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Cross-talk between tissues is critical for intergenerational acclimation to environmental change in *Acanthochromis polyacanthus*

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Organisms' responses to environmental changes involve complex, coordinated responses of multiple tissues and potential parental influences. Here using a multi-tissue approach we determine how variation in parental behavioural tolerance and exposure to elevated CO_2 influences the developmental and intergenerational molecular responses of their offspring in the coral reef fish *Acanthochromis polyacanthus* to future ocean acidification (OA) conditions. Gills and liver showed the highest transcriptional response to OA in juvenile fish regardless of parental OA conditioning, while the brain and liver showed the greatest intergenerational acclimation signals. Developmentally induced signals of OA, such as altered neural function in the brain, were restored to control levels after intergenerational exposure. Intergenerational CO_2 exposure also enabled the offspring to adjust their metabolic processes, potentially allowing them to better meet the energetic demands of a high CO_2 environment. Furthermore, offspring of OA-exposed parents differentially expressed a new complement of genes, which may facilitate intergenerational acclimatory responses. A genetic component of intergenerational plasticity also played a crucial role, with the parental behavioural phenotype largely determining the offspring's transcriptional signals. Overall, our results reveal tissue-specific transcriptional changes underlying intergenerational plastic responses to elevated CO_2 exposure, enhancing understanding of organismal acclimation to OA throughout the whole body.

Given the ongoing rapid human-induced global change, organisms need to acclimate and adapt to the changing environments in order to survive. Ocean acidification (OA), driven by the absorption of anthropogenic CO_2, has increased the amount of dissolved CO_2 in the oceans. Projections estimate a rise in CO_2 levels from a present-day value of 400 µatm to ~900 µatm by the end of the century[1]. Ocean acidification (OA) is reported to negatively impact the physiology and behaviour of various marine organisms including fish[2,3]. However, increasing evidence suggests that multigenerational exposure to elevated CO_2 conditions could influence the adaptive capacity of future generations to OA conditions[4]. In fact, several studies have reported transgenerational acclimation in a number of fish species as well as some invertebrates to OA[3]. Examples include Atlantic silverside, certain species of anemonefish, oysters, mussels, sea urchins, and copepods[3,5–8]. Specifically, transgenerational exposure to elevated CO_2 conditions has been shown to facilitate acclimation of metabolism, growth, survival, neuronal plasticity and behaviour in independent studies[6,9–15], however, we are still learning about the underlying molecular mechanisms of such acclimation processes.

Furthermore, there exists considerable variability both within and across species in the biological responses to OA, due to differences in their evolutionary and environmental history. This variability in sensitivity to elevated CO_2 within populations could be crucial in long-term adaptation by favouring the selection of more tolerant individuals. Indeed, variation in behavioural tolerance to elevated CO_2 exposure has been reported to be heritable and hence could facilitate rapid selection of tolerant genotypes in the population[16,17]. Such selection for CO_2 tolerance has been shown to occur in nature, leading to populations consisting of individuals with greater behavioural tolerance to elevated CO_2[18]. Furthermore, inter-individual

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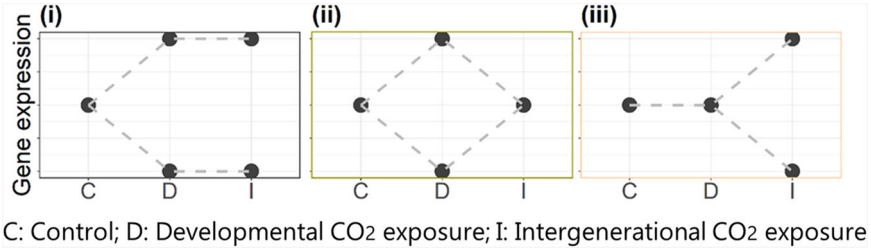

Fig. 1 | Schematic graph representing the gene expression profiles. Expression profile of (i) CO_2-response genes: genes with significantly higher or lower expression in developmental CO_2 treatment (D) and intergenerational CO_2 treatment (I) compared to control (C), (ii) genes showing a rescue pattern: genes with significantly higher or lower expression in developmental CO_2 treatment (D) compared to control (C), but whose expression is not significantly different in intergenerational CO_2 treatment (I) compared to control (C), and (iii) intergeneration-specific genes: genes with significantly higher or lower expression in intergenerational CO_2 treatment (I) compared to control (C), but whose expression is not significantly different in developmental CO_2 treatment (D) compared to control (C).

variation in sensitivity to ocean acidification could have an epigenetic basis[19,20]. Several studies have reported the expression levels of genes involved in epigenetic processes to be altered upon exposure to elevated CO_2 conditions[13,21]. Transfer of epigenetic factors that influence gene expression profiles from parents to offspring (epigenetic inheritance) could be one of the potential mechanisms of inter- and trans-generational acclimation and eventual adaptation to OA.

Adaptive responses of organisms to environmental changes involve integrated activity of various tissues, with each tissue undergoing changes in its transcriptional landscape resulting in the overall response of the organism. However, to date, research has mainly focused on individual tissue functional changes in response to OA with less emphasis on how these changes integrate to create a whole-body response. Several studies have examined the effects of OA on brain and neurosensory systems since the discovery of impaired behavioural responses in various fish species in elevated CO_2 conditions. The altered behavioural responses have been linked to changes in the functioning of the GABAergic signalling pathway[22] and the circadian rhythm in the brain of fish exposed to elevated CO_2[14,23,24]. Previous studies have also focused on the effects of OA on the gill transcriptome as gills are the primary organ involved in acid-base regulation, immune defence, and stress response, and hence play a vital role in maintaining cellular homeostasis under conditions of CO_2 stress[25–27]. These processes are energetically expensive and indeed changes in the aerobic metabolic scope[28–31] and expression levels of key metabolic genes[32] have been reported in fish exposed to elevated CO_2. Therefore, exposure to elevated CO_2 affects various aspects of fish physiology such as metabolism, cellular redox status, ion transport and acid-base homeostasis, neurological functioning and behaviour thereby exerting whole-body functional reprogramming[33]. Therefore, a systematic transcriptomic analysis is needed to determine how the biological processes associated with each tissue interact to drive adaptive responses of the whole organism to elevated CO_2 environments.

