An interplay between plasticity and parental phenotype determines impacts of ocean acidification on a reef fish

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Introductory paragraph

The impacts of ocean acidification will depend on the ability of marine organisms to tolerate, acclimate, and eventually adapt to changes in ocean chemistry. Here, we use a unique transgenerational experiment to determine the molecular response of a coral reef fish to short-term, developmental, and transgenerational exposure to elevated CO_2 and to test how these responses are influenced by variations in tolerance to elevated CO_2 exhibited by the parents. Within-generational responses in gene expression to end of century predicted CO_2 levels indicate that a self-amplifying cycle in GABAergic neurotransmission is triggered, explaining previously reported neurological and behavioural impairments. Furthermore, epigenetic regulator genes exhibited a within-generation specific response, but with some divergence due to parental phenotype. Importantly, we find that altered gene expression for the majority of within-generation responses returns to baseline levels following parental exposure to elevated CO_2 conditions. Our result show that both parental variation in tolerance and cross-generation exposure to elevated CO_2 are crucial factors in determining the response of reef fishes to changing ocean chemistry.

Keywords: Developmental plasticity, Parental effects, Epigenetic regulation, Ocean acidification, Transcriptomics, Adaptation.

Introduction

Increased uptake of anthropogenic CO_2 by the oceans and the seawater acidification it causes will have detrimental effects on many marine organisms[1]. Laboratory experiments have already provided evidence of a diverse range of responses and effects of ocean acidification conditions[2–4], including altered growth rates, survival, and reproduction[5,6]. Fish and other marine organisms can also exhibit behavioural changes that could affect survivorship[7,8], including vital responses to chemical alarm and predator cues[9–14]. The underlying cause of these behavioural impairments is thought to be changed concentrations of acid-base relevant ions to prevent acidosis under elevated CO_2, which in turn affects the function of gamma-aminobutyric acid (GABA) neurotransmitter receptors in the brain[14–16].

To date, most observations regarding impacts of ocean acidification come from short-term experiments that do not account for population heterogeneity and individual variation in tolerance potentially important to adaptive processes[17,18]. Acutely exposing animals to elevated CO_2 for days to weeks cannot predict the potential for long-term acclimation and adaptation[18]. In particular, conditions experienced early in life can affect responses to those conditions later in life (i.e., developmental plasticity), which can be mediated by epigenetic modifications[19]. The environment experienced by the parents can also influence how offspring respond[20–22]. In fact, recent transgenerational studies

47 demonstrate recovery of metabolic and growth rates in juvenile fish when both parents
48 and offspring are exposed to elevated CO_2[23,24]. Finally, individual variation in CO_2
49 tolerance could be heritable, and therefore, variation in parental tolerance to elevated CO_2
50 could influence the tolerance of their offspring[25]. Longer-term developmental studies and
51 multigenerational experiments that incorporate individual variation in tolerance are
52 needed to better understand and predict the effects of elevated CO_2 on populations and
53 their capacity to adapt[17,26].
54
55 A recent brain transcriptome study on juvenile spiny damselfish (*Acanthochromis*
56 *polyacanthus*) exposed to elevated CO_2 revealed phenotypic differences between
57 offspring of parents with behavioural tolerance versus sensitivity to elevated CO_2[27]. This
58 suggests that parental phenotype could influence the expression of developmental and
59 transgenerational plasticity to elevated CO_2 in reef fishes. To further understand the
60 mechanisms that underpin this plasticity, we investigated the effects of acute, long-term
61 developmental, and transgenerational exposure to elevated CO_2 on the molecular
62 response of juvenile spiny damselfish from behaviourally tolerant and sensitive parents.
63 We focus on the brain because altered function of $GABA_A$ neurotransmitter receptors are
64 thought to be responsible for many behavioural changes observed in fish exposed to
65 elevated CO_2[15,16]. Adult spiny damselfish were exposed to a near–future CO_2 level
66 (750μatm) and then tested for their ability to react to chemical alarm cues, a crucial
67 survival mechanism in fish[11]. Based on these results, adults were matched into
68 behaviourally 'tolerant' and 'sensitive' breeding pairs that were maintained under either
69 current-day or elevated CO_2 (Methods & Figure 1). Offspring of these pairs were reared

