved, modest tolerance to low salinity, growing in habitats generally ranging from marine to brackish (Nakov, Beaulieu, and Alverson 2018; Sjöqvist et al. 2015) (Figure 1). *Cyclotella cryptica* is a more robust generalist that grows in salinities ranging from marine to freshwater (Nakov et al. 2020). It is part of a clade with deep freshwater ancestry and repeated traversals across the salinity gradient that gave rise to marine specialists,

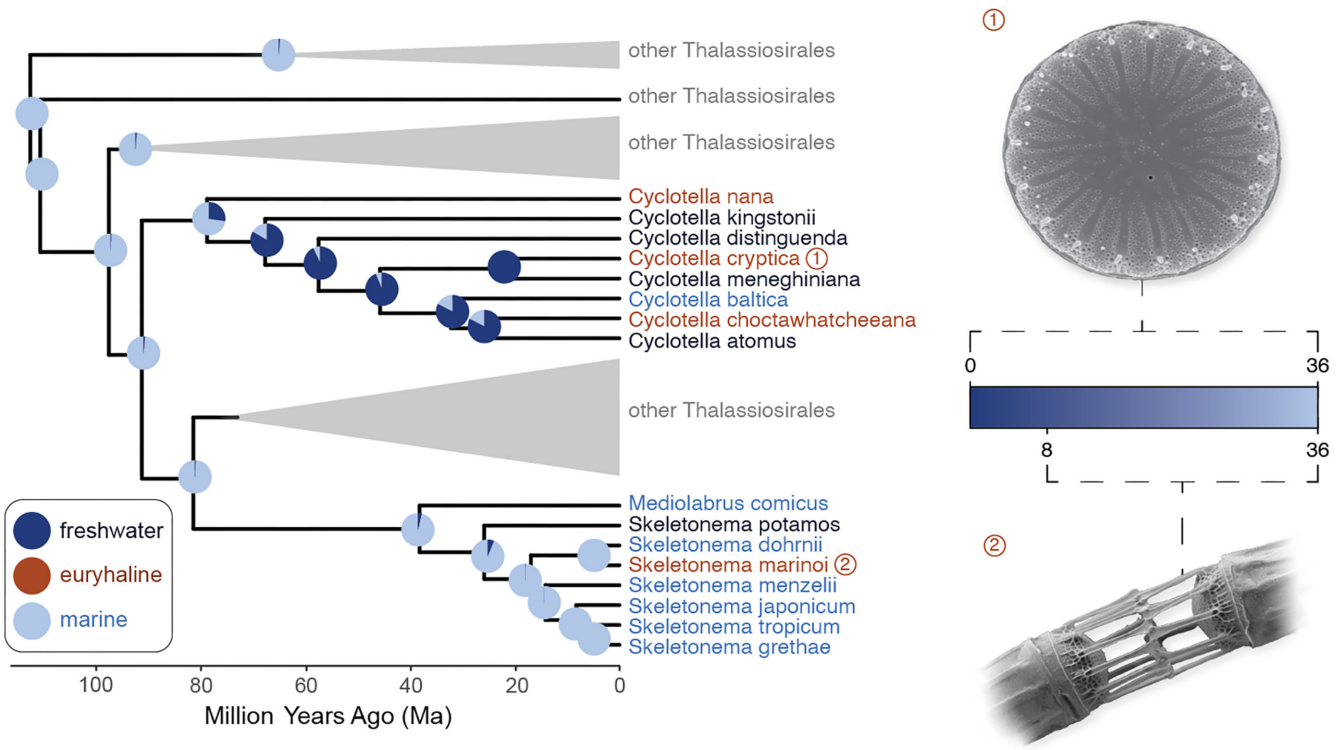


FIGURE 1 | The distinct evolutionary trajectories of two euryhaline diatoms, *Cyclotella cryptica* (pictured on top) and *Skeletonema marinoi* (bottom), following their split from a marine ancestor. *Cyclotella cryptica* is embedded inside a clade with deep freshwater ancestry and several marine–freshwater transitions. *Skeletonema marinoi* has deeper marine ancestry with fewer subsequent transitions. In the right panel, the approximate salinity tolerance of the two focal species is shown by the gradient ranging from freshwater (dark blue) to marine (light blue). On the phylogeny, pie charts represent the probability of ancestral habitat reconstructions (marine or freshwater). Taxon labels are coloured to distinguish freshwater (dark blue), marine (light blue), and euryhaline (orange) species in the focal clades. Figure adapted from Roberts et al. (2023).

freshwater specialists, and salinity generalists (Figure 1). The divergent ecologies and phylogenetic histories of *S. marinoi* and *C. cryptica* combined to offer novel mechanistic insights into the mitigation of hyposaline stress in fluctuating environments and, more broadly, clues about the properties of successful freshwater colonists.

2 | Materials and Methods

2.1 | Sample Collection and Experimental Design

Skeletonema marinoi strain CCMP3694 was germinated from a resting cell collected in the North Sea near Gothenburg, Sweden (sampling year: 2014, germination year: 2017), and grown at 15°C and 21.5 μmol photons·m^{-2}·s^{-1} irradiance under a 16:8 light:dark cycle. Cells were maintained in artificial seawater with 24 g salt per litre (ASW 24), the native salinity of the strain.

Skeletonema marinoi cannot survive freshwaters, so hyposaline stress was induced by transferring cells from ASW 24 to ASW 8. Cells were inoculated into three 1 L flasks with ASW 24 prior to the experiment and growth was monitored with a Fluid Imaging Technologies Benchtop B3 Series FlowCAM particle counter. Upon reaching exponential growth—at which point the cell concentration across the three flasks was approximately 184,000 cells/mL—cells were enumerated with the FlowCam, concentrated by centrifugation (800 rcf, 3 min), and inoculated into 50 mL Falcon tubes containing ASW 24 (control) or ASW 8 (treatment), resulting in 3×10^6 cells/tube (40 mL). Cells were collected for RNA-seq at seven time points: 0 min in ASW 24 (control), and at 0 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 8 h in ASW 8 (treatment). The control and 0 min treatment were collected immediately after inoculation at the start of the experiment. The remaining tubes for later time points were held at 15°C under constant illumination (20 μmol photons·m^{-2}·s^{-1}) and gentle agitation with a Boekel Scientific wave rocker until collection at their respective time points. At each time point, cell pellets were concentrated by light centrifugation (400 rcf for 3 min), flash-frozen in liquid nitrogen, and stored at −80°C. The experiment was performed in biological triplicate across each time series, with each biological replicate performed on separate days to capture day-to-day variation, resulting in a total of 21 RNA-seq samples. Finally, we monitored cellular growth with the FlowCAM in ASW 24 (control) and AWS 8 (treatment) in an additional parallel set of three biological replicates (not collected for RNA-seq), at each of the time points targeted by transcriptomics as well as at 10, 12, 24, 36, 48 h and 7 days posttransfer from ASW 24 to ASW 8.

2.2 | Sequencing and Read Processing

We extracted RNA and prepared Illumina libraries in four batches. To minimise batch effects, samples were randomised using the 'sample' function in R v4.05 (R Core Team, 2020) prior to both RNA extraction and library construction. RNA was extracted with a QIAGEN RNeasy Plant Mini Kit and sequencing libraries were prepared with a KAPA mRNA HyperPrep kit. Indexed libraries were multiplexed and sequenced on a single Illumina HiSeq 4000 lane (paired-end, 100 bp).