In this study, we conducted an intergenerational CO_2 exposure experiment and performed a systematic analysis of gene expression changes in response to elevated CO_2 across three tissues, the brain, the gills, and the liver, using the spiny chromis damselfish *Acanthochromis polyacanthus*. Given the functional specificity of different tissues, we hypothesise that certain molecular pathways would exhibit tissue-specific regulation, while others would show coordinated regulation involving multiple tissues. While *A. polyacanthus* can be sensitive to increases in water temperature and CO_2 levels, it has the potential to acclimate to the changing environmental conditions across multiple generations[13,14,34,35], making it an ideal model to explore the molecular basis of both developmental and intergenerational plasticity. In fact, this species has been used as a model to study the impacts of climate change and investigate the molecular basis of intergenerational plasticity to environmental changes. This is due to its advantageous life-history traits, such as the formation of monogamous breeding pairs, direct development of larvae, and suitability for breeding and rearing in captivity[36]. However, past studies have only focused on single tissues[13,14,19]. Here, by using a multi-tissue transcriptomic approach we demonstrate how dynamic cross-talk between tissues underlies the general and common elevated CO_2 response, as well as the organism's developmental and intergenerational specific response to future ocean acidification conditions (Fig. 1). In particular, we explored genes with three specific expression patterns – (i) genes that show significantly altered expression in both the developmental and intergenerational CO_2 conditions compared to control, (ii) genes that are differentially regulated in the developmental CO_2 condition but whose expression is similar to control levels in the intergenerational CO_2 condition, and (iii) genes whose expression is significantly altered exclusively in the intergenerational CO_2 condition (Fig. 1). This enabled us to tease apart distinct patterns of gene regulation critical for understanding the mechanisms of intergenerational acclimation. Additionally, we also reveal how the acclimation response of offspring, mediated by altered gene expression profiles across multiple tissues, is influenced by variation in parental sensitivity to elevated CO_2 and parental environment. Offspring of behaviourally sensitive parents no longer showed signatures of altered neural activity, observed during within-generation exposure to OA, when the parents had also been previously exposed to OA. Interestingly, offspring of parents with behaviourally tolerant phenotype showed an overall greater magnitude of transcriptional response to elevated CO_2 exposure and increased intergenerational plastic responses compared to offspring with behaviourally sensitive parents. This intergenerational plasticity was evident from the upregulation of cellular stress response genes in the gills and liver with both within- and intergeneration exposure, and additional upregulation in the brain only upon intergenerational exposure. Moreover, several genes associated with energy metabolism were differentially regulated in the brain and liver of offspring with behaviourally tolerant parents only in the intergenerational treatment. Overall, through systemic characterisation of the effects of OA, we show how the molecular responses within each tissue integrate to drive intergenerational acclimation to OA at the organismal level.

Results

Molecular processes affected by all elevated CO_2 treatments

To understand the general effects of elevated CO_2 exposure regardless of the time of exposure to elevated CO_2, we identified genes that were commonly differentially expressed (DE) in both the developmental and intergenerational treatments compared to control (Fig. 1(i)), which are considered the general "CO_2 response genes". Irrespective of the parental environment, the liver showed the greatest transcriptional response to elevated CO_2 exposure in offspring of both sensitive and tolerant parental behavioural phenotypes, with 63 and 986 differentially expressed (DE) genes, respectively (Fig. 2 and Supplementary Table S3 and S4). In the offspring of tolerant parental phenotype, the gills followed with 883 DE genes, while the brain had 44 DE genes (Fig. 2 and Supplementary Table S3). Conversely, in the offspring of

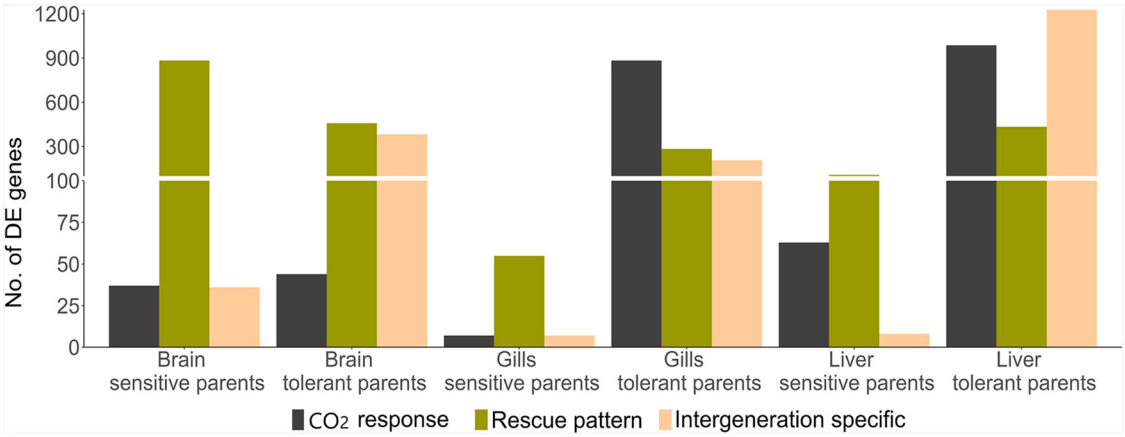

Fig. 2 | Number of differentially expressed genes showing a common CO_2 response, rescue pattern, and intergenerational specific response in all three tissues. Behaviourally sensitive parents showed an impaired response to the chemical alarm cue, while behaviourally tolerant parents showed a normal aversion behaviour to the chemical alarm cue. Note scale break in the *y*-axis at 100 DE genes.