70 under both control and elevated CO_2 conditions for 5 months. Finally, some offspring
71 reared under control conditions from hatching were exposed to elevated CO_2 for the last 4
72 days of the experiment. This produced four different treatments for the two parental
73 phenotypes: a) control CO_2 parents – offspring reared in control conditions (control); b)
74 control CO_2 parents – offspring reared in control conditions, but with a final 4-day
75 elevated CO_2 treatment at the age of 5 months (acute CO_2 treatment), c) control CO_2
76 parents – offspring reared in elevated CO_2 from hatching (developmental CO_2 treatment);
77 d) elevated CO_2 parents – offspring reared in elevated CO_2 from hatching
78 (transgenerational CO_2 treatment) (Figure 1). We measured the genome-wide gene
79 expression in the brains of 72 individuals across all treatments to tease apart the acute
80 response to elevated CO_2 from the responses to longer-term development under elevated
81 CO_2 and differences that occur due to parental exposure to elevated CO_2. Comparing
82 these transcriptomes in offspring from two parental phenotypes allowed us to evaluate
83 how long-term and cross-generational exposure to elevated CO_2 influences the response
84 of fish to future ocean acidification conditions and the influence of individual variation in
85 tolerance to elevated CO_2 on these relationships.
86

Results

Influence of parental phenotype on the response to elevated CO_2

The offspring of behaviourally tolerant and sensitive parents exhibited significant differences in the brain transcriptome. We identified 114 differentially expressed transcripts (DETs) under acute CO_2 exposure and 359 under developmental exposure when comparing offspring from the two parental groups directly, revealing a clear influence of the parental phenotype on the offspring's response to elevated CO_2 (Figure 2, Supplementary Figure 1). The DETs expressed between offspring of the two parental phenotypes upon acute exposure were functionally enriched in pathways controlling haemoglobin and oxygen transport (Supplementary Data 1). No significantly enriched function was found for DETs between parental phenotypes in the developmental treatment.

Besides direct differential expression between offspring of the two parental phenotypes, we also compared expression within each parental group (e.g. acute treatment versus control) in order to identify transcripts with expression profiles that overlap or differ between the two parental phenotypes. While there were similarities, there were also large differences in gene expression patterns among treatments for the offspring of tolerant and sensitive parents (Supplementary Data 2). Offspring of behaviourally tolerant parents exhibited more changes in the transcriptome when acutely exposed to elevated CO_2 (3,669 DETs) compared to the developmentally exposed fish (1,142 DETs) (Figure 2). Interestingly, this pattern was reversed in the offspring of sensitive parents, where the developmental treatment exhibited mores change in gene expression (2,590 DETs)

110 compared with the acute treatment (2,010 DETs). The shared component between the
111 parental phenotypes for these treatments was as low as 27%, and few pathways were
112 commonly enriched in the brains of fish from different parental phenotypes in the
113 developmental treatment (Figure 3). In the developmental treatment, only offspring of
114 tolerant fish showed differential expression of transcripts involved in gluconeogenesis.
115 Several other pathways were enriched only in the offspring of behaviourally sensitive
116 parents, including pathways involved in nervous system development and ion transport
117 (Supplementary Data 3). We therefore found large differences, yet some overlapping
118 transcriptional responses in the offspring of the two parental phenotypes. Nonetheless,
119 the acute and developmental CO_2 treatments had larger overall effects on the
120 transcriptome than did the parental phenotype (Supplementary Figure 1).
121
122 **Short-term and developmental responses to elevated CO_2**
123 Exposure of offspring to a near-future elevated CO_2 level resulted in large differences in
124 gene expression compared with control offspring reared at the current-day CO_2 level
125 (Figure 2). Offspring of behaviourally tolerant parents that were acutely exposed to
126 elevated CO_2 for 4 days exhibited the greatest number of DETs (3,669) compared to
127 control fish (14.5% of the entire brain transcriptome). In this acute treatment, about half
128 of the DETs (51% and 49% for offspring of tolerant and sensitive parents respectively)
129 were expressed at higher levels and resulted in more significant functional enrichments
130 than the transcripts upregulated in controls (Figure 3). Comparing DETs in the acute
131 treatment with those differentially expressed in longer-term treatments enabled us to
132 distinguish rapid, short-term from longer-term transcriptional responses to elevated CO_2.

133 For this comparison we considered the transcripts that were differentially expressed in
134 acutely-treated compared with control fish, but which were not differentially expressed in
135 developmental and transgenerationally treated fish compared to controls. Hence, these
136 DETs were unique to the acute 4-day elevated CO_2 treatment. A total of 184 genes
137 showed a clear pattern of specific short-term response that was common for both parental
138 phenotypes (Supplementary Data 4). These acute-specific genes were significantly
139 enriched in ATPase-related processes (Figure 3 & Supplementary Data 5).
140
141 Fish that were developmentally exposed to elevated CO_2 differentially expressed 1,142
142 and 2,590 transcripts, of which 56% and 78% were upregulated in offspring of tolerant
143 and sensitive parents, respectively (Figure 2). The offspring of sensitive parents had a
144 large number of enriched biological pathways that showed upregulation in the
145 developmental treatment (Figure 3). A total of 698 transcripts were commonly
146 differentially expressed in offspring of both parental phenotypes. Only 27 of these
147 transcripts were uniquely differentially expressed in the developmental CO_2 treatment,
148 regardless of parental phenotype, suggesting developmental treatment specificity
149 (Supplementary Data 6). These transcripts were at control expression levels in acute and
150 transgenerational treatments, but differentially expressed in the developmental treatment.
151 Of these transcripts, 23 showed downregulated expression in the developmental treatment
152 when compared to the controls.
153
154 Importantly, in both the acute and developmental treatments we found a common set of
155 highly upregulated transcripts involved in neurotransmitter secretion, nervous system
156 development, ionotropic glutamate receptor activity, and $GABA_A$ receptor activity