A total of 696,475,182 reads were sequenced. Reads were trimmed with kTrim v1.1.0 (parameters: -t 15 -m 0.5) (Sun 2020) and mapped to the *S. marinoi* reference genome v1.1 using STAR v2.7.3a (Dobin and Gingeras 2015) with default settings and intron sizes '--alignIntronMin 4' and '--alignIntronMax 17105'. Read counts were estimated with HTSeq v0.11.3 in *union* mode (Anders, Pyl, and Huber 2015). Gene annotations and protein localisation predictions were obtained from Pinseel et al. (2022).

2.3 | Differential Expression Analysis

We analysed transcript counts in R v4.0.2. Only genes with at least 1 count per million (CPM) in > 3 samples were retained. We used edgeR v3.30.3 to adjust for variation in library size and composition using the trimmed mean of M-values (TMM) method and to fit a quasi-negative binomial general linear model (GLM) for each gene (Lund et al. 2012; Robinson, McCarthy, and Smyth 2010). We used stageR v1.10.0 (Van den Berge et al. 2017) to identify differentially expressed genes in each contrast between an experimental timepoint and time 0 ($t = 0$ min) at ASW 8 with a false discovery rate (FDR) of 1% (Heller et al. 2009; Van den Berge et al. 2017). Genome-wide differences in gene expression among time points were visualised with multidimensional scaling using limma v3.44.3 (Ritchie et al. 2015), based on the top 500 genes with the greatest log2-fold changes between each pair of samples. Cluster v3.0 and Java Treeview were used to sort genes with similar expression patterns into seven manually delimited clusters (de Hoon et al. 2004; Saldanha 2004). We performed Gene Ontology (GO) term enrichment in topGO v2.40.0 (Alexa and Rahnenführer 2009) using the *elim* algorithm and Fisher's exact test to identify functional similarities within clusters and time points. All GO terms identified in the genome of *S. marinoi* were used as the background set. GO terms with $p < 0.05$ were considered significant, and redundant GO terms were removed using REVIGO (accessed on 22 November 2021) with a similarity cutoff of 0.5 and the SimRel score as similarity measure (Supek et al. 2011).

2.4 | Comparison of the Hypo-Salinity Stress Responses of Two Euryhaline Diatoms

A major goal of this study was to characterise conserved and divergent features of the short-term response to hyposaline stress in diatoms. To this end, we compared the responses of two diatoms, *S. marinoi* (this study) and *C. cryptica* (Downey et al. 2023) (Figure 1). The experiments were carried out simultaneously in the same lab at the same temperature and light intensity, using the same cell concentrations and materials (e.g., falcon tubes), and using the same bioinformatic workflow, allowing for direct comparisons. The major difference between the two experiments was the magnitude of the hypo-salinity shock: *S. marinoi* has a lower salinity tolerance than *C. cryptica* (Figure 1). *Skeletonema marinioi* has a lower boundary of 2.5 g salt per litre, though many strains do not survive below 5–8 salinity, whereas *C. cryptica* has a lower boundary of 0 (Balzano, Sarno, and Kooistra 2010; Liu and Hellebust 1976; Reimann, Lewin, and Guillard 1963). As a result, *S. marinoi* was transferred from ASW 24 to ASW 8 and *C. cryptica* was transferred from ASW 24 to ASW 0. This choice of different salinity treatments was deliberate, as our goal was to compare

the stress response mounted by the respective strains while exposed to their growth limits, so that the magnitude of the stress experienced by the two diatoms would be comparable. If we had exposed the two species to the same salinity treatment (ASW 8), *C. cryptica* would have been much less stressed than *S. marinoi*, making interpretations about the magnitude of expression differences more difficult.

To compare the responses of the two species, we clustered predicted proteins from the genomes of *S. marinoi* and *C. cryptica* with OrthoFinder v.2.2.6 into orthogroups (Emms and Kelly 2019). Orthogroups can contain a mix of orthologues and paralogs, collectively referred to here as homologues. Differences in expression levels between shared homologues were assessed using two-sided, two-sample Wilcoxon tests in base R's *wilcox. test()* function with a significance cutoff of 0.05. We first tested for differences in raw expression levels using an unpaired Wilcoxon rank sum test, evaluating the distribution of LFC values for all significantly expressed homologues by rank, rather than absolute expression, to control for differences in stress treatments between species. Second, assuming the overall physiological response is determined by cumulative expression of all homologues at a given time point, a second test used the summed expression values of all members of an orthogroup in each species, resulting in one expression value per orthogroup per species at each time point, which were compared with a Wilcoxon signed rank test (paired data).

3 | Results and Discussion

3.1 | Hyposalinity Stress Induces Substantial Remodelling of the Transcriptome

We exposed *S. marinoi* to hyposalinity stress by transferring cells from their native salinity at 24 g salt per litre (ASW 24) to low salinity (ASW 8), and sequenced the transcriptome at seven timepoints in the minutes and hours following the transfer. Both the control and stressed cells experienced an initial lag within the first 2 h with little or no growth in the control and some mortality in the treatment (Figure S1). The control increased growth from 2 h onwards, whereas stressed cells resumed growth at 4 h, albeit at a lower rate than the control (Figure S1). By 7 days, the control and treatment cultures had similar chlorophyll *a* fluorescence, suggesting the cultures had reached the same biomass. Alternatively, differences in chlorophyll *a* biosynthesis between treatments could have masked potential differences in biomass that persisted between the stressed and unstressed cultures (Pinseel et al. 2022).

Hyposalinity stress caused profound remodelling of the transcriptome. Of the 22,440 genes in the genome of *S. marinoi*, 14,860 were differentially expressed in at least one time point. The peak response, measured by the number of differentially expressed genes, occurred 2 h following stress exposure (8086 genes) (Figure 2a), coinciding with resumption of growth (Figure S1). The largest number of expression changes occurred 1–4 h post-treatment (Figure 2a). This was supported by multidimensional scaling of the top 500 differentially expressed genes, where the 1–4 h time points were most distant from the control (Figure 2b). Notably, this time window corresponded with the initial decrease in biomass (1–2 h) and resumption of growth (2–4 h) (Figure S1). In contrast, the fewest differentially expressed genes were measured at 8 h, which was most closely aligned to the control in ordination space (Figure 2b), indicating that expression profiles at the beginning and end of the experiment were more similar to each other than to intermediate time points (Figure 2b). By 8 h *S. marinoi* had fully resumed growth and gene expression was returning to baseline levels (Figures 2a and S1). Note that the statistical agreement among technical replicates at each time point was high, as evidenced by both the MDS plot (Figure 2b), and high R^2 values (> 0.9) obtained from a linear regression on the logCPM values among the three replicates at each time point (see Support information for a full overview).

