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Proteomic Responses to Ocean Acidification in the Brain of Juvenile Coral Reef Fish

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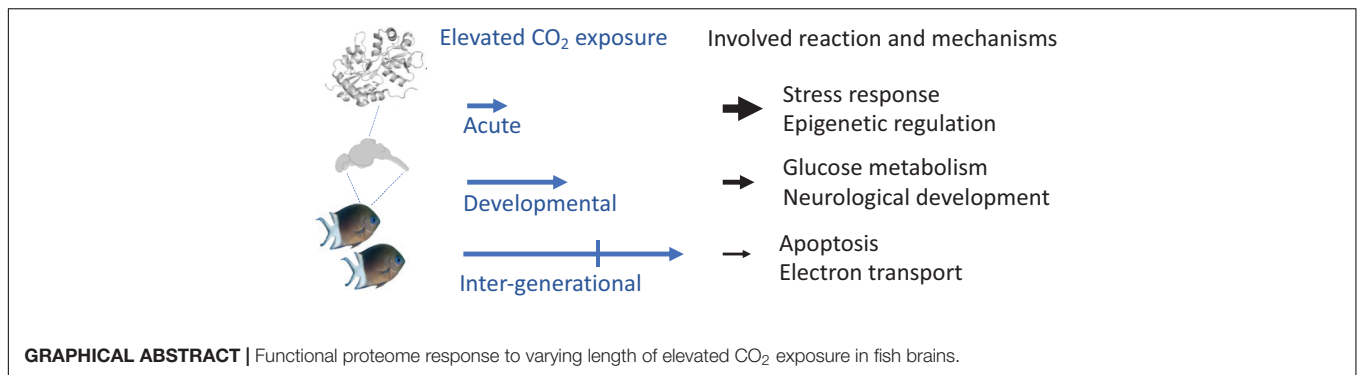
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Elevated CO₂ levels predicted to occur by the end of the century can affect the physiology and behavior of marine fishes. For one important survival mechanism, the response to chemical alarm cues from conspecifics, substantial among-individual variation in the extent of behavioral impairment when exposed to elevated CO₂ has been observed in previous studies. Whole brain transcriptomic data has further emphasized the importance of parental phenotypic variation in the response of juvenile fish to elevated CO₂. In this study, we investigate the genome-wide proteomic responses of this variation in the brain of 5-week old spiny damselfish, *Acanthochromis polyacanthus*. We compared the accumulation of proteins in the brains of juvenile *A. polyacanthus* from two different parental behavioral phenotypes (sensitive and tolerant) that had been experimentally exposed to short-term, long-term and inter-generational elevated CO₂. Our results show differential accumulation of key proteins related to stress response and epigenetic markers with elevated CO₂ exposure. Proteins related to neurological development and glucose metabolism were also differentially accumulated particularly in the long-term developmental treatment, which might be critical for juvenile development. By contrast, exposure to elevated CO₂ in the parental generation resulted in only three differentially accumulated proteins in the offspring, revealing potential for inter-generational acclimation. Lastly, we found a distinct proteomic pattern in juveniles due to the behavioral sensitivity of parents to elevated CO₂, even though the behavior of the juvenile fish was impaired regardless of parental phenotype. Our data shows that developing juveniles are affected in their brain protein accumulation by elevated CO₂, but the effect varies with the length of exposure as well as due to variation of parental phenotypes in the population.

Keywords: environmental proteomics, climate change, ocean acidification, behavior, tolerance

INTRODUCTION

Rising CO₂ levels in the ocean are anticipated to affect the physiology and behavior of marine organisms, with possible impacts on the structure and function of marine ecosystems (Wittmann and Pörtner, 2013; Cattano et al., 2018; Pörtner et al., 2019). Exposure to elevated CO₂ has shown to alter important behaviors, such as navigation, habitat selection, learning and predator-prey



interactions in some fish (Nagelkerken and Munday, 2016; Munday et al., 2019; Paula et al., 2019; Williams et al., 2019) and invertebrates (Watson et al., 2013; Jellison et al., 2016). For example, exposure to elevated CO₂ impairs the natural avoidance behavior of chemical alarm cues (CAC) in some fishes (Ferrari et al., 2011; Chivers et al., 2014; Welch et al., 2014; Ou et al., 2015; Welch and Munday, 2017; Laubenstein et al., 2019). Chemical alarm cues are compounds released from the skin of injured fishes, thereby providing a reliable cue of immediate predation threat (Ferrari et al., 2009). Most fishes respond to the presence of CAC from conspecifics by making behavioral decisions that reduce the risk of predation, such as reducing activity and seeking shelter (Ferrari et al., 2009). However, when exposed to elevated CO₂, some fish fail to exhibit behaviors that reduce their risk of predation. This impaired behavior is hypothesized to be caused by the self-amplifying alteration in the chloride-bicarbonate current in the GABA_A receptor in the brain, following acid-base regulation in reaction to elevated environmental CO₂ (Nilsson et al., 2012; Chivers et al., 2014; Heuer et al., 2016; Schunter et al., 2019).

Studying the response to a change in environmental condition on the molecular level of an organisms can provide insight into the broad response of a tissue to this change. Some molecular processes might change to acclimate to the new condition and prevent the need of a change on the physiological, behavioral or whole organism level. On the other hand, some molecular patterns will be the underlying processes to the whole organisms' physiological changes in response to the environmental alteration (Madeira et al., 2017). Transcriptomics can aid in understanding this molecular reaction whereas the protein response can elucidate possible post-translational modifications that have a more direct effect on cellular responses (López, 2007; Tang et al., 2015).

Previous brain transcriptomic studies on five-month-old spiny damselfish *A. polyacanthus* showed differential gene expression in GABA related pathways especially during short-term elevated CO₂ exposure (Schunter et al., 2018). However, when elevated CO₂ exposure was prolonged across generations, the transcriptional signal differs and shows an overexpression of genes related to sugar and energy production in the brain (Schunter et al., 2016), whereas the behavioral impairments persist (Welch et al., 2014). There is also large variation in the sensitivity of individual *A. polyacanthus* to moderately high CO₂

levels (~700 μatm), with a part of the population exhibiting some resistance to behavioral impairment ("tolerant" individuals) whereas others are behaviourally impaired when exposed to elevated CO₂ ("sensitive" individuals) (Schunter et al., 2016). This variation in sensitivity to behavioral effects of elevated CO₂ has a heritable component as fathers and their offspring show similar sensitivities after short-term exposure to elevated CO₂ (Welch and Munday, 2017).

