

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

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6

7 **Introductory paragraph**

8 The impacts of ocean acidification will depend on the ability of marine organisms to  
9 tolerate, acclimate, and eventually adapt to changes in ocean chemistry. Here, we use a  
10 unique transgenerational experiment to determine the molecular response of a coral reef  
11 fish to short-term, developmental, and transgenerational exposure to elevated CO<sub>2</sub> and to  
12 test how these responses are influenced by variations in tolerance to elevated CO<sub>2</sub>  
13 exhibited by the parents. Within-generational responses in gene expression to end of  
14 century predicted CO<sub>2</sub> levels indicate that a self-amplifying cycle in GABAergic  
15 neurotransmission is triggered, explaining previously reported neurological and  
16 behavioural impairments. Furthermore, epigenetic regulator genes exhibited a within-  
17 generation specific response, but with some divergence due to parental phenotype.  
18 Importantly, we find that altered gene expression for the majority of within-generation  
19 responses returns to baseline levels following parental exposure to elevated CO<sub>2</sub>  
20 conditions. Our result show that both parental variation in tolerance and cross-generation  
21 exposure to elevated CO<sub>2</sub> are crucial factors in determining the response of reef fishes to  
22 changing ocean chemistry.

23

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

26

## 27 **Introduction**

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

38

39 To date, most observations regarding impacts of ocean acidification come from short-  
40 term experiments that do not account for population heterogeneity and individual  
41 variation in tolerance potentially important to adaptive processes<sup>17,18</sup>. Acutely exposing  
42 animals to elevated CO<sub>2</sub> for days to weeks cannot predict the potential for long-term  
43 acclimation and adaptation<sup>18</sup>. In particular, conditions experienced early in life can affect  
44 responses to those conditions later in life (i.e., developmental plasticity), which can be  
45 mediated by epigenetic modifications<sup>19</sup>. The environment experienced by the parents can  
46 also influence how offspring respond<sup>20-22</sup>. 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<sub>2</sub><sup>23,24</sup>. Finally, individual variation in CO<sub>2</sub>  
49 tolerance could be heritable, and therefore, variation in parental tolerance to elevated CO<sub>2</sub>  
50 could influence the tolerance of their offspring<sup>25</sup>. 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<sub>2</sub> on populations and  
53 their capacity to adapt<sup>17,26</sup>.

54

55 A recent brain transcriptome study on juvenile spiny damselfish (*Acanthochromis*  
56 *polyacanthus*) exposed to elevated CO<sub>2</sub> revealed phenotypic differences between  
57 offspring of parents with behavioural tolerance versus sensitivity to elevated CO<sub>2</sub><sup>27</sup>. This  
58 suggests that parental phenotype could influence the expression of developmental and  
59 transgenerational plasticity to elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>A</sub> neurotransmitter receptors are  
64 thought to be responsible for many behavioural changes observed in fish exposed to  
65 elevated CO<sub>2</sub><sup>15,16</sup>. Adult spiny damselfish were exposed to a near-future CO<sub>2</sub> level  
66 (750µatm) and then tested for their ability to react to chemical alarm cues, a crucial  
67 survival mechanism in fish<sup>11</sup>. 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<sub>2</sub> (Methods & Figure 1). Offspring of these pairs were reared

70 under both control and elevated CO<sub>2</sub> conditions for 5 months. Finally, some offspring  
71 reared under control conditions from hatching were exposed to elevated CO<sub>2</sub> for the last 4  
72 days of the experiment. This produced four different treatments for the two parental  
73 phenotypes: a) control CO<sub>2</sub> parents – offspring reared in control conditions (control); b)  
74 control CO<sub>2</sub> parents – offspring reared in control conditions, but with a final 4-day  
75 elevated CO<sub>2</sub> treatment at the age of 5 months (acute CO<sub>2</sub> treatment), c) control CO<sub>2</sub>  
76 parents – offspring reared in elevated CO<sub>2</sub> from hatching (developmental CO<sub>2</sub> treatment);  
77 d) elevated CO<sub>2</sub> parents – offspring reared in elevated CO<sub>2</sub> from hatching  
78 (transgenerational CO<sub>2</sub> 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<sub>2</sub> from the responses to longer-term development under elevated  
81 CO<sub>2</sub> and differences that occur due to parental exposure to elevated CO<sub>2</sub>. 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<sub>2</sub> influences the response  
84 of fish to future ocean acidification conditions and the influence of individual variation in  
85 tolerance to elevated CO<sub>2</sub> on these relationships.  
86