sensitive parental phenotype, the brain displayed a higher number of DE genes (37) compared to the gills (9; Supplementary Table S3). Overall, offspring of parents with tolerant behavioural phenotype had a higher number of DE genes, indicating increased gene expression regulation across all three tissues in response to elevated CO_2 exposure compared to offspring with behaviourally sensitive parents. However, the CO_2 response genes showed high tissue specificity with no genes being commonly DE across the three tissues in the sensitive parental phenotype and only 3 and 81 genes being shared between the brain and gills, and liver and gills respectively in the tolerant parental phenotype (Supplementary Fig. S3a).

Functional enrichment analysis of the CO_2 response genes in offspring with sensitive parental phenotype revealed translation and amino-acid synthesis to be important in the brain, while no significantly enriched functions were found in the gills and liver (Fig. 3 and Supplementary Table S5). For the offspring of tolerant parental phenotype, biosynthetic processes were commonly enriched in the brain and gills (Fig. 3). Few other functional pathways were commonly enriched between the gills and liver, including metabolism, translation, protein transport, immune and stress response (Fig. 3 and Supplementary Table S5). Interestingly, while genes associated with metabolic pathways such as glycolysis, TCA cycle, and mitochondrial electron transport chain were commonly differentially regulated in both the gills and liver, pentose phosphate pathway genes showed upregulation exclusively in the gills. Additionally, immune response and translation showed tissue-specific regulation with downregulation in the liver but upregulation in the gills (Supplementary Table S5). Furthermore, RNA processing and primary active transmembrane transport were enriched only in the liver (Supplementary Table S5). This suggests intricate and tissue-specific regulation of various functions in response to elevated CO_2 exposure.

Parental exposure to elevated CO_2 "rescues" developmental effects

Genes whose expression is altered upon developmental exposure to elevated CO_2 (i.e. are DE in developmental treatment compared to control and intergeneration) but returned to control levels when parents were previously exposed to elevated CO_2 (i.e. are not DE when comparing intergeneration treatment with control) are considered to show a "rescue" pattern suggesting cross-generation plasticity (Fig. 1a(ii)). In offspring with sensitive parental phenotype, a total of 883, 55, and 108 genes in the brain, gills and liver, respectively, showed a "rescue" pattern. Similarly, in offspring with tolerant parental phenotype 458, 284, and 434 genes in the brain, gills, and liver, respectively, showed a "rescue" pattern (Supplementary Tables S3 and S6). Parental conditioning had the largest effect on brain gene expression followed by the liver (Fig. 2). Similar to the CO_2-response genes, there were very few genes commonly DE across tissues (Supplementary Fig. S3b).

Genes exhibiting a rescue pattern in the brains of offspring of behaviourally sensitive parents showed the highest degree of specificity in functional regulation with pathways involved in cytoskeleton organisation, apoptosis, synaptic signalling, sodium and calcium channel activity, transcription regulation, cellular transport, and small GTPase signalling being exclusively enriched only in this tissue (Fig. 4 and Supplementary Table S7). Gills were found to play a key role in regulating ion transport in offspring of both behaviourally sensitive and tolerant parents. Specifically, the expression of genes encoding sodium and potassium channel transporters was altered in the gills upon developmental exposure to elevated CO_2 levels but returned to control levels in intergenerationally exposed fish (Fig. 4 and Supplementary Tables S6 and S7). Additionally, purine ribonucleoside salvage pathway and DNA replication were identified as key functions among the "rescue" genes in the offspring of parents with sensitive phenotype in the gills and liver respectively (Fig. 4 and Supplementary Table S7). This suggests that the molecular responses of the offspring are influenced by their parental environment, with specific functional pathways being selectively regulated in each tissue due to parental conditioning to elevated CO_2 levels.

Intergenerational specific response to elevated CO_2

We found a large transcriptional response to elevated CO_2 that was only seen in the intergenerationally exposed fish and not in fish with only developmental (within-generation) exposure to elevated CO_2. This indicates plasticity of the offspring transcriptome due to parental conditioning to elevated CO_2 and was especially marked in offspring of tolerant parents (Fig. 2). These are genes that were only differentially expressed in the intergenerational treatment (compared to control and development) but were at control levels in the developmental treatment (Fig. 1(iii)). Specifically, 383, 207, and 1226 genes were DE in the brain, gills, and liver respectively in offspring with tolerant parents, while offspring with sensitive parents had 36, 7, and 8 DE genes in the brain, gills, and liver respectively that were specific to the intergenerational treatment (Supplementary Tables S3 and S8). Offspring of both tolerant and sensitive parents showed high tissue specificity in the intergenerational specific transcriptional signature to elevated CO_2 with only one and five genes being commonly DE across all three tissues in the sensitive and tolerant parental phenotypes respectively (Supplementary Fig. S3c).