The protein signal of the tolerant and sensitive parental behavioral phenotype in the offspring has been previously investigated in five-month-old spiny damselfish, by comparing control versus inter-generational CO₂ exposed fish (Schunter et al., 2016). However, the early juvenile developmental period is one of the most critical life stages in fish (DePasquale et al., 2015) and environmental conditions could have strong impacts during this early life stage. Therefore, here we focus on investigating the proteomic responses of five-week-old juvenile *A. polyacanthus* exposed to elevated CO₂ (750 μatm) for different periods of time. In our experimental design we use the variation in the olfactory response behavior of adult fish to study the influence of parental phenotype on brain protein accumulation of small juveniles. Further, these developing offspring of spiny damselfish from behaviourally tolerant and sensitive parents were exposed to different exposure lengths of elevated CO₂: 1) Acute (5-days of elevated CO₂), Developmental (5 weeks of elevated CO₂ since hatching) and Inter-generational (previous parental exposure and 5 weeks of elevated CO₂). The short-term acute treatment allows to tease apart mechanisms involved in the acclimation process, whereas developmental and in particular inter-generational exposure are more relevant on ecological timescales. We investigated the molecular pathways involved in the reaction to the three different CO₂ treatments by analyzing the differential accumulation of the brain proteome of 5-week old developing *A. polyacanthus*.

MATERIALS AND METHODS

Experimental Design

Adult *A. polyacanthus* pairs ($n = 20$) were collected as described in Welch and Munday (2017) and exposed to elevated CO₂ ($754 \pm 92 \mu\text{atm}$), consistent with average atmospheric CO₂ predicted by the end of the century according to the RCP6

emissions trajectory. After a seven-day elevated CO₂ exposure, the reaction toward chemical alarm cues (CAC) in adults was tested as described in Welch and Munday (2017) to define the parental phenotype to be either tolerant (T; less than 30% of the trial time was spent in CAC) or sensitive (S; more than 70% of the trial time in CAC) (Figure 1). Individuals of the same behavioral phenotype (T or S) were paired into breeding pairs and either left at control condition (414 ± 46 μatm) or placed into elevated CO₂ (754 ± 92 μatm) for three months until breeding started. In this study we used offspring from two breeding pairs from each parental phenotype (Tolerant and Sensitive) within each of the two parental CO₂ treatments (Control or elevated CO₂). Offspring clutches from each breeding pair were then placed into different experimental conditions resulting in a total of four treatment groups for each parental behavioral phenotype (T and S) (Figure 1): (1) Parents and offspring were kept at a CO₂ level of 414 ± 46 μatm (Control treatment); (2) Parents and offspring were kept at control condition, and offspring were exposed to elevated CO₂ (754 ± 92 μatm) for five days before dissection (Acute treatment); (3) Parents were kept at control condition and offspring were exposed to elevated CO₂ immediately after hatching for their entire developmental period (Developmental treatment); and (4) both parents as well as offspring were exposed to elevated CO₂ for the entire duration of the study (Inter-generational treatment). Developmental and inter-generational exposures of elevated CO₂ are relevant on an ecological timescale of ocean acidification in the wild. The short-term acute treatment was included to distinguish more immediate physiological responses to elevated CO₂ and to compare with the developmental treatment in order to identify which are specific developmental affects that depend on exposure to elevated CO₂ from early life. After five weeks juvenile fish were tested for their reaction to CAC as described in Welch and Munday (2017) with a sample number of 15–20 individual offspring for each parental behavioral phenotype (T and S; 7–10 per family line) within each of the four CO₂ treatment (N = 120 fish sampled in total). Differences in the percentage of time juveniles spent in CAC was tested by pair-wise comparisons using Tukey's *post hoc* tests in R. Due to the limited sample number that can be included in the proteomic analysis six offspring per treatment and phenotype were randomly selected (without prior knowledge of the outcomes of behavioral tests) and dissected for protein accumulation analysis. This means that three offspring from each of the tolerant and sensitive group coming from two family lines (Figure 1) were euthanized and the brain was dissected out and snapped frozen in liquid nitrogen and then stored in –80°C for further processing. This randomly chosen subset of samples represent the molecular reaction to each of the environmental CO₂ treatments.

Protein Extraction

Proteins from whole brain tissue were extracted along with DNA and RNA with a Qiagen AllPrep DNA/RNA Mini Kit. The flow through at the RNA purification in the manufacturer's instruction was kept and placed on ice for protein extraction. 3.5 μl of Halt protease and phosphatase inhibitor cocktail 100X, Thermo Fisher Scientific, was added, vortexed and split equally into two tubes

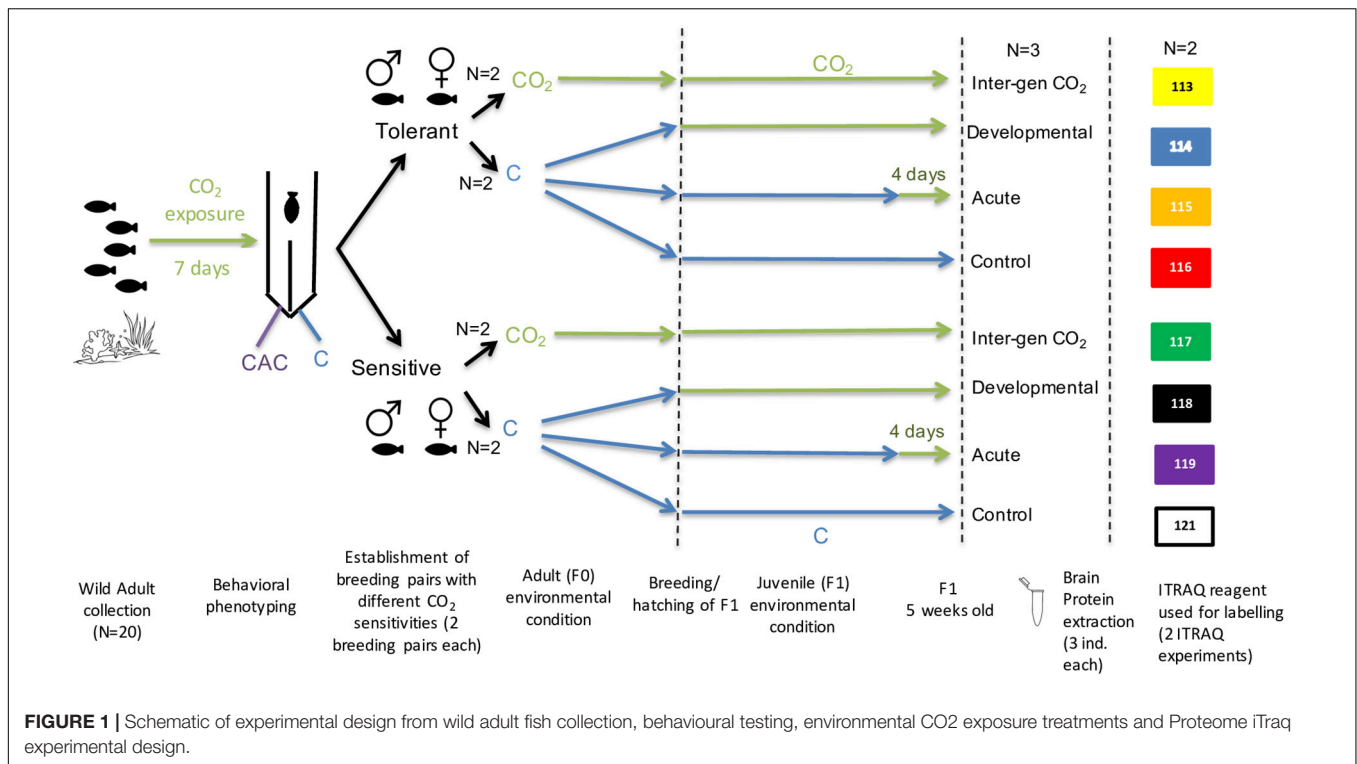
and placed on ice for 15 min. Then 1000 μl of ice-cold acetone were added and placed on ice for 30 min for protein precipitation. Samples were then spun at full speed in a centrifuge at 4°C for 10 min at full speed of 16,873 RCF. The supernatant of acetone was discarded then without disturbing the protein pellet and the samples were left to dry for 15 min and then stored at –80°C.