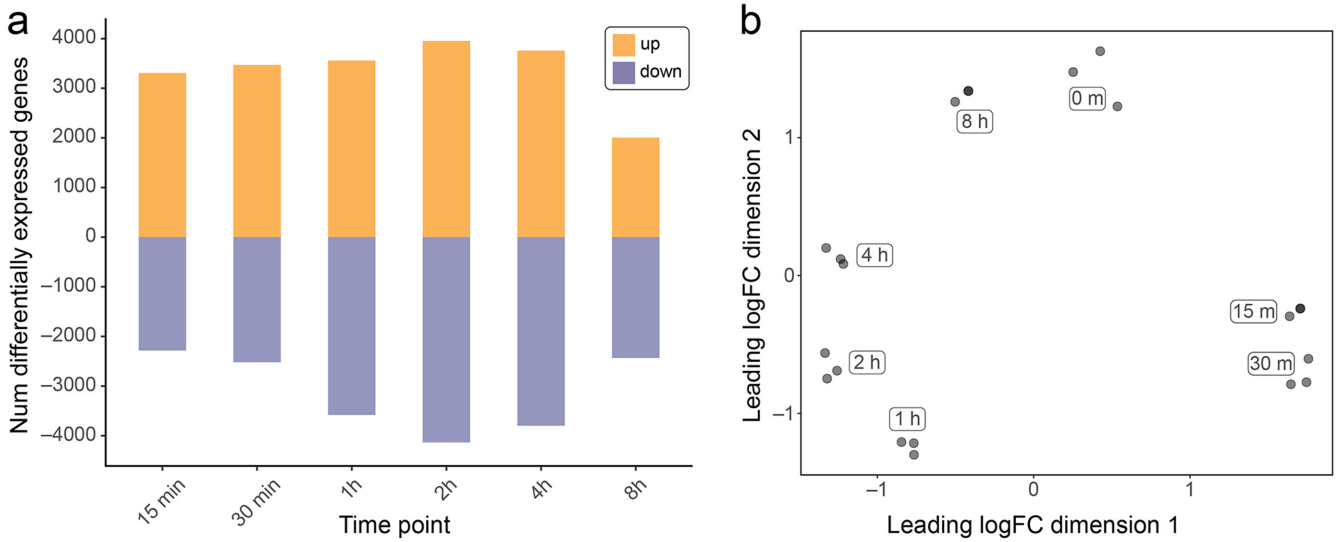


FIGURE 2 | Hyposaline stress remodels the transcriptome of *Skeletonema marinoi*. (a) Number of differentially expressed genes at each time point following exposure to low salinity. The number of significantly upregulated genes is shown in orange, and significantly downregulated genes are shown in purple. (b) Multidimensional scaling plot, showing distinct patterns of gene expression at each time point, based on $\log_2$-fold changes (logFC) in the top 500 differentially expressed genes.

Genes most critical to the stress response should have sustained patterns of differential expression across time (Downey et al. 2023), so we focused on the 10,050 genes differentially expressed at two or more consecutive time points. Hierarchical clustering of these genes revealed seven clusters defined by their distinct temporal dynamics of gene expression (Figure 3). Six of the seven groups showed the largest magnitude of expression responses in the 1–4 h time period, confirming the peak stress response identified by the total number of differentially expressed genes (Figure 3b).

3.2 | Distinct Temporal Dynamics in Expression Patterns During Hyposalinity Stress

The abrupt shift to low salinity induced an immediate transcriptomic response in *S. marinoi* (Figure 3). Within the first hours of hyposaline stress, *S. marinoi* maintained homeostasis through a multiphased stress response, culminating in the onset of acclimation at approximately 8 h (Figure 3). Below, we highlight distinct phases of this response.

3.2.1 | Sustained Increase in Protein Biosynthesis

Skeletonema marinoi increased protein expression throughout the 8 h, reflecting altered metabolism and/or replenishment of stress-damaged proteins. Specifically, most tRNA synthetase genes were upregulated across the time series (Figure S2, $p \approx 0$, Fisher's exact test), and 86 ribosomal proteins were upregulated at all time points ($p \approx 0$, Fisher's exact test). Genes involved in nitrogen metabolism were also mostly upregulated throughout the experiment (Figure S3), including ones involved in the transport and assimilation of ammonium, urea, and nitrate/nitrite. This pattern, alongside upregulation of phosphate transporters in early time points, is consistent with elevated nutrient demands associated with increased protein biosynthesis.

3.2.2 | Stress Mitigation Phase (15–30 Min)

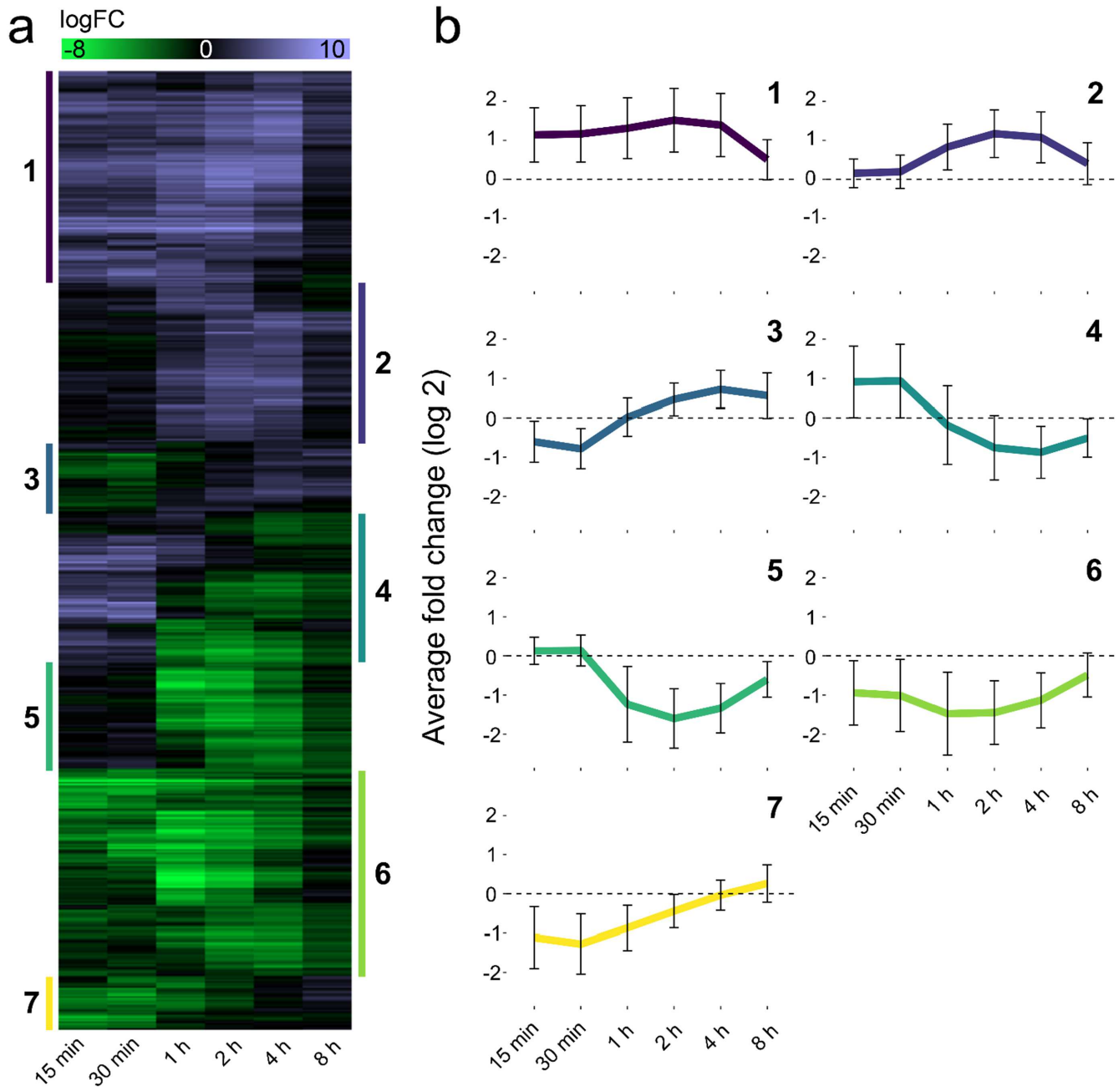
During the first 30 min of hyposalinity stress, *S. marinoi* experienced a reduced growth rate and a marked shift in energy metabolism. Growth halted immediately (Figure S1), which was reflected in the downregulation of DNA replication/recombination. Downregulation of chlorophyll biosynthesis, light-harvesting proteins, Calvin cycle genes, gluconeogenesis, and storage molecule biosynthesis (fatty acids and chrysolaminarin) suggests overall downregulation of photosynthesis and energy storage immediately upon stress exposure (Figure 3b,c, Figures S4–S7). Instead, *S. marinoi* upregulated chrysolaminarin degradation, the last irreversible step of glycolysis (pyruvate kinase), and the TCA cycle (Figure 3b,c, Figure S4). Together, these patterns suggest that stress mitigation hinges on a metabolic shift characterised by rapid energy production through utilisation of storage molecules and metabolic intermediates from glycolysis and the TCA cycle.