The intergeneration specific response was substantially higher in offspring of behaviourally tolerant parents with various functions showing tissue-specific regulation. Stress response and metabolic pathways were commonly differentially regulated in the brain and liver (Fig. 5), but the liver

Fig. 3 | Functions that are significantly enriched (FDR < 0.05) among the DE genes involved in overall CO_2 response. SP indicates offspring of behaviourally sensitive parents and TP indicates offspring of behaviourally tolerant parents. The colour of the circles represents the FDR corrected *p*-value and size of the circles represents the number of genes associated with the respective function.

showed a much larger number of DE genes (~400) associated with metabolism compared to the brain (11 DE genes; Supplementary Table S9). Additionally, signal transduction pathways were enriched only in the brain while several functions such as biosynthetic pathways, endopeptidase activity, lipid binding/ transport, transcriptional regulation, proteolysis, and spindle organisation were exclusive to the liver (Fig. 5, Supplementary Table S9). Interestingly, molecular signatures indicating pH regulation by bicarbonate retention were identified only in the intergenerationally treated fish with tolerant parental phenotype, indicated by the downregulation of *SLC4A1*, *CFTR*, *SLC12A2*, and *SLC9A3* in the gills and upregulation of *SLC4A4* in the brain (Supplementary Table S8). Therefore, offspring of parents with tolerant behavioural phenotype showed increased molecular signatures of intergenerational plasticity and this was especially evident in the liver. This change in transcriptional signature could regulate the above mentioned "rescue" pattern and equip offspring to better cope with OA conditions.

Parental variability in CO_2 sensitivity impacts the offspring transcriptome

Parental behavioural phenotype was found to have a substantial influence on the offspring's transcriptional response. Across all three tissues, offspring with behaviourally tolerant parents when faced with elevated CO_2 had larger changes in gene expression levels (log2FC > 5; Fig. 6) and a greater number of DE genes involved in the overall CO_2 response (common in developmental and intergenerational CO_2 exposure compared to control; Fig. 2). There were also very few genes involved in the overall CO_2 response shared between the two parental phenotypes, specifically only six, three, and eleven common DE genes in the brain, gills, and liver respectively (Supplementary Fig. S3a). When considering genes involved in intergenerational plastic responses, there were more differentially expressed genes in the offspring of tolerant parents compared to those of sensitive parents, except for genes showing a rescue pattern in the brain (Fig. 2). Additionally, none of the DE genes involved in intergenerational plasticity were shared in the liver tissue between the two parental phenotypes while the brain and gills had a small proportion of common DE genes (specifically, 121 and 11 common DE genes showing a rescue pattern in the brain and gills respectively and only two and one common DE genes in the intergeneration specific response in the brain and gills respectively (Supplementary Fig. S3b, c).

The difference in transcriptional response between offspring of the two parental phenotypes was especially pronounced when considering genes showing an intergeneration-specific signature. Offspring of tolerant parental phenotype had more DE genes with a much higher magnitude of gene expression changes (log2FC > 5; Fig. 6), compared to those with behaviourally sensitive parents. While there were some common transcriptional responses between offspring with sensitive and tolerant parents, offspring of tolerant parents showed a much stronger transcriptional response to

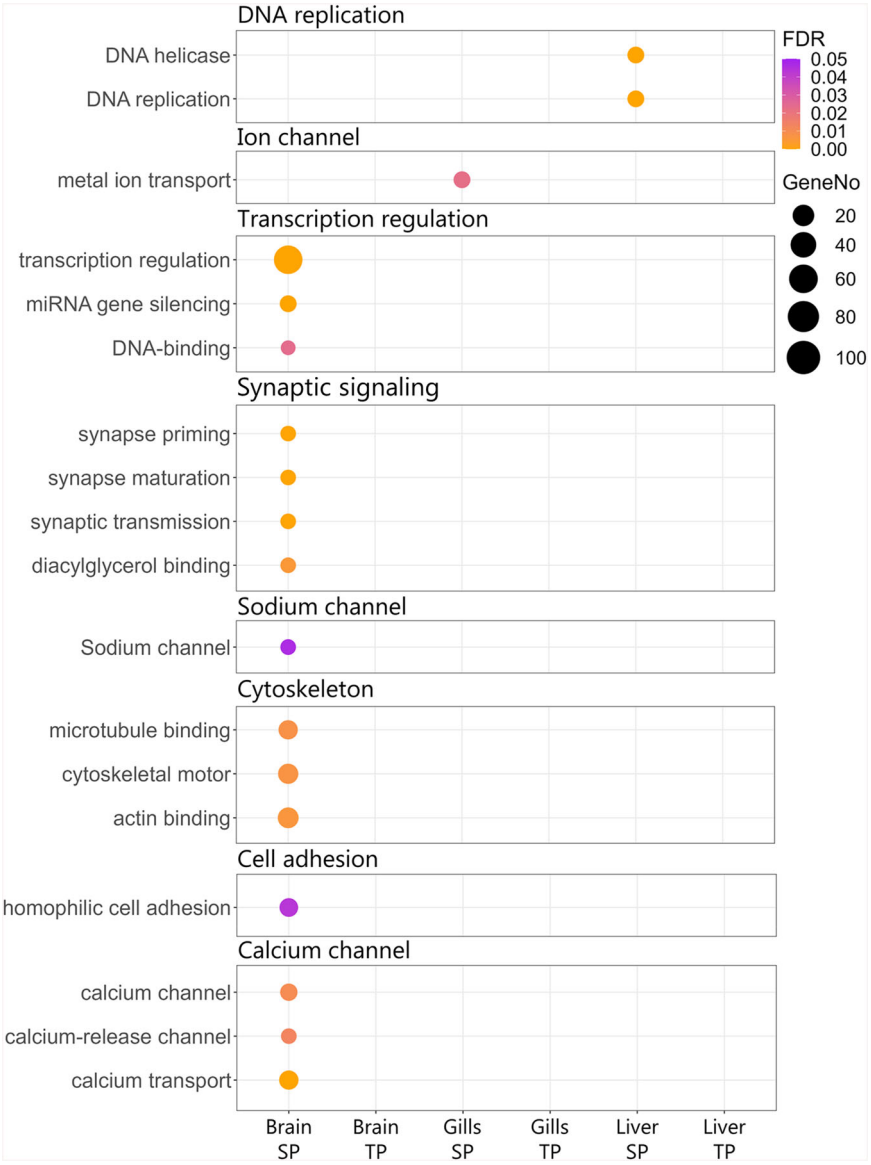

Fig. 4 | Functions that are significantly enriched (FDR < 0.05) among the DE genes showing a rescue pattern. SP indicates offspring of behaviourally sensitive parents and TP indicates offspring of behaviourally tolerant parents. The colour of the circles represents the FDR corrected *p*-value and size of the circles represents the number of genes associated with the respective function.