157 (Figure 3). This upregulation was specific to within-generation treatments, including
158 acutely exposed fish and fish reared under elevated CO_2 for 5 months from hatching.
159 Many of these DETs and associated enriched functions were also found in a weighted
160 correlation network gene cluster (Supplementary Data 7). Hence, both of these
161 independent methods revealed the importance of these genes and functions for fish
162 exposed to elevated CO_2. A clear signature came from GABAergic neurotransmission,
163 with nearly all genes in this pathway overexpressed in the acutely and developmentally
164 treated fish when compared to controls (Figure 4). These included genes involved in
165 GABA production, GABA secretion from presynaptic neurons, all of the $GABA_A$
166 receptor subunits (Supplementary Data 8), and the potassium-chloride co-transporter 2
167 (*kcc2*). Furthermore, we saw a reduction in the expression of GABA transporter 1 (*gat1*).
168
169 Another within-generation specific response involved epigenetic regulation of gene
170 expression. Here we saw common, but also divergent, responses between the parental
171 phenotypes. In the developmental treatment, there were significant differences in the
172 expression of genes involved in methylation between the offspring from different
173 parental groups. Specifically, eight DETs from the direct comparison between the
174 parental groups in the developmental treatment are involved in the control of the DNA,
175 protein, and histone methylation states (*ppme1*, *apex1*, *prmt6*, *setd2*, *kmt2a*, *mecp2*, *kmt2c*
176 & *mrm1*) (Supplementary Data 9). Differences in epigenetic related transcription patterns
177 could also be seen across different CO_2 treatments, as methylation related pathways were
178 significantly enriched in genes that were downregulated in the offspring of tolerant
179 parents, but only when offspring were acutely exposed to elevated CO_2.
180

Transcripts encoding histones also showed treatment-specific expression when considering the parental phenotypes. In the acute treatment, two isoforms of histone 1 (*h1b*, *h10*) were highly expressed in offspring of behaviourally sensitive parents (Figure 5a), but not in the offspring of tolerant parents. However, the expression for other histone variants seemed treatment-specific in fish acutely and developmentally exposed to elevated CO_2, regardless of the parental phenotype (Figure 5a). In general, the expression levels of histones were lower in fish from the developmental treatment for offspring of both parental phenotypes. It is, however, important to note that histone modifiers (e.g., histone-lysine methyltransferases; *setd2*, *kmt2a*, *kmt2c*) were upregulated in the developmental treatment for offspring of tolerant parents (Figure 5b). This suggests that epigenetic factors may play a role in the response to elevated CO_2, and that chromatin and methylation measurements should be included in future studies.

Transgenerational responses to elevated CO_2

The within-generation comparisons revealed a large number of DETs in fish that were acutely or developmentally exposed to elevated CO_2. By contrast, many of these transcripts exhibited expression levels similar to control levels in fish that were transgenerationally exposed to elevated CO_2 (Supplementary Figure 2). A total of 401 DETs in the developmental treatment were at control levels in the transgenerational treatment, regardless of parental phenotype (Figure 3b, Supplementary Data 10). The previously mentioned upregulation of histone expression was generally lower in control and transgenerational treatments and higher in the acute and developmental treatments. Furthermore, altered within-generation gene expression patterns, including the $GABA_A$ related genes, were at control levels in the transgenerational treatment. The transcripts

205 exhibiting recovery patterns, compared with increased expression during developmental
206 exposure, were functionally enriched for microtubule-related pathways (e.g., microtubule
207 proteins; *map1b*, *map4*, *futsch*, microtubule kinases; *mast3*, *mark3*, and microtubule-actin
208 crosslinking factor; *macf1*, Figure 5c). We also identified an opposite pattern of lower
209 expression levels in the developmental treatment for cytoskeleton related genes (e.g.,
210 tubulin alpha 1c; *tub1c* and microtubule associated protein light chain; *map1lc3b*).
211
212 By comparing within-generation and transgenerational CO_2 treatments, we were also able
213 to tease apart a transgenerational-specific transcriptional signature. This refers to
214 transcripts that were at control levels in acute and developmental treatments but were
215 differentially expressed in the transgenerational treatment only. The transgenerational-
216 specific signatures were divergent between offspring from the two parental phenotypes.
217 A larger transgenerational signal was found, represented by 41 transcripts, in offspring of
218 tolerant parents and 8 DETs in offspring of sensitive parents, with none overlapping
219 (Supplementary Data 11). Eleven and one of these transcripts, respectively, showed direct
220 differential expression between the two parental phenotypes in the developmental
221 treatment.
222
223 Finally, independent of the length of exposure, there were only a few brain transcripts
224 commonly differentially expressed in all elevated CO_2 treatments when compared to
225 control fish (Supplementary Figure 3). Only eight and 18 transcripts in offspring of
226 sensitive and tolerant parental phenotypes, respectively, were differentially expressed
227 across all elevated CO_2 treatments. When considering long-term treatments (i.e.,