Transcriptional changes indicated that *S. marinoi* experienced acute stress during the first 30 min. This was evidenced by upregulation of: (i) heat shock proteins (Figure S8), which are molecular chaperones that direct damaged or misfolded proteins to proteases (Guo et al. 2016; Mogk, Huber, and Bukau 2011); (ii) genes involved in mitotic DNA damage and integrity checkpoint signalling, which prevents mitosis in the presence of DNA damage (Rhind and Russell 1998); and (iii) serine-type endopeptidases, which are likely involved in the degradation of stress-damaged proteins (Park and Kwak 2020). Many heat shock proteins were subsequently downregulated after 1 h, suggesting that the acute stress was largely mitigated by that point (Figure S8). During the stress mitigation phase, *S. marinoi* also induced genes that mitigate the effects of reactive oxygen species (ROS), which has been found in other algae (Downs et al. 2009; Rugiu et al. 2020). ROS-mitigating processes included upregulation of: (i) violaxanthin de-epoxidase, involved in the energy-dissipating xanthophyll cycle (Goss and Jakob 2010); (ii) biosynthesis of biliverdin, a scavenger of oxygen radicals (Stocker et al. 1990); (iii) biosynthesis of polyamines that function in ROS management (J.-H. Liu et al. 2015) and osmotic balance (Chen et al. 2018); and (iv) ROS scavengers, including superoxide dismutase, which is a first line of defence against ROS in plants and algae (Alscher, Erturk, and Heath 2002; Kumar et al. 2010) (Figures S7, S9, S10).

At the onset of stress, *S. marinoi* upregulated key pathways involved in osmotic stress mitigation (Figure S10). Osmolytes are low-molecular-weight molecules whose intracellular concentrations maintain osmotic balance (Jackson, Ayer, and Laycock 1992; Kageyama, Tanaka, and Takabe 2018; Lyon et al. 2016). During hyposaline stress, we expect decreased expression of osmolyte genes. Indeed, several osmolyte biosynthesis genes were downregulated and osmolyte degradation genes were upregulated (e.g., taurine dioxygenase) in early time points or, in some cases, across all time points (Figure S10). Many of the strongest expression responses occurred during the stress mitigation phase, including methyltransferases involved in dimethylsulfoniopropionate (DMSP) and glycine betaine biosynthesis (Figure S10). Although proline is a well-characterised osmolyte in diatoms, expression patterns of genes involved in proline metabolism were inconsistent (Figure S10), suggesting proline might not be a universal osmolyte in diatoms (Kamakura, Bilcke, and Sato 2024). Diatoms also maintain osmotic balance by regulating intracellular ion concentrations. Here, 11 of the 26 differentially expressed Na^+ and K^+ transporters were part of clusters upregulated at 15–30 min (Figure S11). Remaining ion transporters were confined to clusters downregulated across the entire time series (Figure S11). In addition, five amino acid ABC transporters were upregulated during the stress mitigation phase only (Figure S12), consistent with removal of osmolyte-functioning amino acids from the cytosol early on (Jackson, Ayer, and Laycock 1992; Scholz and Liebezeit 2012).

3.2.3 | Transition (1 h) and Recovery (2–4 h) Phases

Acute stress mitigation gradually transitioned to recovery, as expression levels at 1 h showed patterns that were a mix between the preceding mitigation and later recovery phases. The latter represents the peak response, as it shows the largest number



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Cluster	# Genes	Enriched process	Associated GO terms
1	2224	translation, TCA cycle	**GO:0006364**, **GO:0006099**, **GO:0006414**
2	1778	redox homeostasis, protein folding	**GO:0045454**, *GO:0006457*
3	614	photosynthesis, DNA replication, protein catabolism	**GO:0009765**, *GO:0031297*, *GO:0006275*, **GO:0006511**
4	1643	transcription factor activity, protein catabolism	**GO:0003700**, **GO:0004252**
5	1057	protein catabolism	**GO:0016567**, **GO:0004252**
6	2174	oxidoreductase activity, proteolysis inhibition	**GO:0016491**, **GO:0004867**
7	560	photosynthesis, recombination, protein catabolism, storage molecule biosynthesis	**GO:0009765**, **GO:0006310**, **GO:0036402**, **GO:0003843**, **GO:0003989**

FIGURE 3 | Legend on next page.

FIGURE 3 | Differentially expressed genes in *Skeletonema marinoi* exhibit distinct temporal dynamics during the hyposalinity stress response. (a) Heatmap of 10,050 genes (*Y* axis) differentially expressed in at least two consecutive time points (*X* axis), sorted by similarity in gene expression across time points. Genes were classified into seven clusters based on shared patterns of gene expression. (b) Average expression of genes assigned to each cluster, corresponding to the cluster numbers on the sides of the heatmap in panel *a*. Error bars indicate ± one standard deviation. (c) Number of genes, enriched biological processes, and specific GO terms for each cluster. GO terms in bold and italic font indicate enrichment at p value <0.001 and <0.01, respectively. The full list of significant GO terms is available as Support information online.

of differentially expressed genes and coincides with upregulation of cell cycle genes and resumption of growth (Figures 2a and 3b). Although many genes involved in osmotic and oxidative stress were no longer differentially expressed during recovery, several transporter and osmolyte genes continued to be differentially expressed (Figures S10–S12). Similarly, many cell-compartment-associated peroxiredoxins and thioredoxins remained upregulated, often at larger magnitudes, at 2–4 h. Most cellular processes that were predominantly upregulated throughout the experiment reached peak upregulation during the recovery phase, most notably protein biosynthesis, chrysolaminarin degradation, and the TCA cycle (Figures S2, S4). In parallel, key irreversible steps of glycolysis were upregulated by 2 h (Figure S4), indicating that storage molecules continued to provide energy during the recovery phase. Genes involved in proteasome activity also became upregulated at 4 h, consistent with clearing of damaged or unnecessary proteins (Figure S13). Altogether, *S. marinoi* carefully balanced stress mitigation and energy production during the recovery phase.

3.2.4 | Pre-Acclimation Phase (8 h)

By 8 h, growth had resumed, several cellular processes that were initially downregulated became upregulated, and the overall magnitude of the gene expression responses declined (Figures 2 and 3b), indicating that *S. marinoi* had begun to acclimate to low salinity (Borowitzka 2018). This is confirmed by the large overlap in differentially expressed genes and pathways between the 8 h time point and a previous study of *S. marinoi* acclimated to ASW 8 for 2 weeks (Pinseel et al. 2022) (Figure 4). Acclimated and pre-acclimated cells increased storage molecule biosynthesis, suggesting *S. marinoi* had fully catabolised storage molecules for energy during the first hours of the response and were replenishing their stocks (Figure 4, Figure S4). Similarly, although the TCA cycle was upregulated during our experiment, downregulation of the *bZIP14* transcription factor, which regulates the TCA cycle in diatoms (Matthijs et al. 2017), from the recovery phase onward suggests the TCA cycle was becoming increasingly downregulated at 8 h and was, in fact, fully downregulated in acclimated cells at 2 weeks (Figures 3 and 4, Figure S4). This suggests that the TCA cycle plays an important role in supplying energy to the cell during acute stress but not acclimation.

