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

Celia Schunter, Megan J. Welch, Göran E. Nilsson, Jodie L. Rummer, Philip L. Munday* and Timothy Ravasi*

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 may be influenced by variations in tolerance to elevated CO_2 exhibited by the parental phenotype. 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 CO_2 by the oceans and the resulting decline in seawater pH (ocean acidification) will have detrimental effects on many marine organisms[1]. Exposing different marine species to projected future CO_2 levels in laboratory experiments has already provided evidence of a diverse range of responses and effects[2–4], including changes in growth rates, survival, and reproduction[5,6]. Fish and other marine organisms can also exhibit behavioural changes that could affect survivorship[7,8], including vitally important responses to chemical alarm cues and predator cues[9–14]. The underlying cause of these behavioural impairments is thought to be changes in the concentration of acid-base relevant ions to prevent acidosis under elevated CO_2 levels, which in turn affects the function of gamma-aminobutyric acid (GABA) neurotransmitter receptors in the brain[14–16].

To date, most observations regarding the impacts of ocean acidification come from short-term experiments that do not account for population heterogeneity and individual variation in tolerance to elevated CO_2 that could be important in adaptive processes[17,18]. Acutely exposing animals to near future CO_2 scenarios for days to weeks is insufficient to predict the potential for acclimation and adaptation over longer time scales[18]. In particular, the environmental conditions experienced early in life can affect responses to

47 those conditions later in life (i.e., developmental plasticity), which can be mediated by
48 environmentally induced epigenetic modification[19], thereby improving performance in a
49 new environment. The environment experienced by the parents can also influence how
50 offspring respond to environmental changes[20–22]. In fact, recent transgenerational studies
51 have demonstrated recovery of metabolic rate and growth rates in juvenile fish when both
52 parents and their offspring are exposed to elevated CO_2[23,24]. Finally, individual variation
53 in tolerance to elevated CO_2 could be heritable, and variation in parental tolerance to
54 elevated CO_2 could therefore influence the tolerance of their offspring to the same
55 conditions[25]. Longer-term developmental studies and multigenerational experiments that
56 incorporate individual variation in tolerance to elevated CO_2 are needed to better
57 understand and predict the effects of ocean acidification on populations and their capacity
58 to adapt[17,26].
59
60 Previous research has shown that changes in the brain transcriptome of juvenile spiny
61 damselfish (*Acanthochromis polyacanthus*) exposed to elevated CO_2 is influenced by the
62 parental phenotype[27]. Differences in the brain transcriptome were found between
63 offspring of parents that had been classified as behaviourally tolerant versus those
64 classified as behaviourally sensitive to elevated CO_2. This suggests that parental
65 phenotype may have a significant influence on the expression of developmental and
66 transgenerational plasticity to elevated CO_2 in reef fishes. Therefore, to further
67 understand the mechanisms that underpin plasticity to ocean acidification, we
68 investigated the effects of acute, long-term developmental, and transgenerational
69 exposure to elevated CO_2 on the molecular response in the brain of juvenile spiny

70 damselfish from behaviourally tolerant and behaviourally sensitive parents. We focus on
71 the brain response because altered function of $GABA_A$ neurotransmitter receptors are
72 thought to be responsible for many of the behavioural changes observed in fish exposed
73 to elevated CO_2[15,16]. Adult spiny damselfish were collected from the Great Barrier Reef
74 in Australia, exposed to near–future levels of CO_2 (754 μatm) for 7 days, and
75 subsequently tested for their ability to react to chemical alarm cues, a crucial survival
76 mechanism in fish[11]. Behaviorally impaired adults were matched into 'sensitive' breeding
77 pairs, while adult fish exhibiting normal behavioural responses to alarm cues when
78 exposed to elevated CO_2 were matched into 'tolerant' breeding pairs (Figure 1). These
79 breeding pairs were then either maintained under current-day control CO_2 or elevated
80 CO_2 conditions for three months until breeding commenced. Offspring of these pairs
81 were reared for 5 months under control or elevated CO_2 conditions. Finally, some fish
82 that were reared under control conditions from hatching were exposed to elevated CO_2
83 for the last 4 days of the experiment. This produced four different treatments for the two
84 parental phenotype groups: a) control parents – offspring reared in control conditions
85 (control); b) control parents – offspring reared in control conditions with a 4-day elevated
86 CO_2 treatment at the age of 5 months (acute CO_2 treatment), c) control parents –
87 offspring reared in elevated CO_2 from hatching (developmental CO_2 treatment); d)
88 elevated CO_2 parents – offspring reared in elevated CO_2 from hatching (transgenerational
89 CO_2 treatment) (Figure 1). We measured the genome-wide gene expression in the brains
90 of 72 individuals across all treatments in order to tease apart the acute molecular response
91 to elevated CO_2 from the responses to longer-term development under elevated CO_2 and
92 transcriptional differences that occur due to parental exposure to elevated CO_2.

93 Comparing these transcriptomes in offspring from the two parental phenotypes allowed
94 us to evaluate how long-term and cross-generational exposure to elevated CO_2 influences
95 the response of fish to future ocean acidification conditions and the influence of
96 individual variation in tolerance to elevated CO_2 on these relationships.
97

Results

Response of adult fish to elevated CO_2

Adult *A. polyacanthus* that were exposed to elevated CO_2 for 7 days 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' and those exhibiting an attraction to CAC under elevated CO_2 to be behaviorally 'sensitive'. About 38% of the randomly collected fish within the population could be assigned to the tolerant or sensitive groups (Table S1).