## 87 **Results**

### 88 **Influence of parental phenotype on the response to elevated CO<sub>2</sub>**

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

99

100 Besides direct differential expression between offspring of the two parental phenotypes,  
101 we also compared expression within each parental group (e.g. acute treatment versus  
102 control) in order to identify transcripts with expression profiles that overlap or differ  
103 between the two parental phenotypes. While there were similarities, there were also large  
104 differences in gene expression patterns among treatments for the offspring of tolerant and  
105 sensitive parents (Supplementary Data 2). Offspring of behaviourally tolerant parents  
106 exhibited more changes in the transcriptome when acutely exposed to elevated CO<sub>2</sub>  
107 (3,669 DETs) compared to the developmentally exposed fish (1,142 DETs) (Figure 2).  
108 Interestingly, this pattern was reversed in the offspring of sensitive parents, where the  
109 developmental treatment exhibited more 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<sub>2</sub> 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<sub>2</sub>**

123 Exposure of offspring to a near-future elevated CO<sub>2</sub> level resulted in large differences in  
124 gene expression compared with control offspring reared at the current-day CO<sub>2</sub> level  
125 (Figure 2). Offspring of behaviourally tolerant parents that were acutely exposed to  
126 elevated CO<sub>2</sub> 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<sub>2</sub>.

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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>A</sub> receptor activity

157 (Figure 3). This upregulation was specific to within-generation treatments, including  
158 acutely exposed fish and fish reared under elevated CO<sub>2</sub> 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<sub>2</sub>. 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<sub>A</sub>  
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<sub>2</sub> 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<sub>2</sub>.

180



181 Transcripts encoding histones also showed treatment-specific expression when  
182 considering the parental phenotypes. In the acute treatment, two isoforms of histone 1  
183 (*h1b*, *h10*) were highly expressed in offspring of behaviourally sensitive parents (Figure  
184 5a), but not in the offspring of tolerant parents. However, the expression for other histone  
185 variants seemed treatment-specific in fish acutely and developmentally exposed to  
186 elevated CO<sub>2</sub>, regardless of the parental phenotype (Figure 5a). In general, the expression  
187 levels of histones were lower in fish from the developmental treatment for offspring of  
188 both parental phenotypes. It is, however, important to note that histone modifiers (e.g.,  
189 histone-lysine methyltransferases; *setd2*, *kmt2a*, *kmt2c*) were upregulated in the  
190 developmental treatment for offspring of tolerant parents (Figure 5b). This suggests that  
191 epigenetic factors may play a role in the response to elevated CO<sub>2</sub>, and that chromatin  
192 and methylation measurements should be included in future studies.

193

#### 194 **Transgenerational responses to elevated CO<sub>2</sub>**

195 The within-generation comparisons revealed a large number of DETs in fish that were  
196 acutely or developmentally exposed to elevated CO<sub>2</sub>. By contrast, many of these  
197 transcripts exhibited expression levels similar to control levels in fish that were  
198 transgenerationally exposed to elevated CO<sub>2</sub> (Supplementary Figure 2). A total of 401  
199 DETs in the developmental treatment were at control levels in the transgenerational  
200 treatment, regardless of parental phenotype (Figure 3b, Supplementary Data 10). The  
201 previously mentioned upregulation of histone expression was generally lower in control  
202 and transgenerational treatments and higher in the acute and developmental treatments.  
203 Furthermore, altered within-generation gene expression patterns, including the GABA<sub>A</sub>  
204 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> response (Supplementary Data 12). These CO<sub>2</sub>-affected  
230 transcripts differed in their expression patterns across parental phenotypes, with the  
231 exception of *fgfl*, *shmt2*, *pck1*, *arhgef*, *phdgh* and *psat* that were differentially expressed  
232 in various CO<sub>2</sub> exposures and common between parental phenotypes (Supplementary  
233 Figure 4 & 5).