Notably, several processes showed similar patterns during the early phases of the stress response and at 2 weeks of acclimation, but without differential expression, or expression in opposite directions, during the pre-acclimation phase (Figure 4). These include the Calvin cycle, transport and assimilation of nitrate/nitrite, the ornithine–urea cycle, genes involved in the biosynthesis of osmolytes and polyamines, ion transporters, proteasome genes, thioredoxins and violaxanthin de-epoxidase (Figure 4, Figures S3, S6, and S9–S13). ROS management strategies were generally downregulated or not differentially expressed during the pre-acclimation phase (except superoxide dismutase) (Figure S9), but became upregulated at 2 weeks of acclimation, whereas the proteasome showed an opposite trend. This suggests that *S. marinoi* reached a new equilibrium for ROS management after full acclimation, namely one that prevented damage to cellular components and allowed for downregulation of proteasome activity. Overall, the contrasting expression patterns between the early time points in our experiment and long-term acclimation on the one hand, and the pre-acclimation phase on the other hand, suggest that the latter represents an 'overcorrection' for some pathways, as has been observed in macrobiota (Stebbing 1981). This might be caused by surplus metabolites, synthesised during the recovery phase, which triggers transient downregulation of their corresponding pathways by feedback inhibition until acclimated cells reach a new equilibrium. Similar feedback mechanisms have been observed for nitrogen metabolism and polyamine biosynthesis in bacteria and algae (Panagiotidis, Huang, and Canellakis 1994; Sanz-Luque et al. 2015).

3.3 | Conserved and Diverged Responses to Hyposaline Stress in Two Euryhaline Diatoms

We compared the response to acute hyposaline stress by *S. marinoi* to another diatom, *C. cryptica* (Figure 1), to reveal patterns of conservation and divergence (Downey et al. 2023). Both experiments were completed simultaneously and used the same design, with the most notable difference being the magnitude of the hyposalinity exposure, which amounted to brackish conditions (ASW 8) for *S. marinoi* and freshwater (ASW 0) for *C. cryptica*. The two species differ in their sensitivity to low salinity (Figure 1), so these salinities were chosen to induce comparable levels of stress at the growth limits of the two strains. Both species underwent substantial transcriptional remodelling in the minutes and hours following hyposaline stress but eventually approached gene expression levels similar to those of the control in ASW 24. Taken together, this suggests that the two species experienced a comparable degree of stress despite differences in the salinity exposure. This is consistent with this strain of *S. marinoi* being a less robust euryhaline diatom, as it does not survive below approximately salinity 4.

3.3.1 | Conserved Features of the Hyposaline Stress Response

Several similarities in the transcriptional responses of the two species identify the conserved features of the response to hyposaline stress by diatoms. Both species experienced an immediate arrest in growth and widespread up- or downregulation of signal transduction kinases, including (i) histidine kinases, which are responsible for signal transduction across cell membranes and facilitate stress

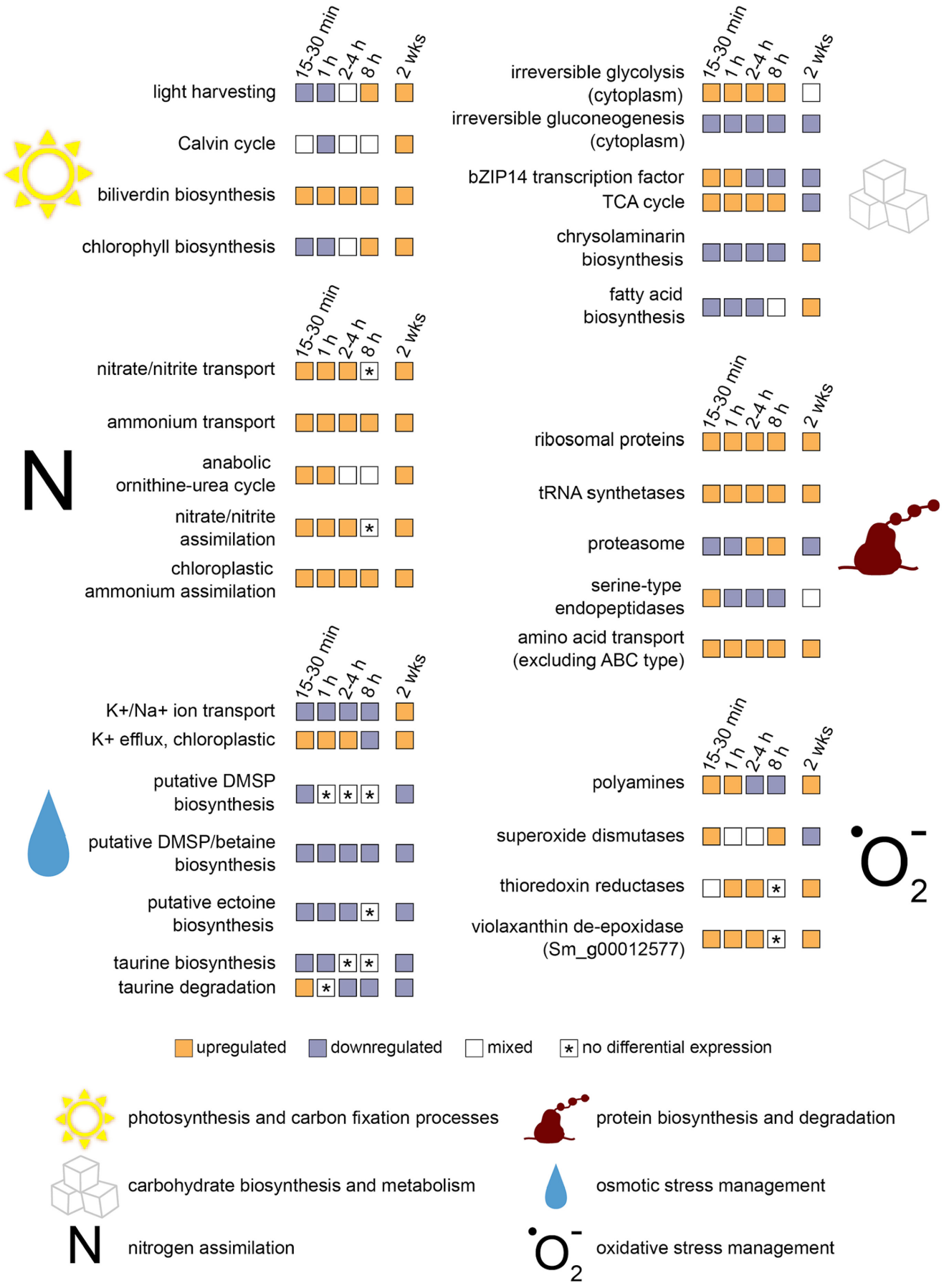


FIGURE 4 | Legend on next page.