Influence of parental phenotype on the response to elevated CO_2

The offspring of the tolerant and sensitive parents exhibited significant differences in the brain transcriptome. We identified 114 differentially expressed transcripts under acute CO_2 exposure and 359 under developmental exposure when comparing offspring from the two parental groups directly, disclosing a clear influence of the parental phenotype on the offspring's response to elevated CO_2 (Figures 2, S1, & Table S14). The transcripts differentially expressed between offspring of the two parental phenotypes upon acute exposure were functionally enriched in pathways controlling haemoglobin and oxygen transport (Table S2). No significant enriched function was found for the transcripts differentially expressed between parental phenotypes in the developmental treatment.

120 Besides direct differential expression between offspring of the two parental phenotypes,
121 we also compared expression within each parental group (e.g. acute treatment versus
122 control) in order to identify transcripts with expression profiles that overlap between the
123 two parental phenotypes as well as those that differ. While there were similarities in the
124 gene expression patterns among treatments for the offspring of tolerant and sensitive
125 parents, there were also large differences in the transcriptomes between offspring of
126 tolerant parents and the offspring of sensitive parents (Table S3). Offspring of
127 behaviourally tolerant parents exhibited more changes in the transcriptome when acutely
128 exposed to elevated CO_2 (3,669 transcripts) when compared to the developmentally
129 exposed fish (1,142 transcripts differentially expressed) (Figure 2). Interestingly, this
130 pattern was inversed in the offspring of sensitive parents, for which the developmental
131 treatment resulted in a larger change in gene expression, with 2,590 differentially
132 expressed transcripts compared with 2,010 transcripts in acute treatment. The shared
133 component between the parental phenotypes for these treatments was as low as 27%
134 (Table S3). In fact, only a few pathways were commonly enriched in the brains of fish
135 from different parental phenotypes in the developmental treatment (Figure 3). In the
136 developmental treatment, offspring of tolerant fish showed differential expression of
137 transcripts involved in gluconeogenesis, which was not seen for the offspring of sensitive
138 parents. Several other pathways were enriched only in the offspring of behaviourally
139 sensitive parents, including pathways involved in nervous system development and ion
140 transport (Table S4). Hence, we found large differences, yet some overlapping
141 transcriptional responses in the offspring of the two parental phenotypes. Nonetheless,

142 the acute and developmental CO_2 treatments had larger overall effects on the
143 transcriptome than did the parental phenotype (Figure S1).
144
145 **Short-term and developmental responses to elevated CO_2**
146 Exposure of offspring to near future CO_2 levels resulted in large differences in gene
147 expression patterns compared with control offspring reared at current-day CO_2 levels
148 (Figure 2). The offspring of tolerant parents that were acutely exposed to elevated CO_2
149 for 4 days exhibited the greatest number of differentially expressed genes (3,669) when
150 compared to control fish (14.5% of entire brain transcriptome). In this acute treatment,
151 about half of the differentially expressed genes (51% and 49% for offspring of tolerant
152 and sensitive parents respectively) were expressed at higher levels, resulting in more
153 significant functional enrichments than the transcripts upregulated in control fish (Figure
154 3). Comparing differentially expressed genes in the acute treatment with those
155 differentially expressed in longer-term treatments enabled us to distinguish rapid, short-
156 term transcriptional responses from longer-term responses to elevated CO_2. For this
157 comparison we considered those genes differentially expressed in acutely-treated fish
158 against control fish, but which were not differentially expressed in developmental and
159 transgenerationally treated fish when compared to control fish. Hence, these differentially
160 expressed genes were unique to the acute 4-day exposure to elevated CO_2. A total of 184
161 genes showed a clear pattern of specific short-term response that was common for both
162 parental phenotypes (Table S5). These acute-specific genes were significantly enriched in
163 ATPase-related processes (Figure 3 and Table S6).
164

165 The fish that were developmentally exposed to elevated CO_2 differentially expressed
166 1,142 and 2,590 transcripts, of which 56% and 78% were upregulated in offspring of
167 tolerant and sensitive parents, respectively (Figure 2). The offspring of sensitive parents
168 had a large number of enriched biological pathways that showed upregulation in the
169 developmental treatment (Figure 3). A total of 698 transcripts were commonly
170 differentially expressed in offspring of both parental phenotypes. Only 27 of these
171 transcripts were uniquely differentially expressed in the developmental CO_2 treatment,
172 regardless of parental phenotype, suggesting a developmental treatment specificity (Table
173 S7). These transcripts were at control expression levels in acute and transgenerational
174 treatments, but differentially expressed in the developmental treatment. Of these
175 transcripts, 23 showed lower expression levels in the developmental treatment when
176 compared to the control, indicating downregulation.
177
178 Importantly, in both the acute and developmental treatments we found a common set of
179 highly upregulated transcripts involved in neurotransmitter secretion, nervous system
180 development, ionotropic glutamate receptor activity, and $GABA_A$ receptor activity
181 (Figure 3). This upregulation was specific to within-generation treatments, including
182 acutely exposed fish and fish reared under elevated CO_2 for 5 months from hatching.
183 Many of these differentially expressed transcripts and associated enriched functions were
184 also found in one module cluster shown through weighted correlation network analysis
185 (Figure S2 & S3, Table S8). Hence, both of these independent methods revealed the
186 importance of these genes and their functions for fish exposed to higher CO_2. A clear
187 signature came from genes involved in GABAergic neurotransmission, with nearly all
188 genes in this pathway overexpressed in the acutely and developmentally treated fish when

compared to control individuals (Figure 4). These included genes involved in GABA production, GABA secretion from presynaptic neurons, all of the GABA receptor subunits (details in Table S9), and the potassium-chloride co-transporter 2 (*kcc2*). Furthermore, we saw a reduction in the expression of GABA transporter 1 (*gat1*).