234

## 235 **Discussion**

236 Fundamental changes in the transcriptional landscape of the brain, displayed by  
237 numerous differentially expressed genes, were observed in all elevated CO<sub>2</sub> treatments.  
238 Nevertheless, the specific functional response depended on the duration of exposure. The  
239 4-day acute CO<sub>2</sub> treatment resulted in the largest treatment-specific response. Several  
240 glycoprotein-encoding genes, including neurexophilin (*nrxph1*, 2 and 4) and ependymin  
241 (*epdl*), were overexpressed in acutely-treated fish. These genes play a role in short-term  
242 neuronal plasticity, and neurexophilin has recently been linked to GABA receptor subunit  
243 expression, revealing an instructive role in configuring GABA receptors<sup>28</sup>. The increased  
244 expression of GABA receptor genes in the acutely treated fish could therefore also be  
245 driven by an upregulation of *nrxph1* and associated inhibitory neural circuits.

246

247 When fish were reared under elevated CO<sub>2</sub> from hatching (i.e., developmental treatment),  
248 fewer treatment-specific responses were observed, with most genes downregulated. This  
249 was the case for reticulon-4 (*rtn4*), a neurite growth regulating factor that, in mammals,  
250 activates the growth-inhibiting Nogo receptor complex in regenerating axons<sup>29</sup>, thus  
251 downregulating growth and inhibiting neuronal plasticity. The function of the Nogo  
252 receptor in fish is still unclear, but it was previously associated with embryonic and brain  
253 development<sup>30</sup>. Another possible negative effect associated with elevated CO<sub>2</sub> during  
254 development was the downregulation of the creatine transporter (*slc6a8*). This could  
255 cause a decrease in intracellular creatine, which plays a central role in energy  
256 homeostasis<sup>31</sup>. Thus, our results indicate that elevated CO<sub>2</sub> exposure early in life could  
257 have detrimental effects on development. This is consistent with previous studies

258 reporting negative effects on growth, development, and survival in juvenile fish exposed  
259 to elevated CO<sub>2</sub><sup>6,16,32-34</sup>.

260

261 Fish exposed to elevated CO<sub>2</sub> regulate their intra- and extracellular pH to avoid acidosis,  
262 primarily via HCO<sub>3</sub><sup>-</sup> accumulation<sup>16</sup>. Nilsson and coauthors<sup>15</sup> suggested this could lead to  
263 altered GABA<sub>A</sub> receptor function. Specifically, changes in transmembrane HCO<sub>3</sub><sup>-</sup> and  
264 Cl<sup>-</sup> gradients could lead to a reversal of ion fluxes through the receptor, which could  
265 explain the behavioural changes observed in fish upon elevated CO<sub>2</sub> exposure<sup>35</sup>. We  
266 observed that many GABA-related genes were highly upregulated after acute and  
267 developmental exposure to elevated CO<sub>2</sub>, showing a common within-generation  
268 response. This pattern included genes involved in GABA production, all GABA<sub>A</sub>  
269 receptor subunits, and transporter genes (Figure 4). If GABA<sub>A</sub> receptor function becomes  
270 excitatory under elevated CO<sub>2</sub>, the inhibitory input in neural circuits are lowered, making  
271 them overactive. This can trigger futile feedback responses aimed to reduce the over-  
272 activity by releasing more GABA and increasing the number of GABA<sub>A</sub> receptors. This  
273 will be counter-productive if GABA has started to act excitatory, thus initiating a self-  
274 amplifying (vicious) cycle. When CLCN3 and VGAT genes are upregulated, as observed,  
275 packing of GABA into synaptic vesicles could increase<sup>36,37</sup>, thereby increasing GABA  
276 release. Exacerbation of this vicious cycle also comes from GAT1 (responsible for  
277 removing extracellular GABA) being downregulated, which would increase GABA in the  
278 synaptic cleft. These changes can explain how a small increase in CO<sub>2</sub>, causing a  
279 moderate change in Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> gradients, can be amplified to cause a significant  
280 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  $\text{Cl}^-$ <sup>38</sup>, which could counteract the excitatory  
283 action of GABA<sub>A</sub> receptors.

284

285 Epigenetic regulation of gene expression could underpin whole-organism responses to  
286 environmental change<sup>39</sup>. Our results suggest regulators influences development under  
287 elevated CO<sub>2</sub> 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<sup>40,41</sup>.