Protein Digestion and iTRAQ Labeling

Dried protein pellets extracted from fish brains were resuspended in 8 M urea lysis buffer with the help of a cooled stick sonicator for ten seconds at an amplitude of 20 (Fisher Scientific Q55) while kept on ice. Protein concentrations were measured using a 2-D Quant kit (GE Healthcare, United Kingdom). ITRAQ labeling allows for the relative comparison of protein accumulation across a maximum of eight labeled samples. To increase the number of biological samples and get a more representative picture we used three biological replicates of the same treatment and parental phenotype and pooled them at equal concentrations to a final volume of 100 μg. The pools of protein samples were then diluted 1:7 with 50 mM triethylammonium bicarbonate and digested using trypsin (Promega, United States) at an enzyme:protein ratio of 1:40 and left overnight at 37°C. Trifluoroacetic acid at 2% was used to inactivate the digestion and peptides were desalted using *Sep-Pak C18 cartridges* (100 mg, Water Corporation, United States). Each sample (of three pooled biological replicates) was then labeled with iTRAQ Reagents-8plex reagents (Applied Biosystems) and incubated for 60 min. All eight samples (four CO₂ treatments for two parental phenotypes) were then mixed together and dried by SpeedVac (Chandramouli et al., 2015). To further increase the representation of biological replicates to six per treatment and parental phenotype (for a total of 48 biological samples) we used two family lines and produced two complete iTraQ experiments, each with eight treatment samples (of three pooled biological samples) for a total of 16 treatment samples.

Peptide Fractionation and Mass Spectrometry Analysis

The labeled pool of peptides was fractionated by strong cation exchange chromatography (SCX)²⁶ using an Accela 1250 LC system (Thermo Scientific, United States). We obtained a total of 15 peptide fractions and then desalted, dried and resuspended them in 20 μL of LC-MS sample buffer (97% H₂O, 3% ACN, 0.1% formic acid). The samples were analyzed through three technical replicates using a Q Exactive HF mass spectrometer (Thermo Scientific, Germany) coupled with an UltiMateTM 3000 UHPLC (Thermo Scientific). Introduction of the sample into mass spectrometer was done through a Nanospray Flex (Thermo Scientific) with an electrospray potential of 1.5 kV and the ion transfer tube temperature was set at 160°C. The Q Exactive was set to perform data acquisition in the positive ion mode. A full MS scan (350–1400 m/z range) was acquired in the Orbitrap at a resolution of 60,000 (at 200 m/z) in a profile mode, a maximum ion accumulation time of 100 ms and a target value of 3 × e⁶. Charge state screening for precursor ion was activated. The ten most intense ions above a 2e4 threshold and carrying multiple charges were selected for fragmentation using higher energy



collision dissociation (HCD) and the resolution was set as 15000. Dynamic exclusion for HCD fragmentation was 20 s. Other setting for fragment ions included a maximum ion accumulation time of 100 ms, a target value of $1 \times e^5$, a normalized collision energy at 28%, and isolation width of 1.8.

Protein Identification and Quantification

Mascot generic format (*mgf*) files was used to convert the raw MS data in the Proteome Discoverer 1.4 software (Thermo Scientific) and submitted to MASCOT v2.4 (Matrix Sciences Ltd., United Kingdom) for database search against an *A. polyacanthus* brain protein dataset developed in-house from the genome assembly (Schunter et al., 2016). The mass tolerance was set to 10 ppm for precursors, and 0.5 Da for the MS/MS fragment ion with a maximum allowance of one missed cleavage. Variable modifications included 8-plex iTRAQ at tyrosine and oxidation at methionine. The fixed modifications were set to methylethanethiosulfonate at cysteine and lysine, and 8-plex iTRAQ at N-terminal. The MASCOT result files were processed using Scaffold v4.1.1 (Proteome Software Inc., United States) software for validation of peptide and protein identifications with a threshold of 95% using the Prophet algorithm with Scaffold delta-mass correction. iTRAQ label-based quantitation of the identified proteins was performed using the Scaffold Q+ algorithm. The intensities of all labeled peptides were normalized across all runs (Zhang et al., 2010) and individual quantitative data acquired in each run were normalized using the i-Tracker algorithm (Shadforth et al., 2005; Tian et al., 2011). Peptide intensity was normalized within the assigned protein. The reference channel was normalized to produce a 1:1 fold change,

and the iTRAQ ratios were then transformed to a log scale. *P*-values were calculated using a paired *t*-test with significance level of 0.05. We allowed for missing data in one of the three technical replicates and accepted differential accumulation at a fold-change level of 1.5 (consistent over technical replicates).