Another important within-generation specific response involved epigenetic regulation of gene expression. Here, however, we saw a common but also divergent response between the parental phenotypes. In the developmental treatment, there were significant differences in the expression of genes involved in methylation between the offspring from different parental groups. Specifically, eight differentially expressed transcripts from the direct comparison between the parental groups in the developmental treatment are involved in the control of the DNA, protein, and histone methylation states (*ppme1*, *apex1*, *prmt6*, *setd2*, *kmt2a*, *mecp2*, *kmt2c* & *mrm1*) (Table S10). Differences in epigenetic related transcription patterns could also be seen across different CO_2 treatments, as methylation related pathways were significantly enriched in genes that were downregulated in the offspring of tolerant parents, but only when offspring were acutely exposed to elevated CO_2.

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 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. Yet, it is 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, which suggests 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 transcripts that were differentially expressed 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 (Figure S4). A total of 401 differentially expressed transcripts in the developmental treatment were at control expression levels in the transgenerational treatment, regardless of parental phenotype (Figure 3b, Table S11). The previously mentioned upregulation of histone expression was generally lower in control and transgenerational treatments and higher in the acute and developmental treatments. Further within-generation specific gene expression patterns, including the $GABA_A$ related genes that were up or downregulated in acutely and developmentally treated fish, were at control levels in the transgenerational treatment. Of the transcripts exhibiting recovery patterns, some increased expression during developmental exposure to elevated CO_2. These transcripts were enriched for genes involved in microtubule-related pathways (e.g., microtubule proteins; *map1b*, *map4*, *futsch*, microtubule kinases; *mast3*, *mark3*, and microtubule-actin crosslinking factor; *macf1*, Figure 5c). We also identified an opposite pattern of expression for cytoskeleton

237 related genes (e.g., tubulin alpha 1c; *tub1c* and microtubule associated protein light chain;
238 *map1lc3b*), which exhibited lower expression levels in the developmental treatment but
239 were to control levels in the transgenerational treatment.
240
241 By comparing within-generation and transgenerational CO_2 treatments, we were also able
242 to tease apart a transgenerational-specific transcriptional signature. Transgenerational-
243 specific responses refer to transcripts that were at control levels in acute and
244 developmental treatments but were differentially expressed in the transgenerational
245 treatment only. The transgenerational-specific signatures were divergent between
246 offspring from the two parental phenotypes. A larger transgenerational signal was found,
247 represented by 41 transcripts, in offspring of tolerant parents and 8 differentially
248 expressed transcripts in offspring of sensitive parents, with none overlapping (Table
249 S12). Eleven and one of these transcripts, respectively, showed direct differential
250 expression between the two parental phenotypes in the developmental treatment.
251
252 Finally, independent of the length of exposure, elevated CO_2 affected only a few brain
253 transcripts commonly differentially expressed when compared to control fish. Only eight
254 and 18 transcripts in offspring of sensitive and tolerant parental phenotypes, respectively,
255 were differentially expressed across all elevated CO_2 treatments (Figure S5). When
256 considering long-term treatments (i.e., excluding acute), 31 and 27 transcripts from
257 offspring of sensitive and tolerant parents, respectively, showed a clear CO_2 response
258 (Table S13). These CO_2 affected transcripts differed in their expression patterns across
259 parental phenotypes, with the exception of *fgf1*, *shmt2*, *pck1*, *arhgef*, *phdgh* and *psat*,

which were differentially expressed in various CO_2 exposures and common between parental phenotypes (Figure S6 and S7).

Discussion

Fundamental changes in the transcriptional landscape in the brain, displayed by numerous differentially expressed genes, were observed in all elevated CO_2 treatments, reflecting an important transcriptional response to near-future CO_2 levels. Nevertheless, the specific functional response depended on the duration of exposure to elevated CO_2. The 4-day acute CO_2 treatment resulted in the largest treatment-specific response in gene expression. One of the upregulated genes specific to the acute CO_2 treatment, stanniocalcin (*stc2*), a glycoprotein involved in calcium and phosphate regulation and thought to be linked to oxidative stress responses through anti-apoptosis, was first discovered in fish[28]. Other glycoprotein-encoding genes (e.g., neurexophilin; *nxph1, 2* and *4* and ependymin; *epd1*) were also overexpressed in acutely treated fish. These genes play a role in short-term neuronal plasticity, and neurexophilin has recently been linked to $GABA_A$ and $GABA_B$ receptor subunit expressions, revealing an instructive role in the configuration of GABA receptors[29]. The increased expression of the GABA receptor genes in the acutely treated fish could therefore be driven by an upregulation of *nxph1* and associated inhibitory neural circuits.

When fish were reared under elevated CO_2 conditions 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

283 factor which, in mammals, activates the growth-inhibiting Nogo receptor complex in
284 regenerating axons[30], thus downregulating growth and inhibiting neuronal plasticity. The
285 function of the Nogo receptor in fish is still unclear, but it was previously associated with
286 embryonic and brain development[31]. Another possible negative effect associated with
287 elevated CO_2 during development was the downregulation of the creatine transporter
288 (*slc6a8*). Decreased expression of this transporter is correlated with a decrease in
289 intracellular creatine, which plays a central role in energy homeostasis[32]. Thus, our
290 results indicate that exposure to near future CO_2 levels early in life could have
291 detrimental effects on the healthy development of juvenile fish. This is consistent with
292 previous studies reporting negative effects on growth, development, and survival in
293 juvenile fish exposed to elevated CO_2 levels[6,16,33–35].
294
295 When exposed to elevated CO_2, fish regulate their intra- and extracellular pH to avoid
296 acidosis, primarily via HCO_3^- accumulation[16]. Nilsson and coauthors[15] suggested that
297 such acid-base regulatory mechanisms could lead to altered $GABA_A$ receptor function.
298 Specifically, changes in the transmembrane gradient for HCO_3^- and Cl^- could lead to a
299 reversal of ion fluxes through the opened receptor, which could explain the behavioural
300 changes observed in fish upon elevated CO_2 exposure[36]. We observed that many GABA-
301 related genes were highly upregulated in the brain in fish that were acutely and
302 developmentally exposed to elevated CO_2, showing a common within-generation
303 response. This pattern included genes involved in GABA production, all GABA receptor
304 subunits, and transporter genes. When $GABA_A$ receptor function switches to being
305 excitatory under elevated CO_2, the inhibitory input in neural circuits are lowered, and the