228 excluding acute), 31 and 27 transcripts from offspring of sensitive and tolerant parents,
229 respectively, showed a clear CO_2 response (Supplementary Data 12). These CO_2-affected
230 transcripts differed in their expression patterns across parental phenotypes, with the
231 exception of *fgf1*, *shmt2*, *pck1*, *arhgef*, *phdgh* and *psat* that were differentially expressed
232 in various CO_2 exposures and common between parental phenotypes (Supplementary
233 Figure 4 & 5).
234

Discussion

Fundamental changes in the transcriptional landscape of the brain, displayed by numerous differentially expressed genes, were observed in all elevated CO_2 treatments. Nevertheless, the specific functional response depended on the duration of exposure. The 4-day acute CO_2 treatment resulted in the largest treatment-specific response. Several glycoprotein-encoding genes, including neurexophilin (*nxph1, 2* and *4*) and ependymin (*epd1*), were overexpressed in acutely-treated fish. These genes play a role in short-term neuronal plasticity, and neurexophilin has recently been linked to GABA receptor subunit expression, revealing an instructive role in configuring GABA receptors[28]. The increased expression of GABA receptor genes in the acutely treated fish could therefore also be driven by an upregulation of *nxph1* and associated inhibitory neural circuits.

When fish were reared under elevated CO_2 from hatching (i.e., developmental treatment), fewer treatment-specific responses were observed, with most genes downregulated. This was the case for reticulon-4 (*rtn4*), a neurite growth regulating factor that, in mammals, activates the growth-inhibiting Nogo receptor complex in regenerating axons[29], thus downregulating growth and inhibiting neuronal plasticity. The function of the Nogo receptor in fish is still unclear, but it was previously associated with embryonic and brain development[30]. Another possible negative effect associated with elevated CO_2 during development was the downregulation of the creatine transporter (*slc6a8*). This could cause a decrease in intracellular creatine, which plays a central role in energy homeostasis[31]. Thus, our results indicate that elevated CO_2 exposure early in life could have detrimental effects on development. This is consistent with previous studies

reporting negative effects on growth, development, and survival in juvenile fish exposed to elevated CO_2[6,16,32–34].

Fish exposed to elevated CO_2 regulate their intra- and extracellular pH to avoid acidosis, primarily via HCO_3^- accumulation[16]. Nilsson and coauthors[15] suggested this could lead to altered $GABA_A$ receptor function. Specifically, changes in transmembrane HCO_3^- and Cl^- gradients could lead to a reversal of ion fluxes through the receptor, which could explain the behavioural changes observed in fish upon elevated CO_2 exposure[35]. We observed that many GABA-related genes were highly upregulated after acute and developmental exposure to elevated CO_2, showing a common within-generation response. This pattern included genes involved in GABA production, all $GABA_A$ receptor subunits, and transporter genes (Figure 4). If $GABA_A$ receptor function becomes excitatory under elevated CO_2, the inhibitory input in neural circuits are lowered, making them overactive. This can trigger futile feedback responses aimed to reduce the over-activity by releasing more GABA and increasing the number of $GABA_A$ receptors. This will be counter-productive if GABA has started to act excitatory, thus initiating a self-amplifying (vicious) cycle. When CLCN3 and VGAT genes are upregulated, as observed, packing of GABA into synaptic vesicles could increase[36,37], thereby increasing GABA release. Exacerbation of this vicious cycle also comes from GAT1 (responsible for removing extracellular GABA) being downregulated, which would increase GABA in the synaptic cleft. These changes can explain how a small increase in CO_2, causing a moderate change in Cl^-/HCO_3^- gradients, can be amplified to cause a significant GABAergic dysfunction leading to altered behaviour. We did see one potentially