Differential Protein Accumulation

We performed two different iTRAQ experiments including two family lines for differential protein accumulation among treatments. The final number of proteins varied between 2,100 to 2,300 with a total of 1,862 proteins overlapping among the two iTRAQ experiments/family lines. Protein quantification was only considered valid if minimum two technical measurements were valid. As we are measuring relative protein accumulation between samples, we used average values of technical replicates and normalized these against the control replicate for each biological replicate and protein. Each labeled iTRAQ replicate was compared to the seven other iTRAQ replicates (each including 3 biological pooled samples) for all commonly measured proteins. Significant differential accumulation was accepted at a fold change level of 1.5 between two replicates. Differential accumulation for each treatment comparison was consolidated among both iTRAQ experiments and was called when both family lines (represented by the two iTRAQ experiments) showed differential accumulation for the particular treatment comparison. In cases where only one of the two families showed differential accumulation but the other technical replicates in the family have a trend of up/down regulation, the overall average of all technical replicates within the two families was considered. Hence, the sum of relative protein accumulation values of all technical replicates

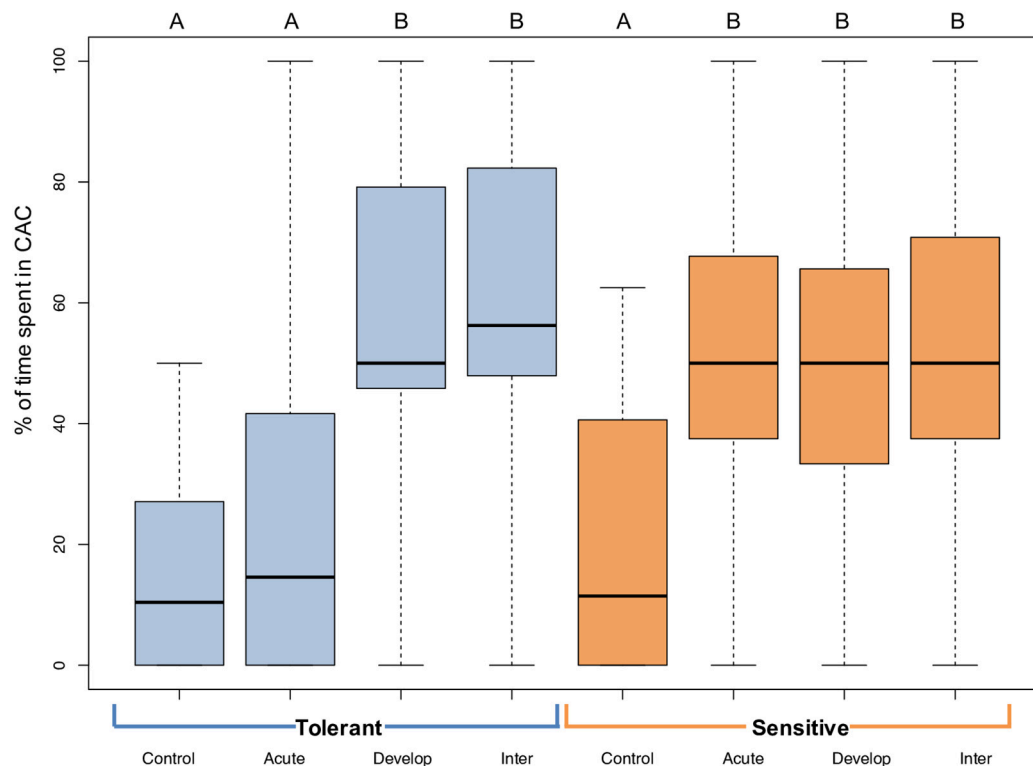


FIGURE 2 | Behavioral responses to chemical alarm cues (CAC) of 5-week-old juvenile *A. polyacanthus* exposed to different CO₂ treatments. Offspring of tolerant parental phenotype are in blue and sensitive in orange. Control, Acute, Develop, and Inter are the four different environmental CO₂ exposures. The thick black line represents the median, the lower line of the colored box is the first quartile and the top line of the box represents the third quartile; whiskers show minimum and maximum values. Different letters represent significant differences between treatments (Tukey, $P < 0.05$). $N = 15$ – 20 for each of the eight treatments - phenotypes combinations.

was divided by the number of valid technical replicates. The proteins were considered to be differentially accumulated if the average of the technical replicates showed 1.5-fold change in accumulation difference. Functional annotation of detected proteins was performed on the whole list of proteins obtained by using the in-house *A. polyacanthus* protein database and annotated with gene ontology terms in Blast2GO using an FDR significance cutoff of 0.05 (Conesa et al., 2005).