306 circuits become overactive. This can trigger futile feedback responses aimed to reduce
307 the over-activity by releasing more GABA and increasing the number of $GABA_A$
308 receptors. This will be counter-productive if GABA has started to act excitatory, thus
309 initiating a self-amplifying (vicious) cycle. When CLCN3 and VGAT genes are
310 upregulated, as we see here in within-generation elevated CO_2 exposed fish, packing of
311 GABA into synaptic vesicles could increase[37,38], thereby increasing GABA release.
312 Exacerbation of this vicious cycle also comes when GAT1 (responsible for removing
313 extracellular GABA) is downregulated, which would increase GABA in the synaptic
314 cleft. These changes can explain how a small increase in CO_2, causing a relatively
315 moderate change in Cl^-/HCO_3^- gradients, can be amplified to cause a significant
316 dysfunction of GABAergic neurotransmission, thus leading to altered behavioural
317 responses. We did see a GABA related transcriptomal change that could be adaptive:
318 upregulation of potassium-chloride co-transporter 2 (*kcc2*), a transporter responsible for
319 removing Cl^- from the cells[39], which could counteract the excitatory action of $GABA_A$
320 receptors.
321
322 Epigenetic regulation of gene expression is an important mechanism that could be
323 underpinning whole-organism responses to environmental change[40]. Our results suggest
324 that epigenetic regulators have an influence on development under elevated CO_2.
325 Moreover, the parental phenotype also influences the expression of epigenetic regulators,
326 as some were differentially regulated between the offspring of the two parental
327 phenotypes. One of the genes that was differentially expressed between offspring of
328 tolerant and sensitive parents, arginine methyltransferase 6 (*prmt6*), is involved in

329 posttranscriptional modification by methylation. It is implicated in the regulation of Hox
330 genes during development via histone methylation[41]. The *prmt6* gene is known to
331 methylate CREB Regulated Transcription Coactivator 2 (CRTC2), a transcriptional
332 activator of the gluconeogenic program[42,43] that is upregulated in the offspring of
333 sensitive parents. Upregulated gluconeogesis through the AMPK signaling pathway,
334 which facilitates glucose uptake, would require glucose transporters. Glucose
335 transporters, such as *gtr1* (*gtr10, 3, & 8*), were indeed upregulated in the offspring of
336 sensitive parents after developmental CO_2 treatment. Hence, differential glucose
337 regulation in fish exposed to elevated CO_2 during development – via selective DNA
338 methylation – could cause differences in the offspring of the two parental groups.
339
340 Changes to the chromatin landscape and the alternative use of histone variants also
341 influence differences between offspring of tolerant and sensitive parents in the
342 developmental treatment (Figure 5a). Histone variants (e.g., *h2az*) that were
343 downregulated in the acute CO_2 treatment in the offspring of tolerant parents and in the
344 developmental treatment in the offspring of sensitive parents have been shown to mediate
345 responses to environmental change (e.g., temperature and season)[44]. In the common carp,
346 such seasonal changes to cold and warm conditions are related to methylation of H2A[45].
347 In general, histones and histone modifications control chromatin dynamics, making
348 transcription factors more or less accessible and therefore regulating gene expression[46].
349 We found that the general pattern for most of the histone variants was a decreased
350 expression in the developmental treatment; this pattern has also been identified in a
351 marine invertebrate upon elevated CO_2 exposure[47]. Additional evidence for reduced

352 transcriptional repression mediated by histones is supported by the downregulation of
353 several polycomb protein encoding transcripts (e.g., Polycomb Group Ring Finger 2;
354 *pcgf2* and SUZ12 Polycomb Repressive Complex 2; *suz12b*) in the acute and
355 developmental treatments. The polycomb repressive complex is shown to chemically
356 modify histones, for instance, by adding methyl groups to the histone tails, thereby
357 repressing transcription of certain genes[48]. Thus, downregulation would result in
358 increased gene expression. Hence, we demonstrate a strong developmental plasticity in
359 gene expression, which is likely controlled in part by DNA methylation and the use of
360 histone variants. We also observed that genetic variation and non-genetic (epigenetic)
361 parental effects can, to a certain extent, influence within-generation control of gene
362 expression of individual fish exposed to elevated CO_2.
363
364 Inheritance of an optimized acid-base regulatory system where key genes are controlled
365 epigenetically could enhance physiological performance in fish living in more acidified
366 oceans[22,24]. However, inheriting a beneficial epigenetic program seems unlikely here
367 because transgenerationally CO_2-treated fish did not exhibit the aforementioned
368 differential expression of epigenetic-related genes when compared to control fish. In fact,
369 it appears that histone genes were downregulated, and many other transcripts specific to
370 within-generation treatments were upregulated and reversed through transgenerational
371 exposure. Such a recovery pattern was also found for multiple microtubule-related genes,
372 implicating cytoskeleton plasticity in response to exposure to near-future CO_2 levels.
373 Cytoskeleton plasticity in response to elevated CO_2 has already been suggested for
374 invertebrates[49,50]. Cytoskeleton plasticity is directly related to neuronal plasticity[51], and it