281 adaptive GABA related change; upregulation of potassium-chloride co-transporter 2
282 (*kcc2*) responsible for removing intracellular Cl^- [38], which could counteract the excitatory
283 action of $GABA_A$ receptors.
284
285 Epigenetic regulation of gene expression could underpin whole-organism responses to
286 environmental change[39]. Our results suggest regulators influences development under
287 elevated CO_2 with an additional effect of parental phenotype. One of the genes that was
288 upregulated in the offspring of sensitive parents compared with tolerant parents, arginine
289 methyltransferase 6 (*prmt6*), is known to methylate CREB Regulated Transcription
290 Coactivator 2 (CRTC2), a transcriptional activator of the gluconeogenic program[40,41].
291 Upregulated gluconeogesis through the AMPK signaling pathway, which facilitates
292 glucose uptake, would require glucose transporters. Glucose transporters, such as *gtr1*
293 (*gtr10, 3, & 8*), were indeed upregulated in developmentally-treated offspring of sensitive
294 parents. Hence, differential glucose regulation – via selective DNA methylation – could
295 cause differences in the offspring of the two parental groups.
296
297 Changes to the chromatin landscape and the alternative use of histone variants also
298 influence differences between offspring of tolerant and sensitive parents. Histone variants
299 (e.g., *h2az*) that were downregulated in the acute CO_2 treatment in offspring of tolerant
300 parents and in the developmental treatment in offspring of sensitive parents have been
301 shown to mediate responses to environmental change in animals including fish (e.g.,
302 temperature and season)[42,43]. In general, histones and histone modifiers regulate gene
303 expression by controlling chromatin dynamics, making transcription factors more or less

304 accessible[44]. We found that the general pattern for most histone variants was a decreased
305 expression in the developmental treatment; this pattern has also been identified in a
306 marine invertebrate upon elevated CO_2 exposure[45]. Additional evidence for reduced
307 transcriptional repression is the downregulation of several polycomb protein encoding
308 transcripts (e.g., Polycomb Group Ring Finger 2; *pcgf2* and SUZ12 Polycomb Repressive
309 Complex 2; *suz12b*) in the acute and developmental treatments. The polycomb repressive
310 complex chemically modifies histones, for instance, by adding methyl groups, thereby
311 repressing transcription[46]. Thus, downregulation would increase gene expression. Hence,
312 the strong developmental plasticity we see in gene expression is likely controlled in part
313 by DNA methylation and use of histone variants. We also observed that genetic variation
314 and non-genetic (epigenetic) parental effects could, to a certain extent, influence within-
315 generation control of gene expression of individual fish exposed to elevated CO_2.
316
317 Inheriting an optimized acid-base regulatory system where genes are controlled
318 epigenetically could enhance physiological performance under ocean acidification[22,24].
319 However, this seems unlikely to occur because transgenerationally CO_2-treated fish did
320 not exhibit the aforementioned differential expression of epigenetic-related genes when
321 compared to controls. In fact, it appears that histone genes and many other transcripts
322 specific to within-generation treatments were reversed through transgenerational
323 exposure. Such a recovery pattern was found for multiple microtubule-related genes,
324 implicating cytoskeleton plasticity in response to exposure to near-future CO_2 levels, a
325 finding already suggested for invertebrates[47,48]. Cytoskeleton plasticity is directly related
326 to neuronal plasticity[49], and it seems that within-generation CO_2 exposure leads to a

cytoskeletal rearrangement that can aid neuronal plasticity to return to a control state during transgenerational exposure. Further responses to stress via downregulation of *nlrc3* and the hypoxia inducible factor prolyl hydroxylase 2 (*egln1*) and upregulation of the hypoxia inducible factor 2 alpha (*epas1*), both important during oxidative stress, could become maladaptive, as we found these expression patterns, even after five months of exposure to elevated CO_2. Importantly, such responses seem to also be reversed with transgenerational exposure.

The long-term response to elevated CO_2, independent of parental phenotype, was linked to glucose metabolism. A role of the brain in regulating glucose homeostasis is becoming evident, but it was only recently shown that increased brain *fgf1* can promote blood glucose reduction[50]. All previously reported genes involved in transgenerational acclimation to elevated CO_2[27] were upregulated in our developmental and transgenerational CO_2 treatments, suggesting a delayed response to prolonged exposure rather than an immediate adaptive response. Therefore, we propose that the capacity for fish to maintain performance in acidified oceans will depend of their ability to cope with long-lasting CO_2 effects. The rebalance of gluconeogenesis and glucose homeostasis, neither of which is compensated for via transgenerational exposure, may be key to adapting to new environmental conditions.

Here, by using an integrative genomics approach coupled with a unique experimental design, we tested the response of a coral reef fish to end-of-century CO_2 levels and provide further evidence for an important role of altered GABA receptor function in the

response to elevated CO_2. In particular we demonstrated a possible vicious feedback cycle exacerbating the GABA pathway reaction to elevated CO_2, which can explain the fast neural impairment. Importantly, we identified numerous transcriptional changes in within-generation treatments that returned to baseline levels in fish that were transgenerationally exposed to elevated CO_2 levels. This emphasizes the influence of environmental exposure on the parents as well as the parental phenotype in the response of fish to future ocean acidification.