RESULTS

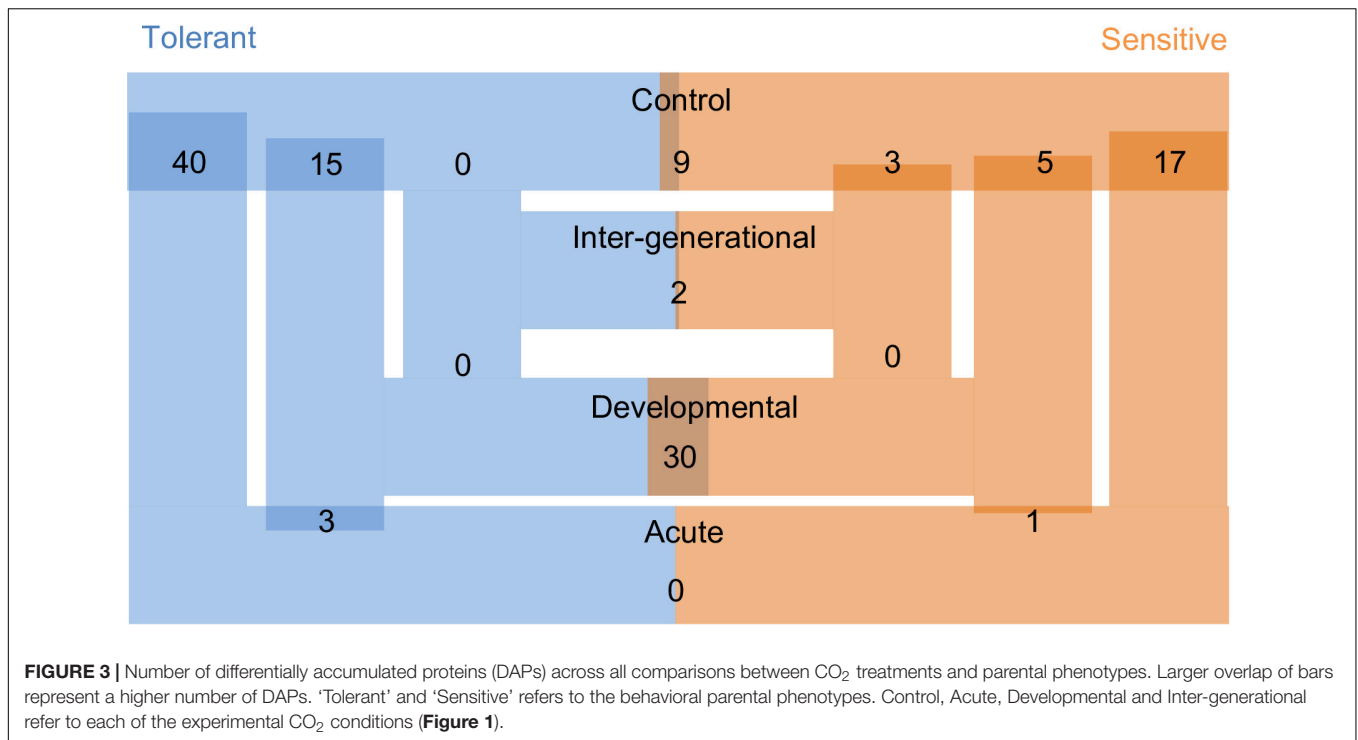
Behavioral Responses

The behavioral response of 5-week-old *A. polyacanthus* to CAC was variable among individuals within the same CO₂ treatment (Figure 2). Nevertheless, there were significant differences among CO₂ treatments and parental phenotypes. At control conditions, the average percent of time spent by offspring of tolerant and sensitive parent in CAC was 14.9 and 20.3%, respectively. In the acute elevated CO₂ treatment, offspring of sensitive parents ($W = 647$, $P \leq 0.001$), but not tolerant parents ($W = 870.5$, $P = 0.243$) spent significantly more time in CAC compared with controls (Figure 2). For both parental phenotypes, the developmentally and the inter-generationally

elevated CO₂ treated offspring showed a significant difference in behavior when compared with the offspring in control condition (T Dev: $W = 155$ and $P \leq 0.001$; T Inter: $W = 334$ and $P \leq 0.001$; S Dev: $W = 833.5$ and $P \leq 0.001$; S Inter: $W = 673$ and $P \leq 0.001$). When comparing directly between parental phenotypes, only the acute elevated CO₂ treatment resulted in a significant difference with more time spent in CAC for the offspring of sensitive parents ($W = 563.5$, $P < 0.001$).

Proteome Responses to Acute Elevated CO₂ Conditions

Over 1,800 proteins were detected and commonly accumulated among the two-family lines. We first considered the offspring of the two parental phenotypes separately to understand the reaction to the different CO₂ treatments against the respective control individuals. We detected between 0 and 40 differentially accumulated proteins among the pair-wise comparisons of different elevated CO₂ exposures (Figure 3). Individuals exposed to acute elevated CO₂ for five days exhibited a total of 17 and 40 differentially accumulated brain proteins in comparison to the control group for the sensitive and tolerant parental group, respectively (Figure 3). Fifteen proteins were found commonly differentially accumulated in both groups, representing 37.5% and 88% in tolerant and sensitive groups, respectively, showing



a common response to acute exposure to elevated CO₂ (Table 1). Fourteen out of the 15 proteins were accumulated at lower levels in the acute treatment than in the control condition. One of these proteins was B-cell receptor-associated protein 31-like isoform 1 (BCAP31), which is involved in the expression of MHC class I protein and the negative regulation of GABAergic synapses density (Elmer and McAllister, 2012). Three down-regulated proteins are involved in histone modifications. This includes the non-histone chromosomal protein HMG-14A-like isoform X2 (HMGN2) which is involved in phosphorylation inhibition of nucleosomal histones H3 and H2A; chromobox protein homolog 1-like (CBX1) which is associated with histone binding; and prothymosin alpha-B-like (PTMAB) which plays a role in histone methylation. Expression of two histone proteins (H1 and H2) were also differentially accumulated in the short-term elevated CO₂ treatment.

Proteome Responses to Longer-Term CO₂ Exposure

The longer-term developmental elevated CO₂ exposure resulted in a less pronounced protein response when compared with the respective control individuals, with fewer differentially accumulated proteins than in the acute CO₂ treatment (Figure 3). There was also no overlap in the differential accumulation among the offspring of sensitive and tolerant parents for this CO₂ treatment (Table 1). In the offspring of sensitive parents, five proteins were differentially accumulated with one accumulated at a lower level and four at a higher level, including CB1 cannabinoid receptor-interacting protein 1 (CNRIP-1), which is related to synaptic plasticity. Fifteen

proteins were differentially accumulated in the offspring of tolerant parents, none of which were differentially accumulated in the offspring of sensitive parents (Figure 3 and Supplementary Table S1). Two down-regulated proteins, vacuolar fusion CCZ1 like protein (CCZ1) and vascular cell adhesion protein (VCAM-1), are functionally related to calcium ion binding and organ development, respectively (Supplementary Table S1). 60S ribosomal protein L37 (RPL37), chromobox protein homolog 1-like (CBX1) and prothymosin alpha-B-like (PTMAB) in the offspring of tolerant parents showed similar down-regulation patterns as seen for fish in the acute CO₂ treatment. Further proteins found to be differentially accumulated between the more longer-term developmental treatment and the acute exposure are several collagen alpha chain isoforms (COL1A1 and COL1A2; Table 1) as well as fructose-biphosphate aldolase C (ALDOC) for the offspring of tolerant parents whereas only one protein was differentially accumulated for the offspring of sensitive parents (IQ motif and SEC7 domain-containing protein).

The acute and developmental CO₂ treatments reflected the within-generational responses. In the offspring of sensitive parents, 17 proteins were differentially accumulated in the acute CO₂ condition while only five were differentially accumulated in the developmental CO₂ condition and none overlap between the two comparisons (Figure 3 and Supplementary Table S1). As for the offspring of tolerant parents, five proteins were commonly differentially accumulated for the acute and developmental CO₂ condition against the control condition which include functions such as immune response and epigenetic control (RPL37, PTMAB, MARCKS, CBX1, and COL1A1; see Supplementary Table S1).

Inter-Generational Exposure to Elevated CO₂

To determine the effects of parental CO₂ exposure on the offspring brain protein accumulation, we compared the inter-generational CO₂ treatment with the control condition for both parental groups. No proteins were found to be differentially accumulated in the tolerant group, whereas three proteins were differentially accumulated in the sensitive group (Table 1). The two down-regulated proteins are prothymosin alpha-B-like (PTMAB) and cytochrome c oxidase subunit 5A mitochondrial-like protein (COX5A). The third (up-regulated) protein, nascent polypeptide-associated complex subunit complex alpha muscle specific form-like protein (NAC-alpha), is functionally related to the TBP-class protein binding.