375 seems that within-generation CO_2 exposure leads to a cytoskeletal rearrangement that can
376 aid neuronal plasticity to return to a control state during transgenerational exposure.
377 Further responses to stress via downregulation of *nlrc3* and the hypoxia inducible factor
378 prolyl hydroxylase 2 (*egln1*) and upregulation of the hypoxia inducible factor 2 alpha
379 (*epas1*), both important during oxidative stress, could become maladaptive, as we found
380 these expression patterns, even after five months of exposure to elevated CO_2.
381 Importantly, such responses seem to also be reversed with transgenerational exposure.
382
383 Differentially expressed genes in within-generation treatments but at control levels in the
384 transgenerational treatment could be reverted back by transgenerational transmittance or
385 by pre-hatching environmental effects. We cannot distinguish between these two
386 mechanisms, but some of the functions of these genes are life-stage restricted. For
387 example, cullin 3 (*cul3*), an important gene that mediates ubiquitination and degradation
388 of proteins, has recently been implicated in the differentiation of embryonic stem cells
389 into neural crest cells and therefore important for early development. Downregulation in
390 the developmental treatment, but not in the transgenerational fish, might be evidence of a
391 very early developmental influence of CO_2 on the expression of this gene. A possible
392 maternal influence can be seen for a ubiquitin conjugating DNA repair enzyme (*ube2a*),
393 also involved in protein ubiquitination. A maternal influence of *ube2a* was shown to be
394 essential for the correct embryonic development in mice[52]. It is possible, therefore, that
395 exposure to elevated CO_2 of the parents during breeding leveled the expression in
396 offspring, thus returning to control levels despite elevated CO_2 in the juvenile
397 environment.

Transgenerational exposure to elevated CO_2 influences the expression of a smaller set of genes with divergent responses in offspring of tolerant and sensitive parents. Among these are the previously described circadian rhythm genes[27] showing a transgenerational pattern for offspring only of tolerant parents. Other genes that are only differentially expressed at a transgenerational level include Activin A Receptor Like Type 1 *(acvrl1)* and Cytochrome P450 Family 27 Subfamily C Member 1 (*cyp27c1).* The activin receptor is an important regulator in vascular blood vessel development and also emerged as an alternative transforming growth factor (TGF) beta-receptor in epithelial cells. Upregulation of the TGF signaling pathway is correlated with brain injury[53] and both receptors (*tgfr1*, *tgfr2*) showed a recovery signal with upregulation in the developmental treatment, but not during transgenerational exposure. The activin receptor, however, is upregulated in offspring of tolerant fish with transgenerational exposure. Expression of *Acvrl1* during brain injury has been suggested to limit consequences of metabolic injury in the nervous system[53]. Although *cyp27c1* function in the brain is still unknown, it was previously connected to Vitamin A_2 production and infrared vision in zebrafish[54] and exhibited a parental phenotype based response in this study.

The long-term molecular response to elevated CO_2, independent of parental phenotype, was linked to glucose metabolism. All previously reported genes involved in transgenerational acclimation to higher CO_2 levels[27] exhibited in our system were upregulated in developmental as well as transgenerational CO_2 treatments, suggesting that this signal is not an immediate adaptive response, but rather a delayed response to prolonged exposure. The role of the brain in regulating glucose homeostasis is becoming

421 more evident, but it was only very recently shown that an increase in brain *fgf1* can
422 promote blood glucose reduction[55]. Therefore, we propose that the capacity for fish to
423 maintain performance in more acidified oceans will depend of their ability to cope with
424 the long lasting effects of CO_2 exposure. The rebalance of gluconeogenesis and glucose
425 homeostasis, neither of which are compensated for via transgenerational exposure, may
426 be key to adapting to new environmental conditions.
427
428 Here, by using an integrative genomics approach coupled with a unique experimental
429 design, we tested the response of a coral reef fish to end-of-century CO_2 levels and
430 provide further evidence for an important role of altered GABA receptor function in the
431 response to elevated CO_2. In particular we demonstrated a possible vicious feedback
432 cycle that exacerbates the way the GABA pathway reacts to elevated CO_2, which can
433 explain the fast and severe neural impairment. Importantly, we identified numerous
434 transcriptional changes in within-generation treatments that returned to baseline levels in
435 fish that were transgenerationally exposed to elevated CO_2 levels. This emphasizes the
436 influence of environmental exposure on the parents as well as the parental phenotype in
437 the response of fish to future ocean acidification conditions.
438

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Affiliations

KAUST Environmental Epigenetic Program (KEEP), Division of Biological and Environmental Sciences & Engineering, King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia

Celia Schunter and Timothy Ravasi

ARC Centre of Excellence for Coral Reef Studies and College of Marine and Environmental Sciences, James Cook University, Townsville, Queensland, Australia

Megan Welch

ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia

Jodie L. Rummer, Philip L. Munday

Section for Physiology and Cell Biology, Department of Biosciences, University of Oslo, Oslo NO-0316, Norway

Göran E. Nilsson

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

Additional information:

RNA-seq transcriptome sequences and the *de novo* assembled reference genome have been deposited in NCBI under BioProject ID PRJNA311159. Correspondence and requests for materials should be addressed to T.Ravasi or P.L.M.

Competing financial interests

The authors declare no competing financial interests.

Methods

Adult collection and phenotyping

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 were then categorized into 'tolerant' and 'sensitive' according to the time spent in the CAC (Table S1). Fish were considered tolerant if they spent less than 30% of the trial in CAC and sensitive if they spent more than 70% of the trial in CAC. Behavioural sensitivity and fish size were then used to form breeding pairs with individuals of the same sensitivity (i.e., tolerant male with tolerant female).