FIGURE 4 | The far-reaching response to low salinity in *Skeletonema marinoi*. Differentially expressed genes are involved in diverse cellular processes during acute stress (15 min to 8 h) and acclimation (2 weeks). Coloured tiles represent four phases of the response: Stress mitigation (15–30 min), transition (1 h), recovery (2–4 h), pre-acclimation (8 h) and acclimation (2 weeks). Vertical spacing of tile rows indicates associations between processes (e.g., the *bZIP14* transcription factor regulates the TCA cycle). Tile colour was determined based on the proportion of genes significantly up- or downregulated for that process. Purple: > 60% downregulated genes, Orange: > 60% upregulated genes, White: 40%–60% up- and downregulated genes, Asterisks: No differentially expressed genes for that process.

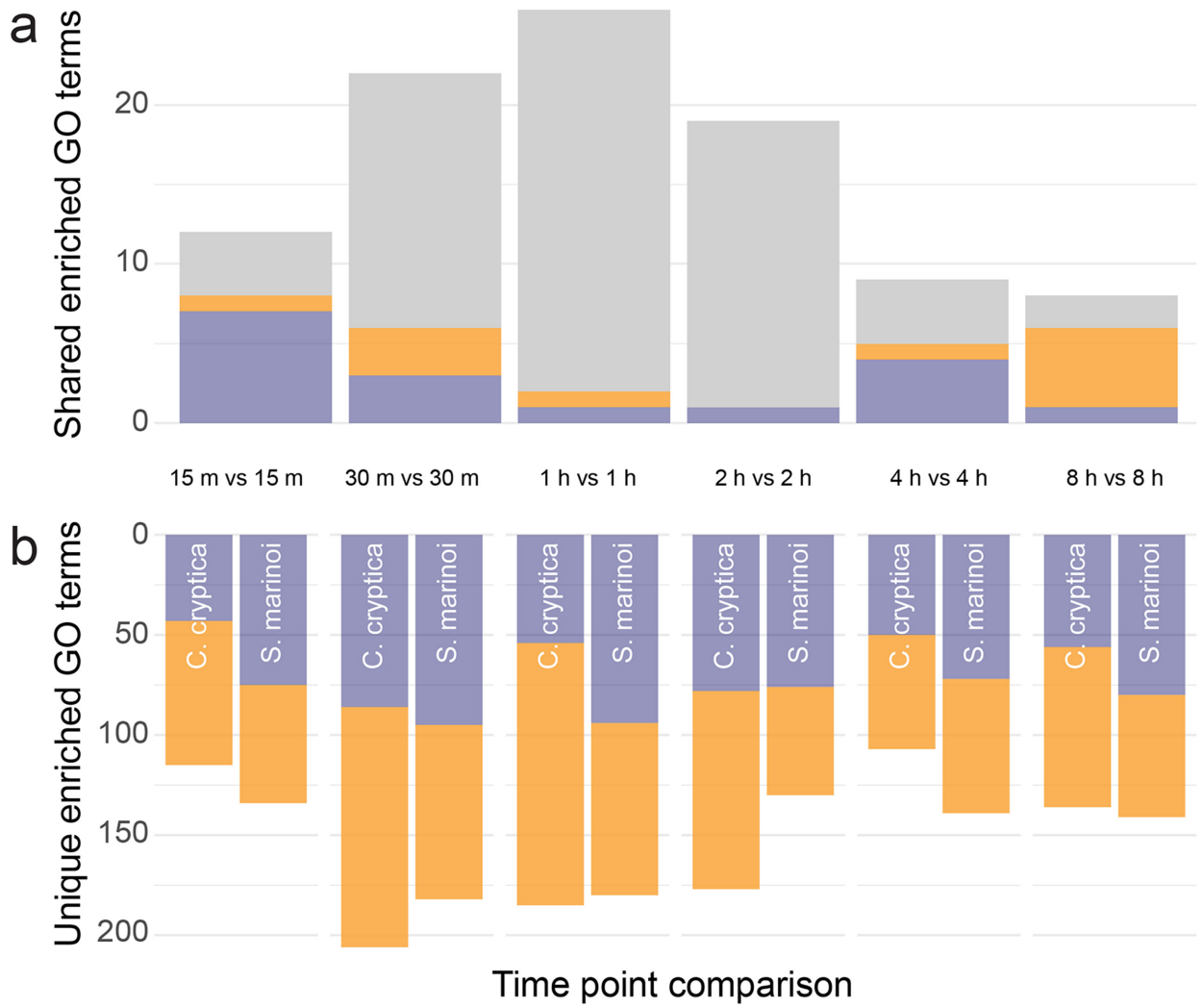
adaptation across the tree of life (Kabbara et al. 2019); (ii) serine/threonine protein kinases, which are involved in stress responses in diatoms (Chen et al. 2014; Pelusi et al. 2023); and (iii) cGMP-dependent protein kinases, which are important for salt stress responses in vascular plants (Shen et al. 2019). Notably, these signal transduction kinases were targets of positive selection associated with adaptation of *S. marinoi* to the Baltic Sea environmental gradients, including low salinity (Pinseel et al. 2023). Conserved features of the early phases of the stress response (15 min–30 min) include (i) oxidative stress management with ROS scavengers and polyamines, (ii) upregulation of heat shock proteins and associated transcription factors, and (iii) downregulation of cell cycle genes and histones. In this early phase, both species also upregulated (i) plastid K^+-efflux antiporters to counter osmotic pressure in the chloroplasts, (ii) phosphate and molybdate transporters, presumably to meet increased demands for resources allocated to damage repair and growth resumption, and (iii) a chitinase. *Cyclotella cryptica* forms B-chitin threads, which might play a role in buoyancy adjustments under acute hyposalinity stress (Downey et al. 2023). However, given that *S. marinoi* does not form such chitin structures, a general chitin response in both species suggests a broader role of chitin in hyposalinity stress mitigation, perhaps involving cell wall remodeling (Davis, Hildebrand, and Palenik 2005; Downey et al. 2023; Durkin, Mock, and Armbrust 2009). Many of the aforementioned processes were still upregulated at 2 h. However, both species now also upregulated cell cycle genes, indicative of resumed growth, whereas most heat shock proteins and associated transcription factors became downregulated. This pattern continued at 4 h, in addition to substantial upregulation of genes involved in protein translation. Finally, at 8 h, both species continued to upregulate translational activity and superoxide dismutase.

3.3.2 | The Swift, Efficient and Orchestrated Response to Hyposaline Stress in a Diatom With Freshwater Ancestry

Despite many similarities, several important differences in the responses to hyposalinity stress between the two species highlighted key features that impart greater overall salinity tolerance in *C. cryptica*. Although *S. marinoi* experienced a milder salinity shift than *C. cryptica*, *S. marinoi* nevertheless had to mount a much stronger response in terms of both the number and magnitude of differentially expressed genes. Specifically, *S. marinoi* differentially expressed a larger fraction of its genes (14,860 genes; 66%) during the time series than *C. cryptica* (10,566; 50%), including at each individual time point. Few enriched GO terms were shared between species at the same time points, and many that were shared were expressed in opposite directions, pointing to fundamentally different responses in the two species (Figure 5). Notably, the majority of these opposite expression patterns were confined to 30–120 min, whereas shared GO terms at 4–8 h tended to be expressed in the same direction in both species, suggesting the responses of both species most strongly diverged during the initial period of acute stress. Considering genes differentially expressed in both species, the magnitude of the response, expressed as logFC, was significantly greater in *S. marinoi* at nearly all time points (Figure 6, Figure S14). Finally, the multiphased response of *S. marinoi*, spread over 8 h, was much quicker in *C. cryptica*, which mounted a stronger immediate response with peak gene expression during the initial stress mitigation phase (30–60 min) and gene expression returning to baseline levels by 4 h for many processes. By contrast, peak gene expression occurred during the recovery phase (2–4 h) in *S. marinoi*, suggesting *C. cryptica* directs the most effort towards immediate stress mitigation, whereas *S. marinoi* invests more of its energy in stress recovery.