Differential Responses due to Parental Phenotype

In our experiment we were also able to directly compare differential brain protein accumulation between the offspring of the two parental phenotypes in the same environmental treatment (Figure 3). In control condition, nine proteins differed significantly in accumulation between offspring of sensitive and of tolerant parents of which five were accumulated at lower levels and four at higher levels for the offspring of sensitive parents in comparison to the offspring of tolerant parents (Supplementary Table S2). Both parental phenotypes reacted similarly in the acute elevated CO₂ treatment as there was no significant difference in protein accumulation directly between the offspring of the two parental phenotypes for this environmental condition (Figure 3). The similar response between offspring of tolerant and sensitive parents in the acute elevated CO₂ treatment is also shown by the previously described overlap of differentially accumulated proteins when comparing between environmental conditions of acute and control CO₂ (Table 1). By contrast, 30 proteins were differentially accumulated between the offspring of the two parental phenotypes in the developmental elevated CO₂ treatment. The majority of the differentially accumulated proteins (83%) were found at a higher level in the offspring of tolerant parents (Supplementary Table S2). As for the inter-generational CO₂ condition, only two proteins were differentially accumulated between the offspring of the two parental phenotypes. The function of one of the proteins is not known. The other protein was AMP deamase 2-like isoform X1 protein (AMPD2), which is functionally related to the cyclic purine nucleotide metabolic process and energy homeostasis.

The different responses on the brain protein level for offspring of both parental phenotypes as well as for the different environmental treatments can be visualized in four recurring differentially accumulated proteins, including ribosomal protein L37 (RPL37), chromobox protein homolog 1-like (CBX1), cytochrome c oxidase subunit 5A (COX5A) and prothymosin alpha-B-like (PTMAB; Figure 4). In the acute elevated CO₂ treatment, all four proteins were down-regulated in offspring of both sensitive and tolerant parental phenotype (Figures 4A–D). However, in the developmental elevated CO₂ treatment, the proteomic responses of the offspring from the two parental

phenotypes diverged. RPL37 and CBX1 were down-regulated in the offspring of tolerant parents, whereas the accumulation of these proteins was at control levels in offspring of sensitive parents (Figures 4A,B). The diverged proteomic responses between the two parental phenotype groups were further detected in the inter-generational elevated CO₂ treatment. COX5A and PTMAB were down-regulated in the offspring of sensitive parents (Figures 4C,D), whereas the accumulation of these proteins was at control levels in the offspring of tolerant parents.

DISCUSSION

Measuring brain proteome accumulation of organisms exposed to elevated CO₂ conditions for different durations, within and between generations, can provide insight into how ocean acidification affects biological processes over relevant timescales. In this study we tested the effects of elevated CO₂ treatment and parental phenotype on protein accumulation in the brain of five-week old developing juvenile *Acanthochromis polyacanthus*. Previous studies have investigated transcriptional effects in the fish brain, but at five months of age, whereas here we particularly focus on the developmental stage of a young juvenile and investigate the protein molecular response. Protein accumulation varied greatly among the different CO₂ treatments, as well as between offspring from the two parental phenotypes, revealing treatment-specific responses to ocean acidification conditions in the brain proteome. This is consistent with previous results on the transcriptional level, where responses largely depended on the fish's life stage and the duration of the exposure (Schunter et al., 2018).

The acute (5 day) elevated CO₂ treatment resulted in the largest change in brain protein abundance of all treatments. Acutely treated fish were also the only group with a partial overlap in proteins response between the parental phenotypes. This reveals a large and partially common stress response to an acute change in environment CO₂. We found changes in the accumulation of stress-related proteins, for example the cytochrome c oxidase subunit 5A (COX5A) which regulates cell activation and differentiation (Tarasenko et al., 2017). Studies in fish have shown that highly oxidative organs such as the brain depend on cytochrome c oxidase for normal functioning as it serves as a marker for neuronal activity (Wong-Riley, 2012) and Cox deficient fish displayed neurological abnormalities (Diaz, 2010). Another stress-related response in the acute treatment was the differential regulation of proteins involved in epigenetic regulation. Amelioration of environmental CO₂ changes by epigenetic regulation might be seen in our juvenile fish through the downregulation of several proteins linked to histone regulation. Despite the relatively small number of differentially accumulated proteins (maximum of 40 proteins) it represents more than 2% of the recovered proteins with this shot-gun proteomics method. We found differential protein accumulation in histone H1-like, together with PTMAB which binds to linker histone H1 (Karetsou et al., 1998; Zakharova et al., 2011), which also exhibited higher transcriptional levels in brains of five-month-old *A. polyacanthus* with elevated CO₂

TABLE 1 | Differentially accumulated proteins (DAPs) in the brain of 5-week-old juvenile *A. polyacanthus* exposed to four different CO₂ treatments: Acute (*n* = 6) vs. Control (*n* = 6), Developmental (*n* = 6) vs. Control (*n* = 6), and Inter-generational (*n* = 6) vs. Control (*n* = 6) for each parental phenotype (sensitive and tolerant).

Comparison	Parental phenotype	
	Sensitive	Tolerant
Acute vs. Control	60S ribosomal protein L37 (RPL37)	
	armadillo repeat protein deleted in velo-cardio-facial syndrome homolog (ARVCF)	
	B-cell receptor-associated protein 31-like isoform 1 (BCAP31)	
	brain acid soluble protein 1 homolog (BASP1)	
	chromobox protein homolog 1-like, partial (CBX1)	
	cold-inducible R-binding protein A-like isoform X1 (CIRBP)	
	cytochrome c oxidase subunit 5A, mitochondrial-like (COX5A)	
	high mobility group nucleosome-binding domain-containing protein 3 isoform X3 (Hmgn3)	
	histone H2A.x-like (H2AX)	
	non-histone chromosomal protein HMG-14A-like isoform X2 (HMGN2)	
	prothymosin alpha-B-like (PTMAB)	
	ras GTPase-activating protein-binding protein 2 isoform X1 (G3BP2)	
	ras-related protein Rab-5A (RAB5A)	
	THO complex subunit 4-like isoform X1 (THO4)	
	histone H1-like (HCTB)	
	ERC protein 2-like isoform X1 (ERC2)	
	protein KIAA1045 homolog (PHF24)	
		palmdelphin-like (PALMD)
		Phosphorylase b kase gamma catalytic chain, skeletal muscle isoform (PHK-gamma-M)
		Potassium voltage-gated channel subfamily H member 4 (KCNH4)
		activated RNA polymerase II transcriptional coactivator p15 (SUB1)
		catenin alpha-2-like isoformX1 (CTNNA2)
		chromatin target of PRMT1 protein (CHTOP)
		collagen alpha-1(I) chain-like isoform X1 (COL1A1)
		collagen alpha-2(I) chain isoform X1 (COL1A2)
		C-terminal-binding protein 1-like (CTBP1)
		cytochrome c oxidase subunit 5A, mitochondrial-like isoform X1 (COX5A)
	gelsolin-like (EWAM)	
	histone-lysine N-methyltransferase setd3 (hSETD3)	
	IQ motif and SEC7 domain-containing protein 1-like (BRAG2)	
	IST1 homolog isoform X1 (hIST1)	
	myristoylated alanine-rich C-kise substrate (MARCKS)	
	neuromodulin (GAP43)	
	neuropilin-2-like isoform X1 (NRP2)	
	protein NDRG3 isoform X2 (NDRG3)	
	protein phosphatase 1H (PPM1H)	
	protein SOGA3-like (SOGA3)	
	protein transport protein Sec61 subunit beta (SEC61B)	
	ras-related protein Rab-5C-like (RAB5C)	
	spectrin beta chain, erythrocytic-like (SPTB1)	
	talin-1 (TLN1)	
	WD repeat-containing protein 37 (WDR37)	
Acute vs. Development	IQ motif and SEC7 domain-containing protein 1-like (IQEC1)	collagen alpha-1(I) chain-like isoform X1 (COL1A1) collagen alpha-2(I) chain isoform X1 (COL1A2) fructose-bisphosphate aldolase C-like (ALDOC)
Developmental vs. Control	tropomyosin beta chain isoform X2 (TPM2) 40S ribosomal protein S17, partial (RPS17) actin, cytoplasmic 2-like isoform X1 (ACTG) CB1 cannabinoid receptor-interacting protein 1 (CNRIP-1) serine/threonine-protein kinase MRCK alpha isoform X1 (MRCK alpha)	