674 *Experimental design*

675 Breeding pairs were held in 40 L aquaria, with 3 tolerant and 3 sensitive pairs in control
676 conditions (414 ± 46 μatm) and 2 tolerant and 3 sensitive pairs in elevated CO_2 conditions
677 (754 ± 92 μatm, Table S1). Breeding pairs were acclimated to their respective conditions
678 for three months prior to the breeding season. Offspring clutches from breeding pairs
679 were immediately removed from parental tanks after hatching and placed into control or
680 elevated CO_2 conditions. A total of four combinations between parental and offspring
681 conditions were processed with several parental pairs for each combination to avoid a
682 family effect (Main text, Figure 1, Table S1). Offspring conditions were: a) control
683 conditions, b) acute elevated CO_2 treatment, in which offspring developed in control
684 conditions but were acutely exposed to elevated CO_2 for the last 4 days before
685 sacrificing, c) developmental elevated CO_2 treatment, in which offspring were
686 immediately placed into elevated CO_2 after hatching and d) transgenerational elevated
687 CO_2 treatment where parents and offspring were exposed to elevated CO_2. Offspring were
688 kept in their respective conditions (Figure 1) and sacrificed at the age of 5 months.

689

690 *CO_2 treatment*

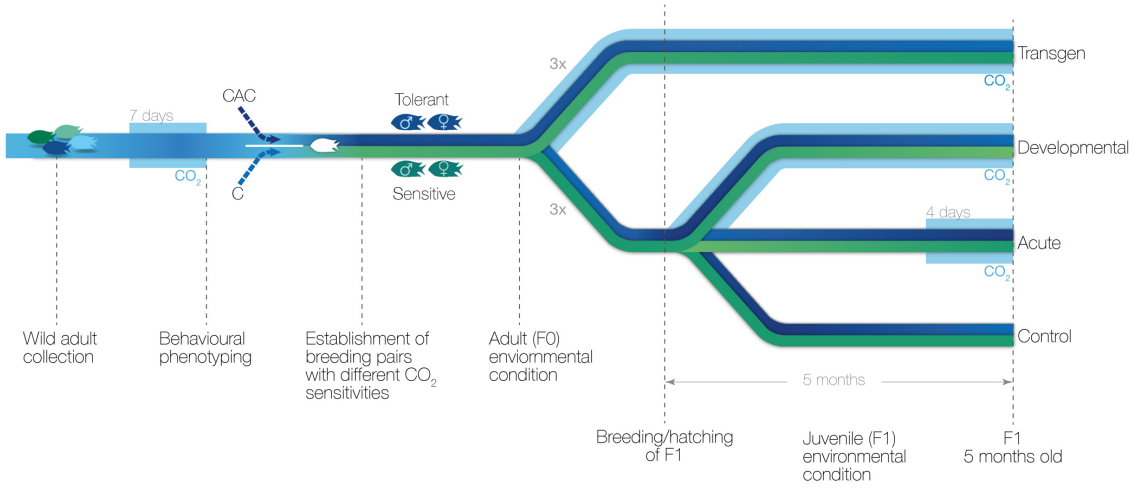
691 Experimental procedures followed those described by Welch and Munday (2017)[25].
692 Briefly, two 10,000 L recirculating aquarium systems were each set to a different pH and
693 corresponding CO_2 level: a current-day control (414 ± 46 μatm) and an end of century
694 elevated CO_2 treatment (754 ± 92 μatm)[56,57]. An Aqua Medic AT Control System (Aqua
695 Medic, Germany) was used to dose CO_2 into a 3,000 L sump to maintain the desired pH
696 in the elevated CO_2 treatment. An identical sump on the control system was not dosed

697 with CO_2. Control and elevated CO_2 water were then delivered to the holding aquaria at
698 1.5 L per minute. Temperature and pH_{NBS} were measured daily in randomised tanks.
699 Salinity and total alkalinity were measured weekly. Total alkalinity was measured by
700 Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) using
701 certified reference material from Dr. A.G. Dickson (Scripps Institution of
702 Oceanography). pCO_2 was then calculated in CO2SYS[58], using constants from Dickson
703 and Millero (1987)[59].
704
705 *RNA and transcriptome expression analyses*
706 Fish brains were immediately dissected out after euthanization, snap frozen with liquid
707 nitrogen, and stored at -80^{o}C. Whole frozen fish brains were then homogenized in RT-
708 Plus Buffer for 30 second in a Fisher bead beater with single-use silicon beads, and total
709 RNA was extracted with AllPrep DNA/RNA Mini Kits (Quiagen). The RNA quality was
710 evaluated on the nanodrop and the Agilent Tape reader, and only minimum RNA
711 integrity values (RIN) of 8 were accepted. Extracted RNA was converted into cDNA and
712 prepped for Illumina sequencing with a TruSeq RNA Illumina Library Prep Kit. Libraries
713 were then sequenced on an Illumina HiSeq 2500 paired end to the length of 100bp at
714 Macrogen, South Korea. Raw reads were inspected and quality trimmed to a minimum
715 Phred score of 30 with FastQC and Trimmomatic respectively[60,61]. High quality reads
716 were mapped against the *de novo* assembled genome reference using Tophat 2[62] with
717 bowtie2 very-sensitive mode and providing the coordinates of the reference based
718 annotated transcriptome. The *A. polyacanthus de novo* genome assembly and annotation
719 have been previously described[27]. The bam files resulting from the mapping step were

720 then sorted with samtools[63] and read counts were extracted by using an HT-seq script[64]
721 adding exon read counts to receive transcript-based read count values. Differential
722 expression was statistically evaluated with DEseq2[65] in Bioconductor version 3.2 in R
723 3.2.1 through pair-wise treatment comparisons. Comparisons between the different
724 treatments were performed by comparing the expression of acute, developmental, and
725 transgenerational samples for each parental phenotype separately against the control
726 samples. Differential expression was evaluated between the different treatments, but the
727 expression levels of the two parental phenotypes were also directly compared for each
728 CO_2 treatment. The significance level for differential expression was set to an FDR
729 adjusted p-value of <0.05 with additional filters of a minimum log 2 fold expression of
730 0.3 and standard deviation correction (SD<Mean). Gene expression patterns across
731 different treatments were based on significant differential expression in all pairwise
732 comparisons.
733
734 To evaluate a potential family effect within the parental phenotypes, we compared
735 treatments in which full siblings were exposed (comparison of control and acute as well
736 as developmental treatments for offspring of tolerant and sensitive parents). We used a
737 model comparison approach. First, differential expression was measured accounting for
738 treatment effect only, then family line was added as a factor and differential expression
739 compared. Finally, the full (treatment+family) model was compared directly with the
740 reduced model (treatment only) (Supplementary Materials Table S14).
741