291 Upregulated gluconeogenesis 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<sub>2</sub> 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)<sup>42,43</sup>. In general, histones and histone modifiers regulate gene  
303 expression by controlling chromatin dynamics, making transcription factors more or less

304 accessible<sup>44</sup>. 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<sub>2</sub> exposure<sup>45</sup>. 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<sup>46</sup>. 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<sub>2</sub>.

316

317 Inheriting an optimized acid-base regulatory system where genes are controlled  
318 epigenetically could enhance physiological performance under ocean acidification<sup>22,24</sup>.  
319 However, this seems unlikely to occur because transgenerationally CO<sub>2</sub>-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<sub>2</sub> levels, a  
325 finding already suggested for invertebrates<sup>47,48</sup>. Cytoskeleton plasticity is directly related  
326 to neuronal plasticity<sup>49</sup>, and it seems that within-generation CO<sub>2</sub> exposure leads to a

327 cytoskeletal rearrangement that can aid neuronal plasticity to return to a control state  
328 during transgenerational exposure. Further responses to stress via downregulation of  
329 *nirc3* and the hypoxia inducible factor prolyl hydroxylase 2 (*egln1*) and upregulation of  
330 the hypoxia inducible factor 2 alpha (*epas1*), both important during oxidative stress,  
331 could become maladaptive, as we found these expression patterns, even after five months  
332 of exposure to elevated CO<sub>2</sub>. Importantly, such responses seem to also be reversed with  
333 transgenerational exposure.

334

335 The long-term response to elevated CO<sub>2</sub>, independent of parental phenotype, was linked  
336 to glucose metabolism. A role of the brain in regulating glucose homeostasis is becoming  
337 evident, but it was only recently shown that increased brain *fgf1* can promote blood  
338 glucose reduction<sup>50</sup>. All previously reported genes involved in transgenerational  
339 acclimation to elevated CO<sub>2</sub><sup>27</sup> were upregulated in our developmental and  
340 transgenerational CO<sub>2</sub> treatments, suggesting a delayed response to prolonged exposure  
341 rather than an immediate adaptive response. Therefore, we propose that the capacity for  
342 fish to maintain performance in acidified oceans will depend of their ability to cope with  
343 long-lasting CO<sub>2</sub> effects. The rebalance of gluconeogenesis and glucose homeostasis,  
344 neither of which is compensated for via transgenerational exposure, may be key to  
345 adapting to new environmental conditions.

346

347 Here, by using an integrative genomics approach coupled with a unique experimental  
348 design, we tested the response of a coral reef fish to end-of-century CO<sub>2</sub> levels and  
349 provide further evidence for an important role of altered GABA receptor function in the



350 response to elevated CO<sub>2</sub>. In particular we demonstrated a possible vicious feedback  
351 cycle exacerbating the GABA pathway reaction to elevated CO<sub>2</sub>, which can explain the  
352 fast neural impairment. Importantly, we identified numerous transcriptional changes in  
353 within-generation treatments that returned to baseline levels in fish that were  
354 transgenerationally exposed to elevated CO<sub>2</sub> levels. This emphasizes the influence of  
355 environmental exposure on the parents as well as the parental phenotype in the response  
356 of fish to future ocean acidification.

357

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543 **Author contributions:** M.J.W. and P.L.M designed and managed the fish rearing  
544 experiments. M.J.W. performed the adult fish behavioural phenotyping. C.S. prepared the  
545 samples for RNA sequencing and analysed transcriptome expression data and performed  
546 quantitative real-time PCR expression validation. G.E.N. and J.L.R. assisted in  
547 interpreting the expression data. C.S., P.L.M. and T.Ravasi wrote the paper and all  
548 authors read, revised, and approved the final manuscript.