(Continued)

TABLE 1 | Continued

Comparison	Parental phenotype
	myosin heavy chain, fast skeletal muscle
	vascular cell adhesion protein 1-like (VCAM-1)
	60S ribosomal protein L37 (RPL37)
	protein LSM12 homolog isoform X1 (LSM12)
	myristoylated alanine-rich C-kise substrate (MARCKS)
	CAAX prenyl protease 1 homolog (FACE1)
	myosin heavy chain, fast skeletal muscle-like
	myosin heavy chain, fast skeletal muscle-like
	vacuolar fusion protein CCZ1 like protein (CCZ1)
	myosin heavy chain, fast skeletal muscle-like
	glycogen synthase kise-3 beta isoform X1 (GSK-3 beta)
	prothymosin alpha-B-like (PTMAB)
	chromobox protein homolog 1-like, partial (CBX1)
	collagen alpha-1(I) chain-like isoform X1 (COL1A1)
cyclin-dependent kise-like 5 (CDKL5)	
Transgenerational vs. Control	prothymosin alpha-B-like (PTMAB)
	cytochrome c oxidase subunit 5A, mitochondrial-like (COX5A)
	nascent polypeptide-associated complex subunit alpha, muscle-specific form-like (NAC-alpha)(skNAC)

Colored background represents higher levels of accumulation in the elevated CO₂ treatment when compared to Control. For the comparison between Developmental and Acute the colored background represent higher accumulation in the Developmental treatment. Differential accumulation analysis was performed separately for each parental phenotype against the respective control individuals. Top rows in the table show DEPs for each comparison significantly differentially accumulated for offspring of both parental phenotypes.

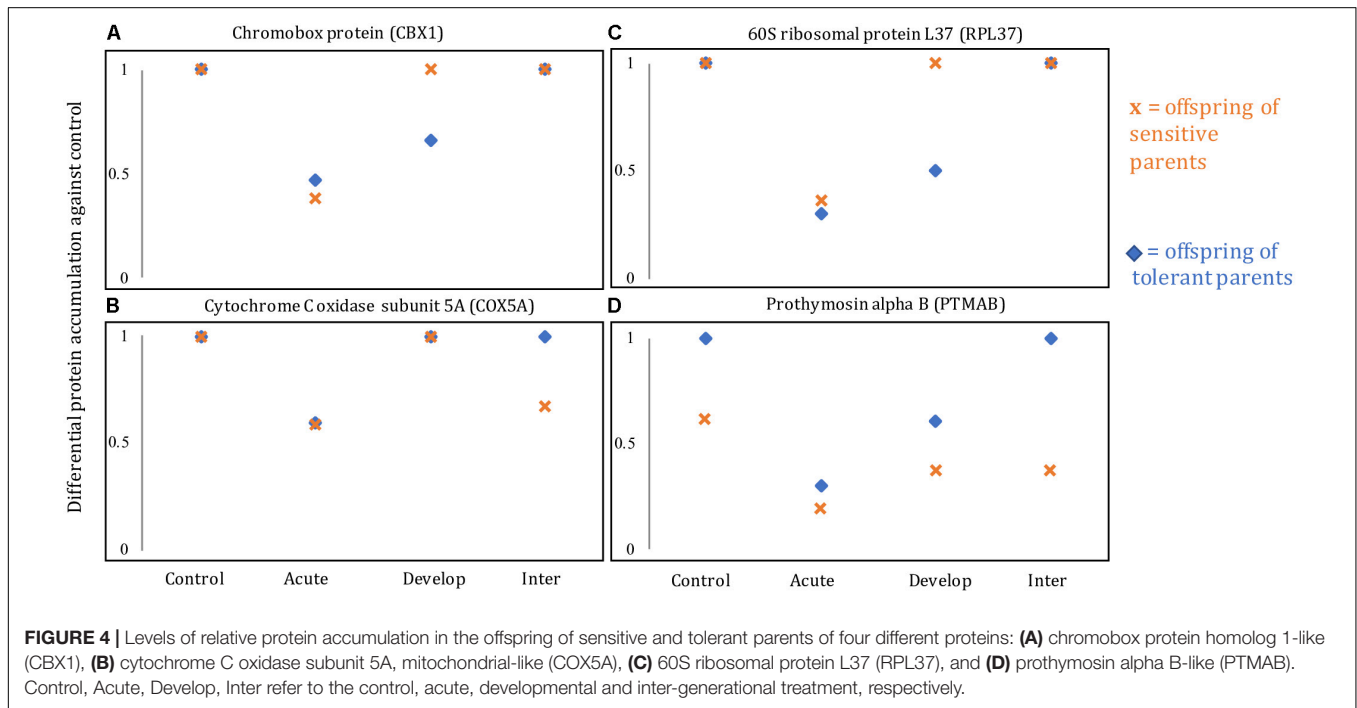


FIGURE 4 | Levels of relative protein accumulation in the offspring of sensitive and tolerant parents of four different proteins: (A) chromobox protein homolog 1-like (CBX1), (B) cytochrome C oxidase subunit 5A, mitochondrial-like (COX5A), (C) 60S ribosomal protein L37 (RPL37), and (D) prothymosin alpha B-like (PTMAB). Control, Acute, Develop, Inter refer to the control, acute, developmental and inter-generational treatment, respectively.

exposure (Schunter et al., 2016). The differential regulation of the genome, including silencing, enhancing and splicing of genes, relies heavily on these histone modifiers (Bird, 2002) and has been shown to enable plastic responses to environmentally induced changes (Turner, 2009). The regulation patterns of epigenetic markers at different life stage reveals an important molecular

mechanism by which individuals respond to acute environmental change in CO₂ conditions.