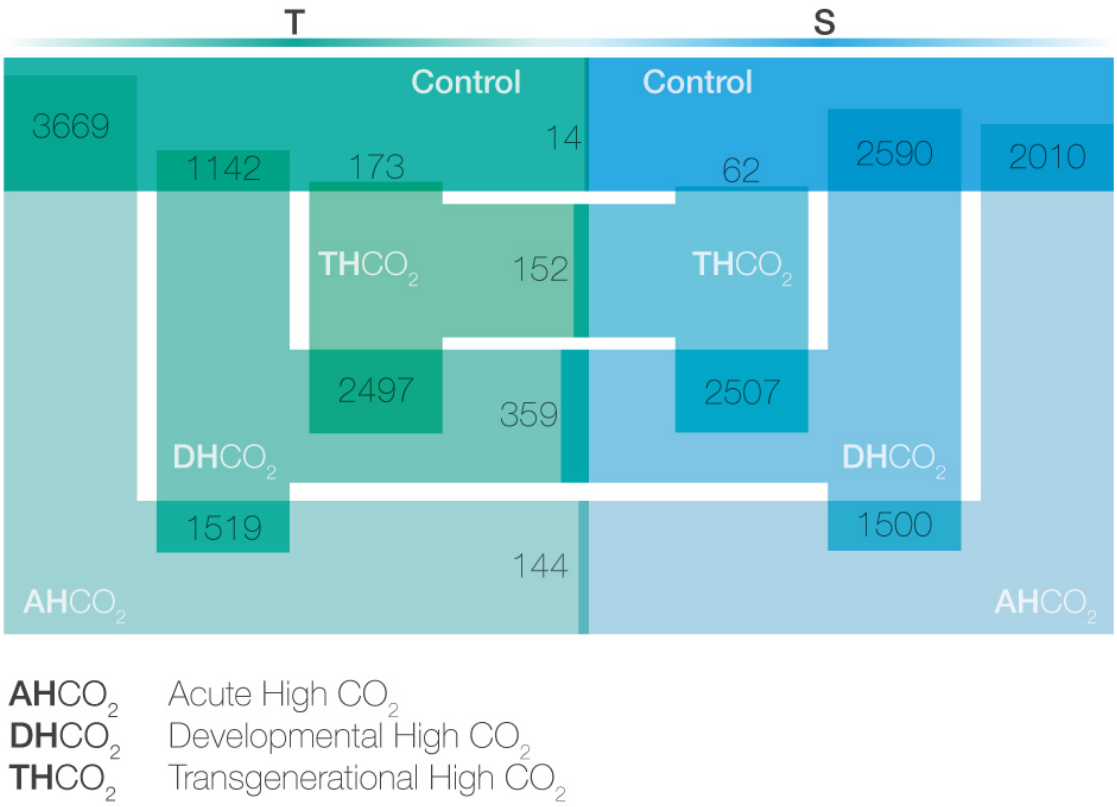
742 After stringent filtering of significant differential expression assignment, we further
743 accounted for false positive assignment through randomization. This was done on the
744 acutely and developmentally treated samples comparing the two different parental
745 phenotypes. For each CO_2 treatment parental phenotype was randomly assigned to a gene
746 expression profile and gene expression analysis was rerun. This was repeated 10 times for
747 the acute and the developmental treatments (Supplementary Materials Table S15).
748 To improve the insight into the complex dataset, we performed a weighted gene-
749 correlation network analysis with the WGCNA package (version 1.6) in R[66]. We used the
750 DEseq2 normalized dataset of raw counts of all 72 samples included in the study. Gene
751 expression data was then variance stabilized, and transcripts with low read counts were
752 removed. Soft-thresholding power was evaluated and the highest value was accepted for
753 network construction (pow=9). This approach was used to approximate a scale free
754 topological network (TOM), which was constructed following these parameters:
755 TOMtype= "assigned', minModuleSize= 30, mergeCutHeight= 0.25. TOM was then used
756 to create a cluster dendogram. Transcripts clustered within one colour module were then
757 extracted if the module had more than 500 transcripts and compared with the
758 differentially expressed gene analysis.
759
760 Blast annotations of the reference-based transcriptome and an Interpro scan were
761 imported into Blast2GO[67] to retrieve Gene Ontology terms and KEGG pathways.
762 Functional enrichment analyses were performed for differentially expressed genes as well
763 as global network clusters with Fisher's exact tests (FDR < 0.05). All tests were
764 performed on the different differential gene expression models, and results presented

765 were significantly enriched functions found with both models. Graphical representations
766 (i.e., heat maps, bubble graphs, and bar plots) were produced in R 3.3.1. A Principle
767 Component Analysis (PCA) was performed with the cloud platform WebMeV[68] using the
768 normalized expression of acutely and developmentally treated samples.
769
770 *qRT-PCR validation of RNA-seq results*
771 Quantitative Realtime PCR was performed on two sets of samples to evaluate all the
772 different experimental treatment groups. We compare control samples with
773 transgenerational elevated CO_2 exposed fish from behaviourally tolerant as well as
774 sensitive parents. We also examined the qPCR gene expression for acutely and
775 developmentally elevated CO_2 treated fish for both parental pairs and compare the
776 relative expression between treatments with the RNAseq expression differences. For each
777 treatment group, two biological samples were, which were from the same treatment, but
778 additional biological individuals than those sequenced via RNAseq. Primers were
779 designed using the genome sequence of the respective transcript of interest with
780 Primer3Plus[69], which was checked in NCBI Primer-BLAST for specificity and HPSF
781 purified by Sigma (Sigma-Aldrich, Germany). Using the high capacity reverse
782 transcription kit by ABI (Applied Biosystems) 550ng of RNA for each sample were
783 reverse transcribed and 15ng of cDNA was used for each reaction with three replicate
784 reactions with specified reaction details[27]. For analysis, the livak method was used and
785 Delta Delta CTs were calculated by normalizing the CTs against three housekeeping
786 genes. Further details and results on the validation can be found in the Supplementary
787 Materials.
788