elevated CO_2 in general and also had a stronger signature of intergenerational plasticity. As these differences between the parental behavioural phenotype already suggest, we also found an interaction between parental phenotype and offspring gene expression response to the different CO_2 conditions. Specifically, 158 genes in the brain, 157 in the gills, and 1635 in the liver exhibited a significant interaction effect (Supplementary Table S10).

Discussion

Changes in the offspring transcriptional landscape in response to elevated CO_2 exposure exhibited tissue-specific signatures which were largely influenced by previous parental exposure to elevated environmental CO_2, but also by the parental behavioural phenotype. In all elevated CO_2 treatments, we found that gills are critical in activating stress and immune response pathways and the brain and liver had the greatest signal of intergenerational plastic response. In fact, intergenerationally treated fish no longer showed molecular signatures of altered neural signalling in the brain that were seen in the developmental (within-generation) treatment. Additionally, differential regulation of a new complement of metabolic genes exclusively in the brain and liver of offspring from parents with prior exposure to elevated CO_2 suggests that parental conditioning may influence the offspring's capacity to regulate metabolic processes in response to elevated CO_2 exposure. *A. polyacanthus* is known to have a highly plastic genome enabling it to respond and acclimate to environmental changes[37,38] and our results show that this persists across generations. One limitation of our study is the relatively small number of family lines, which may confound treatment effects with inherent family line differences. However, our findings suggest that this species has the potential to rapidly acclimate to the changing ocean environments.

Genes that are always differentially expressed in elevated CO_2 conditions, regardless of the timing of exposure (within-generation or intergeneration), are key genes in the general response to ocean acidification (OA). The gills and liver exhibited a higher transcriptional response in all elevated CO_2 treatments suggesting that these tissues play an important role in the overall response of the fish to elevated CO_2. In both the developmental and intergenerational CO_2 treatments, these tissues exhibited differential regulation of key functional pathways such as cellular metabolism, stress response, and immune function. However, while stress response genes were upregulated in both the gills and the liver, genes involved in immune response were upregulated in the gills but downregulated in the liver. This suggests tissue-specific regulation of key pathways, with the gills and liver working in synergy to mitigate oxidative stress and maintain cellular redox balance. Similar differences in tissue-specific regulation of specific metabolic pathways were observed between the gills and liver in elevated CO_2

Fig. 5 | Functions that are significantly enriched (FDR < 0.05) among the intergeneration specific DE genes. SP indicates offspring of behaviourally sensitive parents and TP indicates offspring of behaviourally tolerant parents. The colour of the circles represents the FDR corrected *p*-value and size of the circles represents the number of genes associated with the respective function.

treatments. While TCA cycle genes were elevated in both tissues, the pentose phosphate pathway was upregulated only in the gills. Furthermore, the cytochrome complex and glycolytic pathway were downregulated in the hepatic transcriptome. This suggests a shift in metabolic priorities in response to elevated CO_2 exposure potentially redirecting carbon fluxes to support cellular demands for various metabolites and cofactors needed for other biological processes such as biosynthetic pathways and the cellular stress response machinery[39–41]. Overall, our results indicate a common functional response in the gills and liver that are always activated in elevated CO_2 conditions irrespective of previous parental conditions.

Parental exposure to altered environmental conditions can pre-acclimate the offspring transcriptome to these new conditions via intergenerational plasticity. In juvenile *A. polyacanthus*, parental conditioning to elevated CO_2 was found to "rescue" the effects of developmental exposure to the same conditions. This "rescue pattern" was predominantly evident in the brains of offspring with sensitive parental phenotype. Specifically, the expression of genes involved in synaptic plasticity and calcium channel activity, initially altered in the developmental treatment, were similar to control levels in the intergenerationally treated fish. Exposure to OA conditions has been shown to affect neural plasticity and neurogenesis in some fish species resulting in changes in neural circuitry and signalling[42,43], which could result in behavioural alterations[22]. However, the "rescue pattern" of synaptic plasticity genes along with cell adhesion and cytoskeleton-related genes, which mediate synaptic plasticity[44,45], in intergenerationally treated fish hints at the restoration of altered neural signalling in the offspring facilitated by previous parental exposure to elevated CO_2. This suggests an enhanced capacity for intergenerational plasticity in *A. polyacanthus*. In fact, a comparison of six species living in wild CO_2 seeps with long-term elevated CO_2 exposures revealed *A. polyacanthus* to have substantially larger brain transcriptional plasticity compared to the other species[37]. Therefore, intergenerational CO_2 exposure potentially restores the dynamic equilibrium of cytoskeletal proteins, thereby re-establishing synaptic signalling processes to control levels.