549

550 **Data availability:**

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

554

555 **Competing financial interests**

556 The authors declare no competing financial interests.

557

558 **Methods**

559 *Adult collection and response of adult fish to elevated CO<sub>2</sub>*

560 Adult *Acanthochromis polyacanthus* (spiny damselfish) were collected as described in  
561 Schunter *et al.* (2016)<sup>27</sup> in the central Great Barrier Reef, Australia (18°38'24,3"S,  
562 146°29'31,8"E) and exposed to 754 ± 92 µatm CO<sub>2</sub> levels for 7 days before behavioural  
563 testing. The behavioural phenotype was determined by exposing the adult fish to  
564 conspecific chemical alarm cues (CAC) in a two-chamber flume (30 cm x 13 cm), where  
565 time spent in the CAC was recorded. A 1:1 ratio of adult CAC donor fish to adult test fish  
566 was used. Donor fish were held in control conditions until it was euthanized by a quick  
567 blow to the head. To generate CAC, superficial cuts to both sides of the body were made  
568 after euthanization of the donor fish. The fish was then rinsed with 60 ml of control  
569 water<sup>27</sup>, and the rinse water was added to 10 L of elevated CO<sub>2</sub> seawater. Elevated CO<sub>2</sub>  
570 water including CAC and elevated CO<sub>2</sub> control water were fed into the flume at a  
571 constant rate of 450 ml per minute. Each behavioural trial was run for 9 minutes (2  
572 minutes habituation, 2 minutes recording, 1 minute switch for water sides, where the fish  
573 was recentered at the end of this minute. The 2 minutes habituation and 2 minutes  
574 recording was then repeated), and the location of the fish was recorded every 5 seconds.  
575 Adult fish exhibited a large variation in behavioural responses when tested for chemical  
576 alarm cue (CAC) recognition. These responses ranged from a normal aversion behaviour  
577 with little time spent in the CAC to the opposing behavior, where fish spent most of their  
578 time in CAC. We considered those fish exhibiting an aversion to CAC to be behaviorally  
579 'tolerant' (< than 30% of the trial in CAC) and those exhibiting an attraction to CAC  
580 under elevated CO<sub>2</sub> 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 ( $414 \pm 46 \mu\text{atm}$ ) and 2 tolerant and 3 sensitive pairs in elevated  $\text{CO}_2$  conditions  
590 ( $754 \pm 92 \mu\text{atm}$ , 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  $\text{CO}_2$  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  $\text{CO}_2$  treatment, in which  
597 offspring developed in control conditions but were acutely exposed to elevated  $\text{CO}_2$  for  
598 the last 4 days before sacrificing, c) developmental elevated  $\text{CO}_2$  treatment, in which  
599 offspring were immediately placed into elevated  $\text{CO}_2$  after hatching and d)  
600 transgenerational elevated  $\text{CO}_2$  treatment where parents and offspring were exposed to  
601 elevated  $\text{CO}_2$ . Offspring were kept in their respective conditions (Figure 1) and sacrificed  
602 at the age of 5 months.

603

604 *CO<sub>2</sub> treatment*

605 Experimental procedures followed those described by Welch and Munday (2017)<sup>25</sup>.  
606 Briefly, two 10,000 L recirculating aquarium systems were each set to a different pH and  
607 corresponding CO<sub>2</sub> level: a current-day control (414 ± 46 µatm) and an end of century  
608 elevated CO<sub>2</sub> treatment (754 ± 92 µatm)<sup>51,52</sup>. An Aqua Medic AT Control System (Aqua  
609 Medic, Germany) was used to dose CO<sub>2</sub> into a 3,000 L sump to maintain the desired pH  
610 in the elevated CO<sub>2</sub> treatment. An identical sump on the control system was not dosed  
611 with CO<sub>2</sub>. Control and elevated CO<sub>2</sub> water were then delivered to the holding aquaria at  
612 1.5 L per minute. Temperature and pH<sub>NBS</sub> 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<sub>2</sub> was then calculated in CO2SYS<sup>53</sup>, using constants from Dickson  
617 and Millero (1987)<sup>54</sup>.

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°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