Major differences between species fell into two main categories: (i) activity of osmolytes and ion channels, and (ii) management of oxidative stress, together showing that the two species have different strategies in managing the transition to low salinity. *Cyclotella cryptica* restores osmotic balance by transporting K^+ into the cytosol, and Na^+ and H^+ out of the cytosol, whereas osmolytes play only a minor role (e.g., biosynthesis of DMSP and glycine-betaine were not differentially expressed). *Cyclotella cryptica* upregulates 32 K^+ or Na^+ transporters across multiple time points, and regulates an additional 66 K^+ or Na^+ transporters at single time points with roughly equal distribution of up- and downregulation across transporters, consistent with subfunctionalisation of paralogs. *Skeletonema marinoi*, by contrast, regulates osmotic stress by reducing osmolyte levels and much less so through ion transport. In *S. marinoi* fewer K^+ and Na^+ transporters were differentially expressed at consecutive time points ($n = 26$, out of a total of 75), and most of these ($n = 17$) were primarily downregulated (Figure S11). Moreover, only 11 ion transporters were differentially expressed at single time points, with near equal distribution of up- and downregulation. Strong upregulation of amino acid transporters in *S. marinoi*, including five ABC transporter-binding proteins at 15–30 min, further suggests a dominant role for osmolytes in osmoregulation by *S. marinoi*, as we expect these transporters to actively expel excess osmolytes from the cell. *Cyclotella cryptica* either downregulated or did not differentially express most of its amino acid transporters throughout the time series.

The canonical response to environmental stress in many organisms involves transient repression of ribosome biogenesis and upregulation of stress defence genes (Brostrom and Brostrom 1997; Gasch et al. 2000). This was also observed in the response to hyposaline stress by *C. cryptica* (Downey et al. 2023), but *S. marinoi* instead upregulated both translation and protein degradation across the entire time series. Thus, *C. cryptica* shows a canonical response to freshwater stress where the reduction in translation coincides with growth arrest. In *S. marinoi*, the non-canonical response of increased translation and protein



c

Time point	Direction		Notable terms
	S. marinoi	C. cryptica	
15 min	down	down	ion binding, spermidine biosynthetic process
1 h	up	down	structural constituent of ribosome, RNA binding
1 h	down	up	photosynthesis, light harvesting, oxidoreductase activity
8 h	up	up	rRNA processing, ribosome

FIGURE 5 | The distinct responses of two salinity generalists, *Skeletonema marinoi* and *Cyclotella cryptica*, to hyposaline stress. (a) Number of enriched GO terms ($p < 0.05$) shared between the two species in the minutes and hours following exposure to hyposaline stress. (b). Number of enriched GO terms ($p < 0.05$) unique to each species. (c) Shared enriched GO terms with the greatest number of shared GO terms. A full list of enriched GO terms by category is available as Support information online.

degradation throughout the experiment may reflect a sustained need to degrade and then replenish ROS-damaged proteins. This suggests that protein damage was more extensive and prolonged in *S. marinoi* compared to *C. cryptica*. Furthermore, most light-harvesting proteins were upregulated in *C. cryptica*, whereas more than half were downregulated in *S. marinoi* (Figure S5). One possibility is that indirect quenching of over-excited chlorophyll prevented ROS generation in *C. cryptica* (Latowski, Kuczyńska, and Strzałka 2011), whereas *S. marinoi* limited ROS generation by decreasing photosynthesis altogether (Figure 3b,c), which is a strategy used by some plants (Dalal and Tripathy 2018). These results suggest that the oxidative stress induced by low salinity in *S. marinoi* may be too severe to be managed by increased photoprotection mechanisms alone.

The long-term acclimation strategies, here defined as having reached stable growth rates in exponential phase, also differed substantially between *S. marinoi* and *C. cryptica*. For *C. cryptica*, the acclimated state (at 4 months, approximately 96 generations) was highly distinct from the short-term stress response, as most genes and pathways differentially expressed during short-term stress were not so in acclimated cells, and for those genes that

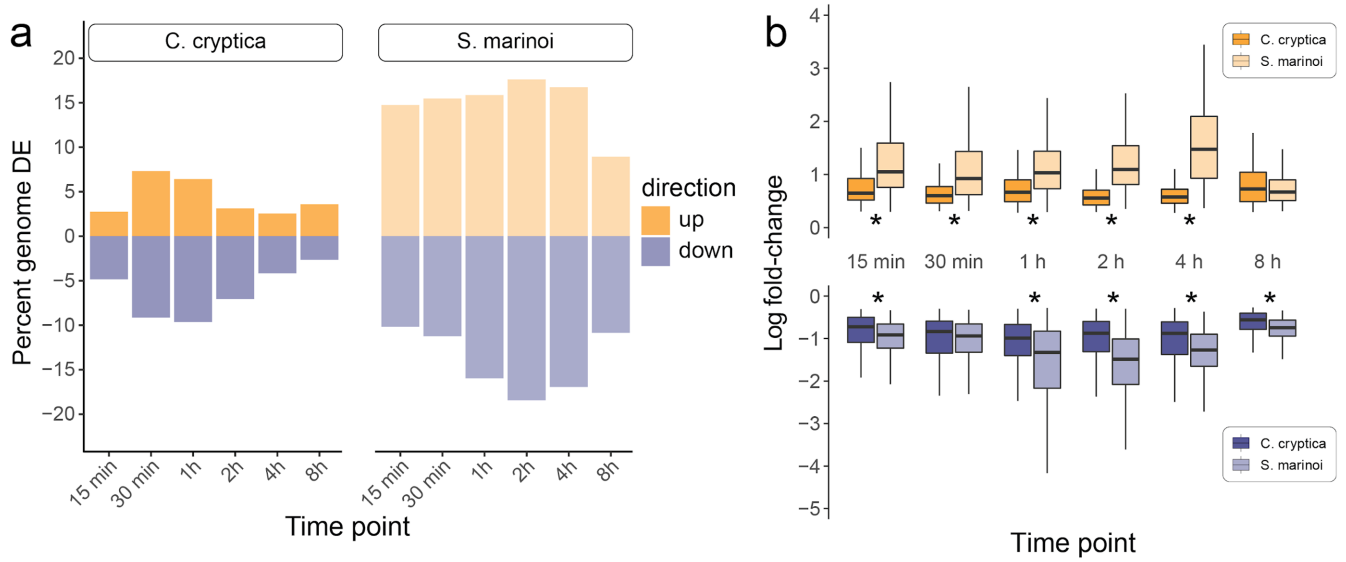


FIGURE 6 | Differences in the strength of the hyposalinity stress response in two diatoms. Despite milder stress exposure, *S. marinoi* expresses more genes (a) at greater magnitudes (b) than *C. cryptica*. Values in (b) show the distribution of $\log_2$-fold changes for shared homologues with significant differential expression in both species. Upregulated genes are on the top panel, downregulated on the bottom. If paralogs were present, their $\log_2$-fold changes were included individually. Stars indicate a significant difference ($p < 0.05$) in population mean ranks by a two-sided, two-sample Wilcoxon rank sum test. Outliers not shown.

were, most were expressed in opposite directions in stressed versus acclimated cells (Downey et al. 2023). In *S. marinoi*, by contrast, many genes and processes differentially expressed under short-term stress remained so in acclimated cells (at 2 weeks, approximately 7–14 generations) (Figure 4). This indicates that acclimated *C. cryptica* reaches a new homeostasis with limited energy requirements, whereas *S. marinoi* mounts a prolonged stress response to continue growth in hyposaline conditions. It is worth noting here that the length of acclimation differed between the two species, so we cannot rule out that some of the differences reflect further changes in the acclimated state between 2 weeks (*S. marinoi*) and 4 months (*C. cryptica*). For instance, 4 months could have provided enough time for *C. cryptica* to begin adapting to low salinity, where changes in the genotype partially underlie its response. Rapid adaptation in diatom cultures has been suggested to occur through mitotic recombination (Bulankova et al. 2021; Schaum et al. 2018).