This "rescue pattern" in the brain was also associated with transcriptional regulation such as transcription factors and RNA-mediated gene silencers. Changes in the external environment can trigger reprogramming of transcriptional networks resulting in dynamic regulation of gene expression[46] as also suggested in wild fish populations naturally exposed to elevated CO_2[47]. Therefore, while these regulatory genes play a key role in developmental plastic responses to elevated environmental CO_2 levels, these are no longer needed in intergenerationally treated fish revealing acclimation. Similarly, in the gills, this 'rescue' pattern was seen for genes encoding ion transporters including potassium channels which are known for their significance in pH regulation and association with CO_2 chemoreception[48]. While the differential expression in the developmental treatment is likely triggered by hypercapnia prompting downstream compensatory responses to maintain pH homeostasis[48,49], the restoration of expression levels to control levels in intergenerationally treated fish suggests that intergenerational exposure may enhance and facilitate improved pH regulatory mechanisms. As pH defence is a key mechanism in the gills[50], this "rescue pattern" may reveal an acclimation process in the offspring facilitated by parental conditioning to elevated CO_2.

To facilitate this intergenerational acclimation, other adjustments specific to the intergenerational treatment may be needed. Changes in lipid

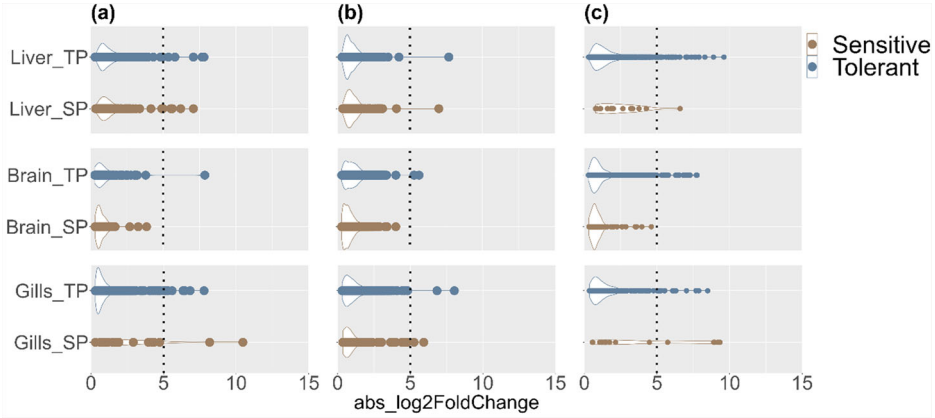

Fig. 6 | Log2 fold change in expression of all differentially expressed genes. Log2 fold change profiles for genes involved in (**a**) overall CO_2 response, (**b**) rescue pattern, and (**c**) intergenerational specific response across all three tissues. SP indicates samples with a sensitive parental phenotype and TP indicates samples with a tolerant parental phenotype.

metabolism have been shown to facilitate acclimation to elevated CO_2 as well as temperature[3,35,51] and here we see metabolic adjustments such as upregulation of fatty-acid metabolism in the brain and liver and increased lipid synthesis in the liver only with previous parental exposure. Metabolic changes have also been seen in juvenile *A. melanopus* exposed to elevated CO_2 when their parents were also exposed to the same CO_2 conditions[6]. Therefore, an increase in lipid metabolism in both the brain and liver in intergenerationally treated fish combined with the observed redirection of metabolic carbon fluxes when considering the overall CO_2 response, might indicate a similar switch from carbohydrate to lipid metabolism as the primary metabolic pathway for ATP production in elevated CO_2 conditions. This could present a key intergenerational acclimation process, where parental exposure equips their offspring to better manage the increased metabolic demands associated with changing environments.

Our data suggests a certain degree of predictability of offspring plasticity from the parental behavioural phenotype, which suggests that plasticity could partially be a genetic trait. Offspring of tolerant parental phenotype showed a stronger transcriptional response to elevated CO_2 exposure and increased intergenerational plastic responses, predominantly in the brain and liver, compared to offspring of behaviourally sensitive parents. Additionally, an interaction between parental behavioural phenotype and offspring transcriptional responses to the different CO_2 treatments was observed, with the liver having the highest number of genes with an interaction effect. Parental behavioural phenotype has been shown to influence the offspring's brain transcriptional response to elevated CO_2 in *A. polyacanthus*[10], with behavioural tolerance being heritable[17]. Selection experiments have indicated a genetic basis for individual variation in OA induced responses in a variety of animals[52–55], including the behavioural phenotype to chemical alarm cues in elevated CO_2 in *A. polyacanthus*[16]. Therefore, there exists a complex interplay between parental phenotype and environmental CO_2 conditions in shaping the offspring's molecular responses. This intraspecific variation in organismal response is key in driving future adaptive evolution. Our results suggest that offspring of parents with a tolerant behavioural phenotype have an increased capacity for intergenerational plasticity and potential acclimation to future ocean acidification conditions, with genetic variation possibly playing a role.

This study used a systematic approach considering parental behaviour, environment, and multiple tissues to assess molecular responses of a coral reef fish to future ocean conditions. The gill and liver transcriptomes exhibited substantial changes, with a certain level of tissue specificity in the regulation of key cellular processes, such as stress and immune responses, in both developmental and intergenerational elevated CO_2 conditions. This highlights their fundamental roles in acclimatory responses to OA, regardless of previous parental conditions. Additionally, parental conditioning to elevated CO_2 facilitated intergenerational acclimation responses in the offspring, as evidenced by the "rescue" of neural activity in the brain and pH regulation in the gills. This implies that intergenerational CO_2 exposure facilitates efficient pH regulation upon within-generation elevated CO_2 exposure. Previous parental exposure to OA conditions also equipped their offspring to better manage metabolic needs associated with a new environment with elevated CO_2. While plastic acclimatory processes with previous parental exposure may facilitate coping mechanisms with OA, these vary depending on the parental phenotype also suggesting a genetic component to the future survival of the species. Overall, our study reveals how intergenerational plasticity is facilitated from a whole-organism perspective and illustrates how transcriptional changes across multiple tissues integrate to drive the plastic responses of fish to the changing ocean chemistry.