627 were then sequenced on an Illumina HiSeq 2500 paired end to the length of 100bp at  
628 Macrogen, South Korea. Raw reads were inspected and quality trimmed to a minimum  
629 Phred score of 30 with FastQC and Trimmomatic respectively<sup>55,56</sup>. High quality reads  
630 were mapped against the *de novo* assembled genome reference using Tophat 2<sup>57</sup> with  
631 bowtie2 very-sensitive mode and providing the coordinates of the reference based  
632 annotated transcriptome. The *A. polyacanthus de novo* genome assembly and annotation  
633 have been previously described<sup>27</sup>. The bam files resulting from the mapping step were  
634 then sorted with samtools<sup>58</sup> and read counts were extracted by using an HT-seq script<sup>59</sup>  
635 adding exon read counts to receive transcript-based read count values. Differential  
636 expression was statistically evaluated with DEseq2<sup>60</sup> in Bioconductor version 3.2 in R  
637 3.2.1 through pair-wise treatment comparisons. Comparisons between the different  
638 treatments were performed by comparing the expression of acute, developmental, and  
639 transgenerational samples for each parental phenotype separately against the control  
640 samples. Differential expression was evaluated between the different treatments, but the  
641 expression levels of the two parental phenotypes were also directly compared for each  
642 CO<sub>2</sub> treatment. The significance level for differential expression was set to an FDR  
643 adjusted p-value of <0.05 with additional filters of a minimum log 2 fold expression of  
644 0.3 and standard deviation correction (SD<Mean). Gene expression patterns across  
645 different treatments were based on significant differential expression in all pairwise  
646 comparisons.

647

648 To evaluate a potential family effect within the parental phenotypes, we compared  
649 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<sub>2</sub> 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<sup>61</sup>. 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 dendrogram. 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<sup>62</sup> 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<sup>63</sup> 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<sub>2</sub> 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<sub>2</sub> 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<sup>64</sup>, 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<sup>27</sup>. 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<sub>2</sub> levels, 2) acute high CO<sub>2</sub> levels, 3)  
703 developmentally high CO<sub>2</sub> levels and 4) transgenerational high CO<sub>2</sub> 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 transgenerational 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<sub>2</sub> (green) was set at 750uatm, simulating end**  
721 **of century CO<sub>2</sub> projections.** Behaviourally tolerant and sensitive parents were phenotyped  
722 based on their response to chemical alarm cues (CAC) after exposure to elevated CO<sub>2</sub>: tolerant  
723 adults exhibited a normal response to CAC in an elevated CO<sub>2</sub> environment whereas sensitive  
724 parents exhibited an impaired response to CAC. Offspring of parental pairs were then reared  
725 in three different CO<sub>2</sub> treatments until the age of 5 months These three treatments were: current  
726 day CO<sub>2</sub> levels as the control (control), fish reared under control conditions with 4 days  
727 exposure to elevated CO<sub>2</sub> at 5 months of age (acute treatment), and fish reared under elevated  
728 CO<sub>2</sub> 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<sub>2</sub> from hatching until 5 months of age that were from parents  
732 maintained in elevated CO<sub>2</sub> 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<sub>2</sub>  
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

741 **Figure 3. Functional enrichment analysis of differentially expressed genes across**  
742 **CO<sub>2</sub> rearing treatments that were significant in both differential gene expression**  
743 **models** (C = control, A = acute, DEV = developmental, TRANS = transgenerational) and  
744 different behavioural parental phenotypes, (T = tolerant, S = sensitive). A)  
745 Overrepresented gene ontologies and B) underrepresented gene ontologies (significantly  
746 more or less of this GO category in comparison to the compared treatment). The colour of  
747 the circles represents the enrichment significance, and size of circles is proportional to the  
748 number of enriched genes.

749

750 **Figure 4. Gamma-aminobutyric acid (GABA) signaling pathway in the synapse**  
751 **between a pre- and postsynaptic neuron.** Many pathway components showed  
752 differential expression in response to CO<sub>2</sub> treatments. The insert highlights the proposed  
753 increase of GABA release due to increased GABA packing in synaptic vesicles<sup>37</sup>.  
754 (Adapted from KEGG pathways). GAD= Glutamate decarboxylase 1, VGAT= GABA  
755 and glycine transporter, CLCN3=Chloride voltage-gated channel 3, KCC2= Neuronal K-  
756 Cl cotransporter, GAT1= GABA transporter 1, CACNA1A= Brain calcium channel 1,  
757 GABAAR= GABA<sub>A</sub> receptor subunits alpha, beta & gamma.

758

759 **Figure 5. Expression pattern of histone-related transcripts across all CO<sub>2</sub>**  
760 **treatments.** Expression levels of a) core histones, b) differential expression of histone-  
761 related transcripts between developmentally CO<sub>2</sub> treated fish from tolerant and sensitive  
762 offspring and c) microtubule-related transcripts. S=sensitive, T=tolerant, C=control,  
763 A=acute, DEV=developmental, TRANS=transgenerational.