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Data availability:

All data generated, analysed or used during this study such as the RNA-seq transcriptome sequences and the *de novo* assembled reference genome have been deposited in NCBI under BioProject ID PRJNA311159

Competing financial interests

The authors declare no competing financial interests.

Methods

Adult collection and response of adult fish to elevated CO_2

Adult *Acanthochromis polyacanthus* (spiny damselfish) were collected as described in Schunter *et al.* (2016)[27] in the central Great Barrier Reef, Australia (18°38'24,3"S, 146°29'31,8"E) and exposed to 754 ± 92 µatm CO_2 levels for 7 days before behavioural testing. The behavioural phenotype was determined by exposing the adult fish to conspecific chemical alarm cues (CAC) in a two-chamber flume (30 cm x 13 cm), where time spent in the CAC was recorded. A 1:1 ratio of adult CAC donor fish to adult test fish was used. Donor fish were held in control conditions until it was euthanized by a quick blow to the head. To generate CAC, superficial cuts to both sides of the body were made after euthanization of the donor fish. The fish was then rinsed with 60 ml of control water[27], and the rinse water was added to 10 L of elevated CO_2 seawater. Elevated CO_2 water including CAC and elevated CO_2 control water were fed into the flume at a constant rate of 450 ml per minute. Each behavioural trial was run for 9 minutes (2 minutes habituation, 2 minutes recording, 1 minute switch for water sides, where the fish was recentered at the end of this minute. The 2 minutes habituation and 2 minutes recording was then repeated), and the location of the fish was recorded every 5 seconds. Adult fish exhibited a large variation in behavioural responses when tested for chemical alarm cue (CAC) recognition. These responses ranged from a normal aversion behaviour with little time spent in the CAC to the opposing behavior, where fish spent most of their time in CAC. We considered those fish exhibiting an aversion to CAC to be behaviorally 'tolerant' (< than 30% of the trial in CAC) and those exhibiting an attraction to CAC under elevated CO_2 to be behaviorally 'sensitive' (> than 70% of the trial in CAC). About

581 38% of the randomly collected fish within the population could be assigned to the
582 tolerant or sensitive groups (Supplementary Data 13). Behavioural sensitivity and fish
583 size were then used to form breeding pairs with individuals of the same sensitivity (i.e.,
584 tolerant male with tolerant female). This project was completed under James Cook
585 University (JCU) ethics permit A1828.
586
587 *Experimental design*
588 Breeding pairs were held in 40 L aquaria, with 3 tolerant and 3 sensitive pairs in control
589 conditions (414 ± 46 μatm) and 2 tolerant and 3 sensitive pairs in elevated CO_2 conditions
590 (754 ± 92 μatm, Supplementary Data 13). Breeding pairs were acclimated to their
591 respective conditions for three months prior to the breeding season. Offspring clutches
592 from breeding pairs were immediately removed from parental tanks after hatching and
593 placed into control or elevated CO_2 conditions. A total of four combinations between
594 parental and offspring conditions were processed with several parental pairs for each
595 combination to avoid a family effect (Figure 1, Supplementary Data 13). Offspring
596 conditions were: a) control conditions, b) acute elevated CO_2 treatment, in which
597 offspring developed in control conditions but were acutely exposed to elevated CO_2 for
598 the last 4 days before sacrificing, c) developmental elevated CO_2 treatment, in which
599 offspring were immediately placed into elevated CO_2 after hatching and d)
600 transgenerational elevated CO_2 treatment where parents and offspring were exposed to
601 elevated CO_2. Offspring were kept in their respective conditions (Figure 1) and sacrificed
602 at the age of 5 months.
603

604 *CO_2 treatment*

605 Experimental procedures followed those described by Welch and Munday (2017)[25].
606 Briefly, two 10,000 L recirculating aquarium systems were each set to a different pH and
607 corresponding CO_2 level: a current-day control (414 ± 46 μatm) and an end of century
608 elevated CO_2 treatment (754 ± 92 μatm)[51,52]. An Aqua Medic AT Control System (Aqua
609 Medic, Germany) was used to dose CO_2 into a 3,000 L sump to maintain the desired pH
610 in the elevated CO_2 treatment. An identical sump on the control system was not dosed
611 with CO_2. Control and elevated CO_2 water were then delivered to the holding aquaria at
612 1.5 L per minute. Temperature and pH_{NBS} were measured daily in randomised tanks.
613 Salinity and total alkalinity were measured weekly. Total alkalinity was measured by
614 Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) using
615 certified reference material from Dr. A.G. Dickson (Scripps Institution of
616 Oceanography). pCO_2 was then calculated in CO2SYS[53], using constants from Dickson
617 and Millero (1987)[54].