Methods

Sample collection, behavioural testing, and experimental design

All sample collection and the experimental procedures were carried out in accordance with institutional and national law guidelines. We have complied with all relevant ethical regulations for animal use. Ethics approval for the study was granted by James Cook University with the approval number A1828.

Adult *Acanthochromis polyacanthus* were collected from the wild on the Great Barrier Reef, Australia (18°38'24.3"S, 146°29'31.8"E) and transported to the James Cook University's Experimental Marine Aquarium Facility as described previously[14,17]. Briefly, upon arrival, the adult fish were left to acclimate to the tank environment for five days after which they were exposed to elevated CO_2 (754 ± 92 µatm) for seven days. To obtain the desired CO_2 conditions, three header tanks (60 L) fed water into 32 L aquaria where fish were held across three separate systems. 100% CO_2 was diffused in the header tanks (for control ambient air was diffused). pH controllers (Aqua Medic, Germany) maintained the desired pH in the header tanks that supplied the tanks in each system with pH_{NBS} (National Bureau of Standards. Temperature measurements were taken daily in each tank using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and a temperature probe (Cormark C26, Norfolk, UK). Further details are described in Welch & Munday[17]. Following the CO_2 exposure we tested their behavioural sensitivity to conspecific chemical alarm cues (CAC) using a two-chamber flume as described previously[14,17]. The behavioural trials lasted nine minutes in total (2 min acclimatisation period, 2 min testing, 1 min water switch, 2 min acclimatisation, 2 min testing). The fish were classified as being behaviourally sensitive or tolerant to elevated CO_2 based on the amount of time spent in water containing the CAC[10,13,14,17]. Sensitive individuals spent ≥ 70% (89.69 ± 15.69%) of time in CAC whereas tolerant individuals spent ≤ 30% of time (7.35 ± 11.1%) in CAC, with approximately 38% of collected fish falling into each category. Individuals of similar size displaying the same behavioural phenotype (sensitive or tolerant) were then grouped into breeding pairs. Three sensitive and three tolerant pairs were held in control conditions (414 ± 46 µatm; similar to the pCO_2 levels in the wild[56]) and two sensitive and two tolerant pairs were held in elevated CO_2 conditions (754 ± 92 µatm) for three months prior to the breeding season. Each parental pair formed a distinct family line, and five different family

lines for tolerant as well as sensitive pairs were used to ensure that the effects seen were not due to a single parental pair (Supplementary Table S1). To further minimise potential genetic bias, one sensitive and one tolerant parental pair, initially exposed to control conditions for three months and bred at control levels, were subsequently exposed to elevated CO_2 for three months after which they bred. Therefore, control and developmentally treated fish are siblings from three different parental pairs for both sensitive and tolerant behavioural phenotypes. Additionally, one parental pair has offspring in control, developmental and intergenerational CO_2 treatment. In this experiment, similar to previous studies, we did not observe any significant effect of elevated CO_2 on reproductive output or juvenile mortality[10]. Offspring clutches from each parental pair were placed into three different experimental treatments resulting in three combinations of parent-offspring conditions for each parental phenotype: (1) Control treatment – Parents and offspring held at control condition (414 ± 46 µatm; $N = 9$ each for sensitive and tolerant (3 from each parental pair)); (2) Developmental treatment – Parents held at control condition and offspring exposed to elevated CO_2 (754 ± 92 µatm; $N = 9$ each for sensitive and tolerant (3 from each parental pair)) immediately after hatching; and (3) Intergenerational treatment – Parents and offspring exposed to elevated CO_2 (754 ± 92 µatm; $N = 9$ each for sensitive and tolerant (three from each parental pair)). The offspring were held in their respective conditions until they were five months old after which nine fish from each parental behavioural phenotype (sensitive or tolerant), from each treatment condition ($N = 27$ from each parental phenotype; $N = 54$ total fish sampled) were euthanized and the brain, gills and liver were dissected, snap frozen in liquid nitrogen and stored at −80 °C until further processing (Supplementary Fig. S1). On average all the sampled fish weighed 2.04 ± 0.43 g. A one-way ANOVA revealed no significant difference in the weight of the offspring across the three CO_2 conditions.

RNA extraction, sequencing, and gene expression analyses

Total RNA was extracted from the fish brains, livers, and gills using the AllPrep DNA/RNA Mini kit from Qiagen following the manufacturer's instructions, with a slight modification for the liver tissues where a lower ethanol concentration (50%) was used in the washing step. RNA quality was determined using nanodrop and Agilent Bioanalyzer and samples having an RNA integrity value (RIN) ≥ 8 were sequenced using Illumina HiSeq 2500 to get paired-end reads of 100 bp at Macrogen Inc., South Korea. A total of 1614.25 ± 3.05, 2367.03 ± 5.19, and 2227.23 ± 6.37 million raw paired-end reads were obtained from the 162 sequenced libraries from brain, gills, and liver, respectively which included nine control, nine developmental, and nine intergenerational samples for each parental phenotype for each tissue (Supplementary Table S1). The quality of the raw reads was examined using FastQC[57] v0.11.8 and adaptors and low quality sequences were trimmed using Trimmomatic[58] v0.39 (ILLUMINACLIP: adaptors.fa:2:30:15:8:true; SLIDINGWINDOW:4:20; MINLEN:32). Only those sequences ≥ 32 bp in length with both the forward and reverse reads retained after trimming were used for further analysis. Potential contaminant sequences were identified using kraken[59] v2.0.8-beta, with a confidence score of 0.3, using the bacteria, fungi and virus RefSeq genomic libraries as reference and removed from further analyses. A total of 1510.51 ± 2.62, 2254.16 ± 4.95, and 2116.25 ± 6.04 million high-quality sequences were retained after the filtering process (Supplementary Table S1). These sequences were mapped to the *Acanthochromis polyacanthus* reference genome (unpublished) using HISAT2[60] v2.1.0. On average, 84 ± 1.83%, 91.22 ± 0.66%, and 93.33 ± 0.81% reads mapped to the reference genome from the brain, gills, and liver respectively (Supplementary Table S1). Raw read counts per gene were obtained using featureCounts[61] v2.0.0 (parameters: -B -J -M –fraction), assigning fractional counts to multi-mapped reads. Exploring the gene expression patterns across the whole dataset (162 samples) using principal component analysis (PCA) revealed a clear clustering of samples by tissues indicating that tissues vary greatly in their gene expression patterns (Supplementary Fig. S2). Subsequent analysis of differences in gene expression levels was therefore carried out separately for each tissue using the DESeq2[62] v1.32.0 package in R[63] v4.2.1.