In contrast, during the longer-term developmental exposure to elevated CO₂ the functions of differentially accumulated proteins were related to neurological development in juvenile *A. polyacanthus*. The CB1 cannabinoid receptor-interacting

protein 1 (CNRIP1), for example, is a protein involved in synaptic plasticity and modulates neurotransmission (Howlett et al., 2010) and was differentially accumulated in the offspring of parents that were behaviourally sensitive to elevated CO₂. Previous studies on zebrafish revealed that CNRIP1 is highly expressed in the nervous system, particularly in developing fish, and CNRIP1 interacts with the cannabinoid receptor 1, a receptor critical for the functional development of vision and locomotion (Fin et al., 2017; Oltrabella et al., 2017). In the offspring of tolerant parents, we further found differentially accumulated proteins, such as the vascular cell adhesion protein (VCAM-1), to be involved in cellular process and organ development (Kong et al., 2018). One of the few studies on proteomics in fish in the context of ocean acidification found glycolysis to be a key process that is altered with elevated CO₂ treatment (Araújo et al., 2018) and for *Acanthochromis* offspring of tolerant parents glycogen synthase is differentially accumulated in the brain. Between the acute and developmental treatments we also detected differentially accumulated proteins that are involved in glucose metabolism (ALDOC) as well as proteins connected to connective tissue, such as collagen and tropomyosin, which is important in brain development as well as brain repair and nerve regeneration (Gregorio et al., 2018) revealing differences among a short-term and longer-term treatment. Our results show that glycolysis as well as some neurological pathways and the structure in the brain could be affected by the differential accumulation of relevant proteins in particular when exposed to elevated CO₂ during development.

Few proteins were differentially accumulated when offspring experienced developmental exposure to elevated CO₂ in combination with previous exposure of the parents to elevated CO₂, revealing an effect of parental exposure on the proteome of the offspring. Only two proteins, prothymosin alpha-B-like (PTMAB) and cytochrome c oxidase subunit 5A (COX5A), were down-regulated independent of parental exposure, but only in the offspring of sensitive parents. Similarly, in the European seabass an immune gene stayed upregulated in the olfactory epithelium despite previous parental exposure to ocean acidification (Mazurais et al., 2020). However, in our study all other differentially accumulated proteins in the within-generational treatments (acute and developmental) were at control levels in the inter-generational treatment. This hints to a non-genetic effect exerted by parents on the offspring through maternal provisioning and/or epigenetic inheritance (Bonduriansky et al., 2012; Salinas et al., 2013). Such parental effects have widely been recognized and has, in some cases, been directly proven to increase offspring's fitness (Evans et al., 2014). The molecular adjustments in fish exposed to ocean acidification conditions are of lesser magnitude when their parents were also exposed to elevated CO₂, both on the transcriptional level (Schunter et al., 2018) as well as seen here on the protein level. Despite this influence of previous parental exposure to elevated CO₂ on the proteome, behavioral responses to chemical alarm cues (CAC) remained impaired in the inter-generational treatment. This is consistent with a previous study on the effect of elevated CO₂ on behavior, based on a larger sample size, where previous parental treatment revealed no difference in the

reaction to CAC (Welch and Munday, 2017). Hence, we see a change in the proteome response of juvenile damselfish with parental exposure, but this is not reflected in the behavioral response. It has to be noted that although the iTraQ method allows for shot-gun proteomics in non-model organisms, there are constraints to the methodology, in particular the limitation to compare only eight labeled protein libraries. Therefore, the measured protein response is based on pooled biological samples and represents averages for the environmental treatments and parental phenotypes. Nonetheless, we exhibit inter-generational acclimation on the molecular level in the brains of *A. polyacanthus*, but this does not alleviate the behavioral impairments to ocean acidification.

Previous studies have linked behavioral impairments in fish under elevated CO₂ conditions to the altered functions of GABA neurotransmitter receptor (Nilsson et al., 2012; Schunter et al., 2019). One previous study in *A. polyacanthus* found highly up-regulated expression of GABA related genes in acute and developmental exposure to elevated CO₂ (Schunter et al., 2018). In our study of five-week-old fish, there were no differentially accumulated proteins related to GABA receptor functions. Our experiment recovered a subset of approximately 2,000 proteins, in which we did detect gamma-aminobutyric acid receptor-associated proteins and aldehyde dehydrogenase family 9 member A1-B-like protein, previously found to be differentially expressed on the transcript level in older fish (Schunter et al., 2016); however, in our 5-week old fish brains these proteins were not differentially accumulated. Our behavioral trials showed that after acute CO₂ exposure our five-week old fish differed in their behavioral responses to CAC between the two different parental phenotypes. Despite this difference in behavior, these fish exhibited no differentially accumulated proteins, when directly compared, revealing that under short-term elevated CO₂ fish of both parental phenotypes exhibit some similarity in their reaction at the brain protein level. For any other CO₂ treatment (developmental or inter-generational), we do find distinct protein accumulation patterns between offspring from the different parental phenotypes. Hence in our five-week old fish the signal found in the brain proteome and the behavior does not show a highly concordant pattern. The connection between impaired behavior and protein responses to environmental elevated CO₂ therefore remains unclear and it is possible that the brain protein accumulation is not representative of behavioral patterns due to the need for biological sample pooling in this proteomics method or the extended time needed for protein synthesis. Nevertheless, our results show that: (1) developing juveniles are affected in their brain proteins accumulation by near-future elevated CO₂ conditions, (2) the impact on protein accumulation depends on the length of exposure to elevated CO₂ exposure, with fewer differentially accumulated proteins being exhibited in longer-terms and inter-generational treatments, and (3) variation of parental sensitivity to behavioral effects of elevated CO₂ affect the brain proteome of juveniles, especially under developmental exposure to elevated CO₂. This variation in molecular response in a juvenile fish reveals a wide repertoire of plastic and inter-generational responses to elevated CO₂ which could aid a coral reef fish to adjust to near-future ocean acidification conditions.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data acquired during this experiment have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020350 and doi: 10.6019/PXD020350.

ETHICS STATEMENT

The animal study was reviewed and approved by James Cook University (JCU) ethics permit A1828.

AUTHOR CONTRIBUTIONS

MW and PM designed and managed the fish rearing experiments. MW performed the fish behavioral phenotyping. CS extracted the proteins, designed the protein accumulation experiment, prepared the i-TRAQ libraries, and identified the proteins. HT and CS analyzed protein accumulation data and interpreted the results. HT, CS, PM, and TR wrote the manuscript and all authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00605/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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