618

619 *RNA and transcriptome expression analyses*

620 Fish brains were immediately dissected out after euthanization, snap frozen with liquid
621 nitrogen, and stored at -80^{o}C. Whole frozen fish brains were then homogenized in RT-
622 Plus Buffer for 30 second in a Fisher bead beater with single-use silicon beads, and total
623 RNA was extracted with AllPrep DNA/RNA Mini Kits (Quiagen). The RNA quality was
624 evaluated on the nanodrop and the Agilent Tape reader, and only minimum RNA
625 integrity values (RIN) of 8 were accepted. Extracted RNA was converted into cDNA and
626 prepped for Illumina sequencing with a TruSeq RNA Illumina Library Prep Kit. Libraries

were then sequenced on an Illumina HiSeq 2500 paired end to the length of 100bp at Macrogen, South Korea. Raw reads were inspected and quality trimmed to a minimum Phred score of 30 with FastQC and Trimmomatic respectively[55,56]. High quality reads were mapped against the *de novo* assembled genome reference using Tophat 2[57] with bowtie2 very-sensitive mode and providing the coordinates of the reference based annotated transcriptome. The *A. polyacanthus de novo* genome assembly and annotation have been previously described[27]. The bam files resulting from the mapping step were then sorted with samtools[58] and read counts were extracted by using an HT-seq script[59] adding exon read counts to receive transcript-based read count values. Differential expression was statistically evaluated with DEseq2[60] in Bioconductor version 3.2 in R 3.2.1 through pair-wise treatment comparisons. Comparisons between the different treatments were performed by comparing the expression of acute, developmental, and transgenerational samples for each parental phenotype separately against the control samples. Differential expression was evaluated between the different treatments, but the expression levels of the two parental phenotypes were also directly compared for each CO_2 treatment. The significance level for differential expression was set to an FDR adjusted p-value of <0.05 with additional filters of a minimum log 2 fold expression of 0.3 and standard deviation correction (SD<Mean). Gene expression patterns across different treatments were based on significant differential expression in all pairwise comparisons.

To evaluate a potential family effect within the parental phenotypes, we compared treatments in which full siblings were exposed (comparison of control and acute as well

650 as developmental treatments for offspring of tolerant and sensitive parents). We used a
651 model comparison approach. First, differential expression was measured accounting for
652 treatment effect only, then family line was added as a factor and differential expression
653 compared. Finally, the full (treatment+family) model was compared directly with the
654 reduced model (treatment only) (Supplementary Data S14).
655
656 After stringent filtering of significant differential expression assignment, we further
657 accounted for false positive assignment through randomization. This was done on the
658 acutely and developmentally treated samples comparing the two different parental
659 phenotypes. For each CO_2 treatment parental phenotype was randomly assigned to a gene
660 expression profile and gene expression analysis was rerun. This was repeated 10 times for
661 the acute and the developmental treatments (Supplementary Data S15).
662
663 To improve insight into the complex dataset, we performed a weighted gene-correlation
664 network analysis with the WGCNA package (version 1.6) in R[61]. We used the DEseq2
665 normalized dataset of raw counts of all 72 samples included in the study. Gene
666 expression data was then variance stabilized, and transcripts with low read counts were
667 removed. Soft-thresholding power was evaluated and the highest value was accepted for
668 network construction (pow=9). This approach was used to approximate a scale free
669 topological network (TOM), which was constructed following these parameters:
670 TOMtype= "assigned', minModuleSize= 30, mergeCutHeight= 0.25. TOM was then used
671 to create a cluster dendogram. Transcripts clustered within one colour module were then

672 extracted if the module had more than 500 transcripts and compared with the
673 differentially expressed gene analysis (Supplementary Figure 6 & 7).
674
675 Blast annotations of the reference-based transcriptome and an Interpro scan were
676 imported into Blast2GO[62] to retrieve Gene Ontology terms and KEGG pathways.
677 Functional enrichment analyses were performed for differentially expressed genes as well
678 as global network clusters with Fisher's exact tests (FDR < 0.05). All tests were
679 performed on the different differential gene expression models, and results presented
680 were significantly enriched functions found with both models. Graphical representations
681 (i.e., heat maps, bubble graphs, and bar plots) were produced in R 3.3.1. A Principle
682 Component Analysis (PCA) was performed with the cloud platform WebMeV[63] using the
683 normalized expression of acutely and developmentally treated samples.
684
685 *qRT-PCR validation of RNA-seq results*
686 Quantitative Realtime PCR was performed on two sets of samples to evaluate all the
687 different experimental treatment groups. We compare control samples with
688 transgenerational elevated CO_2 exposed fish from behaviourally tolerant as well as
689 sensitive parents. We also examined the qPCR gene expression for acutely and
690 developmentally elevated CO_2 treated fish for both parental pairs and compare the
691 relative expression between treatments with the RNAseq expression differences
692 (Supplementary Figure 8). For each treatment group, two biological samples were
693 selected, which were from the same treatment, but additional biological individuals than
694 those sequenced via RNAseq. Primers were designed using the genome sequence of the