3.4 | Hyposalinity Stress in Diatoms and Other Eukaryotes

To the best of our knowledge, no other studies have characterised hyposalinity stress responses at the temporal resolution of our study. This complicates comparison with research performed on other taxa, as it is now clear that sampling time and frequency matters greatly when characterising the cellular machinery deployed during hyposalinity stress (Downey et al. 2023). Nevertheless, some general strategies have emerged. First, organisms as diverse as green algae (Komsic-Buchmann, Wöstehoff, and Becker 2014), ciliates (Ishida et al. 1993) and parasitic protists (Rohloff and Docampo 2008), use contractile vacuoles for osmoregulation, though such vacuoles are generally absent from diatoms (Hausmann and Patterson 1984). Indeed, our data, as well as previous work (Downey et al. 2023; Pinseel et al. 2022), have found no apparent role for aquaporins in osmoregulation. Instead, it appears that diatoms rely more on ion channels and osmolyte biosynthesis for osmoregulation (Downey et al. 2023; Kageyama et al. 2018; Lyon et al. 2016; Nakov et al. 2020; Pinseel et al. 2022). Yet, the relative importance of these mechanisms differs among taxa and likely contributes to different levels of osmotic tolerance, as was apparent from the divergent osmoregulatory strategies deployed by *S. marinoi* and *C. cryptica*. In addition, ROS-management has emerged as a universal strategy to overcome salinity stress. Specifically, photoprotection provided by the xanthophyll cycle helps protect the photosynthetic machinery during both hypo- and hypersalinity stress in vascular plants (Latowski, Kuczyńska, and Strzałka 2011; Misra, Latowski, and Strzalka 2006; Qiu, Lu, and Lu 2003), green algae (Masojídek et al. 2000), brown algae (Xie et al. 2016) and diatoms (Downey et al. 2023; Pinseel et al. 2022). In vascular plants, polyamines have been found to play a critical role in maintaining cellular ROS homeostasis under salt stress through their dual roles as antioxidant and modulator for ion transport (Chen et al. 2018; Saha et al. 2015). Polyamines play a similarly important role in hyposalinity stress management in diatoms (Downey et al. 2023; Pinseel et al. 2022), further underscoring their fundamental role in both hypo- and hypersalinity stress mitigation. It is clear from our study that diatoms rely on several phylogenetically conserved strategies for mitigation of salinity stress, but the contrasting responses of *S. marinoi* and *C. cryptica* also revealed fundamental, lineage-specific differences as well. This underscores the importance of evolutionary history in shaping taxon-specific responses, and thus ultimately their distributions across ecosystems.

4 | Conclusions

Using time-resolved transcriptomics, we found fundamental differences in the acute short-term stress responses and long-term

acclimation strategies of two euryhaline diatoms exposed to low salinity. Despite considerably milder exposure, the weaker of the two generalists, *S. marinoi*, mounted a stronger and more prolonged response to hyposaline conditions than *C. cryptica*. In cases where successful stress management requires higher levels of gene expression, like in *S. marinoi*, its physiological limits and maximum energy demands will be reached at a lower dose of environmental stress, allowing for less tolerance to environmental extremes than species able to survive with a smaller and less energy-demanding response. This was evident in the divergent strategies for mitigating oxidative and osmotic stress, which offer important clues about the comparatively broader salinity tolerance of *C. cryptica*. The greater efficiency of ROS management in *C. cryptica* appears to have reduced the period of acute stress, allowing it to recover and resume growth sooner than *S. marinoi*. In addition, we hypothesise that the regulation of osmotic pressure through increased ion transport in *C. cryptica* is faster and more efficient than the osmolyte-dominated response of *S. marinoi*. Finally, the distinct gene expression profile of fully acclimated *C. cryptica* cells suggests it is better able to settle in comfortably to lower salinities than *S. marinoi*, where the expression profiles of acclimated cells more closely resemble stressed cells. Taken together, our data suggest that early and efficient responses to oxidative and osmotic stress, together with a tailored acclimation state, confer overall broader salinity tolerance.

Marine–freshwater transitions have occurred many times and in both directions throughout diatom evolution (Alverson, Jansen, and Theriot 2007; Nakov, Beaulieu, and Alverson 2019; Roberts et al. 2023), including in *Cyclotella*, which includes salinity generalists as well as marine and freshwater specialists (Figure 1). Ancestral state reconstructions highlight a deep freshwater colonisation event, followed by tens of millions of years of freshwater ancestry that subsequently gave rise to marine specialists and salinity generalists (Figure 1) (Alverson et al. 2011; Roberts et al. 2023). We hypothesise that these repeated transitions are examples of adaptive phenotypic plasticity (Ghalambor et al. 2007), in which the cellular mechanisms underlying the broad plasticity of *C. cryptica* are the same ones that allowed *Cyclotella* to become established in freshwaters originally. Long-term retention of key mechanisms—the ones distinguishing *C. cryptica* from *S. marinoi*—have allowed *Cyclotella* to subsequently, and repeatedly, go on to specialise in marine or freshwaters (Figure 1) through genetic assimilation (Ghalambor et al. 2007; Waddington 1942). The longer marine ancestry of *S. marinoi* suggests its strategies to manage hyposaline stress are more recently evolved, less refined, and less plastic. Our experimental strain of *S. marinoi* originated from the marine North Sea, but strains locally adapted to low-salinity reaches of the Baltic Sea (Sefbom et al. 2023; Sjöqvist et al. 2015) might be able to mount stress responses more resemblant of *C. cryptica*. Indeed, *S. marinoi* exhibits both genomic and transcriptional variation associated with the salinity gradient across the Baltic Sea (Pinseel et al. 2022, 2023).

These results suggest that similarities in environmental stress responses across species are likely limited to shared ancestral mechanisms constituting part of a core stress response, whereas lineage-specific aspects may better predict survival to environmental perturbations on short timescales, and successful colonisation of new habitats on longer timescales. These questions have taken on increased urgency in the context of climate change, where evolutionary history will play a role in determining which lineages survive and adapt to changing ocean conditions (Cavicchioli et al. 2019).

Author Contributions

K.J.J., K.M.D., A.J.A. and J.A.L. conceived and designed the study. K.J.J. and K.M.D. conducted the experiments. K.J.J. performed data analysis with support from E.P. and K.M.D. K.J.J., E.P. and A.J.A. wrote the manuscript. A.J.A., E.P. and J.A.L. supervised the study. J.A.L. edited the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The RNA-seq reads are available from the Sequence Read Archive (NCBI) under project number PRJNA1055154. The diatom strain used in this study is available from the Bigelow laboratory, National Center for Marine Algae and Microbiota under accession CCMP3694. The bioinformatics code and data files for this study are available as online Support information from Zenodo (https://zenodo.org/records/13755773).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.