Principal component analysis (PCA) using the regularised log transformed (rlog) counts was done in R v4.2.1 to detect and remove outlier samples. For samples with sensitive parental phenotype, two developmental CO_2 treatment samples were outliers - one in the gills (SF1-1-D) and one in the liver (SF2-1-D) and three different samples from the intergenerational CO_2 treatment were outliers in the brain, gills and liver (SF1-1-T in brain, SF3-2-T in gills, SF2-3-T in liver). Additionally, for samples with tolerant parental phenotype, three outlier samples were identified in the liver – one from control (TF3-2-C), one from the developmental CO_2 treatment (TF2-3-D), and one from the intergenerational CO_2 treatment (TF1-1-T). The outlier samples were distributed across all five family lines, with no single family line being predominant. A likelihood ratio test (LRT) using a model comparison approach was then used to determine the effect of the family line in driving the gene expression patterns and to determine the best design formula for the final DE analysis. First, significant differences in gene expression were measured by comparing a model including treatment and family line against a reduced model without the family line factor separately for each tissue. For a total of 924, 910, and 923 genes in the brain, gills, and liver respectively, the model including family line better explained the observed differences in gene expression compared to the reduced model excluding this factor (FDR corrected p-value < 0.05; Supplementary Table S2). Pair-wise comparisons between the control, developmental and intergenerational treatment were then carried out in DESeq2 (accounting for the family effect, using Wald test (design = ~ family + condition) separately for each parental phenotype for each tissue to determine the effect of parental environment and parental tolerance to CO_2 on the molecular responses of the offspring to elevated CO_2. Despite accounting for family effects, the limited number of families in our study may still confound treatment effects with family line differences. For each pair-wise comparison, the genes were considered to be significantly differentially expressed (DE) if the False Discovery Rate (FDR) adjusted p-value was <0.05, the absolute log 2-fold change in expression was >0.3 and baseMean was >10, as done in previous studies[10,13,14]. Functional enrichment analysis of the significant DE genes was carried out in OmicsBox[64] (https://www.biobam.com/omicsbox) v1.4.11 using Fisher's Exact Test (FDR corrected p-value < 0.05) with the option of reducing to most specific GO terms to reduce redundancy. The genes associated with the enriched GO terms were further categorised into broader functional groups based on their functional description from the UniProt knowledgebase (UniProtKB; https://www.uniprot.org/). All figures are made using ggplot in R v4.2.1. We also conducted a Likelihood Ratio Test to assess the interaction between parental phenotype and CO_2 exposure conditions of the offspring. Genes with a FDR adjusted p-value <0.05 and baseMean >10 were considered to exhibit a significant interaction effect.

Statistics and reproducibility

The details about experimental design, bioinformatic software tools, and statistics performed in this study are described in detail in the methods section. Sample sizes for each experimental treatment, along with the final counts remaining after the removal of outliers are described in detail in the methods section. Replicates are defined as individual fish.

All statistical analyses were performed in R v4.2.1. Firstly, a one-way ANOVA was used to confirm that there were no significant differences in weight of the fish across all experimental treatments. Outlier samples were identified through principal component analysis (PCA) of regularised log transformed (rlog) counts in R. Differential gene expression analysis was performed using DESeq2 v1.32.0 in R, beginning with a likelihood ratio test (LRT) to assess whether family line influenced the gene expression patterns. Subsequently, pairwise comparisons between control, developmental and intergenerational treatments were

conducted for each parental phenotype and tissue separately, using the Wald test in DESeq2 with the design formula ~ family + condition. Lastly, functional enrichment analysis of all significantly differentially expressed genes was conducted in OmicsBox using Fisher's Extact Test.

Data availability

All supplementary tables are in the Supplementary Data 1 file. The brain RNA-Seq raw sequences are deposited in NCBI under BioProject ID PRJNA311159. The gills and liver RNA-Seq raw sequences are deposited in NCBI under BioProject ID PRJNA989422. The data can be accessed via SRA Entrez (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA989422) or SRA Run selector (https://trace.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA989422).

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Author contributions

The experiment was designed and run by M.J.W. and P.L.M. Molecular lab work was performed by C.S. and sequenced by T.R.; S.S. carried out the transcriptome expression analysis with input from C.S.; S.S. led the writing of the manuscript with input from C.S. and all authors read, edited and approved the final manuscript.

Competing interests

All authors declare they have no competing interests.

Ethical approval

Sample collection was carried out following all institutional and national law guidelines. The experiment was completed under James Cook University ethics approval A1828.

Additional information

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