695 respective transcript of interest with Primer3Plus[64], which was checked in NCBI Primer-
696 BLAST for specificity and HPSF purified by Sigma (Sigma-Aldrich, Germany). Using
697 the high capacity reverse transcription kit by ABI (Applied Biosystems) 550ng of RNA
698 for each sample were reverse transcribed and 15ng of cDNA was used for each reaction
699 with three replicate reactions with specified reaction details[27]. For analysis, the livak
700 method was used and Delta Delta CTs were calculated by normalizing the CTs against
701 three housekeeping genes. Eight comparisons were performed: Offspring of tolerant and
702 sensitive parents were compared at the 1) control CO_2 levels, 2) acute high CO_2 levels, 3)
703 developmentally high CO_2 levels and 4) transgenerational high CO_2 levels. Treatments
704 effect were compared between acute and developmental treatments for 5) offspring of
705 tolerant parents and 6) offspring of sensitive parents. Control levels and
706 transgenereational treatment were compared for 7) offspring of tolerant parents and 8)
707 offspring of sensitive parents. Six out of eight genes used for validation were highly
708 correlated and hence showed the same expression pattern in qRT-PCR as found with
709 RNAseq (Pearson's product-moment correlation, $p<0.001$). Transcript expression of *nfil3*
710 showed an almost significant correlation (Pearson's product-moment correlation, $p<0.08$),
711 whereas *shmt1* did not correlate (Pearson's product-moment correlation, $p=0.5$).
712 However, correlation improves when removing one comparison (HC_S, Pearson's
713 product-moment correlation, $p=0.1$). This high percentage of validation shows that the
714 RNAseq results can be replicated not only with a different method, but also with different
715 biological samples from the same treatment and therefore the observed RNAseq
716 expression pattern is clearly linked to the treatment.
717
718

719 **Figure legends**

720 **Figure 1. Experimental design. Elevated CO_2 (green) was set at 750uatm, simulating end**
721 **of century CO_2 projections.** Behaviourally tolerant and sensitive parents were phenotyped
722 based on their response to chemical alarm cues (CAC) after exposure to elevated CO_2: tolerant
723 adults exhibited a normal response to CAC in an elevated CO_2 environment whereas sensitive
724 parents exhibited an impaired response to CAC. Offspring of parental pairs were then reared
725 in three different CO_2 treatments until the age of 5 months These three treatments were: current
726 day CO_2 levels as the control (control), fish reared under control conditions with 4 days
727 exposure to elevated CO_2 at 5 months of age (acute treatment), and fish reared under elevated
728 CO_2 from hatching until 5 months of age (developmental treatment). Control, acute, and
729 developmentally treated fish were siblings from three different parental pairs for both tolerant
730 and sensitive parental phenotypes. The final treatment (transgenerational treatment) consisted
731 of offspring reared in elevated CO_2 from hatching until 5 months of age that were from parents
732 maintained in elevated CO_2 for breeding.

733

734 **Figure 2. Global differential gene expression patterns between treatments.** Numbers
735 of significantly differentially expressed transcripts between pairwise comparisons of CO_2
736 treatments as well as between different parental behavioural phenotypes (T=tolerant
737 parents, S=sensitive parents). The overlap between blue and green (T and S) represent the
738 transcripts that are directly differentially expressed between the offspring of different
739 parental phenotypes.

740

Figure 3. Functional enrichment analysis of differentially expressed genes across CO_2 rearing treatments that were significant in both differential gene expression models (C = control, A = acute, DEV = developmental, TRANS = transgenerational) and different behavioural parental phenotypes, (T = tolerant, S = sensitive). A) Overrepresented gene ontologies and B) underrepresented gene ontologies (significantly more or less of this GO category in comparison to the compared treatment). The colour of the circles represents the enrichment significance, and size of circles is proportional to the number of enriched genes.

Figure 4. Gamma-aminobutyric acid (GABA) signaling pathway in the synapse between a pre- and postsynaptic neuron. Many pathway components showed differential expression in response to CO_2 treatments. The insert highlights the proposed increase of GABA release due to increased GABA packing in synaptic vesicles[37]. (Adapted from KEGG pathways). GAD= Glutamate decarboxylase 1, VGAT= GABA and glycine transporter, CLCN3=Chloride voltage-gated channel 3, KCC2= Neuronal K-Cl cotransporter, GAT1= GABA transporter 1, CACNA1A= Brain calcium channel 1, GABAAR= $GABA_A$ receptor subunits alpha, beta & gamma.

Figure 5. Expression pattern of histone-related transcripts across all CO_2 treatments. Expression levels of a) core histones, b) differential expression of histone-related transcripts between developmentally CO_2 treated fish from tolerant and sensitive offspring and c) microtubule-related transcripts. S=sensitive, T=tolerant, C=control, A=acute, DEV=developmental, TRANS=transgenerational.