789

790 **Figure 1. Experimental design. Elevated CO_2 (green) was set at 750uatm, simulating end**
791 **of century CO_2 projections.** Tolerant and sensitive parents were phenotyped based on their
792 response to chemical alarm cues (CAC) after exposure to elevated CO_2: tolerant adults
793 exhibited a normal response to CAC in an elevated CO_2 environment whereas sensitive parents
794 exhibited an impaired response to CAC. Offspring of parental pairs were then reared in three
795 different CO_2 treatments until the age of 5 months The three treatments included: current day
796 CO_2 levels as the control (control), fish reared under control conditions with 4 days exposure to
797 elevated CO_2 at 5 months of age (acute treatment), and fish reared under elevated CO_2 from
798 hatching until 5 months of age (developmental treatment). Control, acute, and developmentally
799 treated fish were siblings from three different parental pairs for both tolerant and sensitive
800 parental phenotypes. Offspring reared in elevated CO_2 from hatching until 5 months of age that
801 were from parents maintained for breeding in elevated CO_2 provided a fourth transgenerational
802 treatment.



803

804 **Figure 2. Global differential gene expression patterns between treatments.** Numbers
805 of significantly differentially expressed transcripts between pairwise comparisons of CO_2
806 treatments as well as between different parental behavioural phenotypes (T=tolerant
807 parents, S=sensitive parents). The overlap between blue and green (T and S) represent the
808 transcripts that are directly differentially expressed between the offspring of different
809 parental phenotypes.

810

811

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

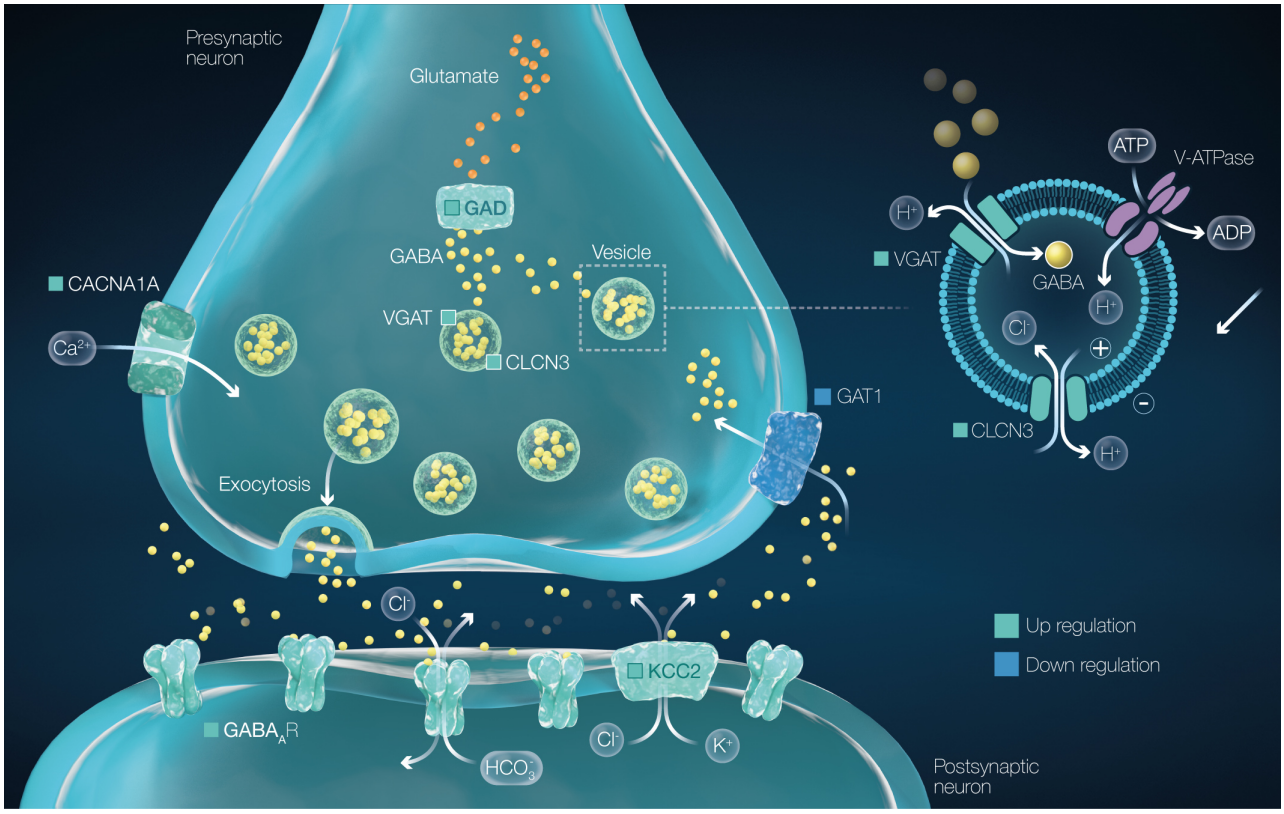


Figure 4. Gamma-aminobutyric acid (GABA) signaling pathway in the synapse between a pre- and postsynaptic neuron. Many pathway components showed differential expression in response to CO_2 treatments. The insert highlights the proposed increase of GABA release due to increased GABA packing in synaptic vesicles[38]. (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.

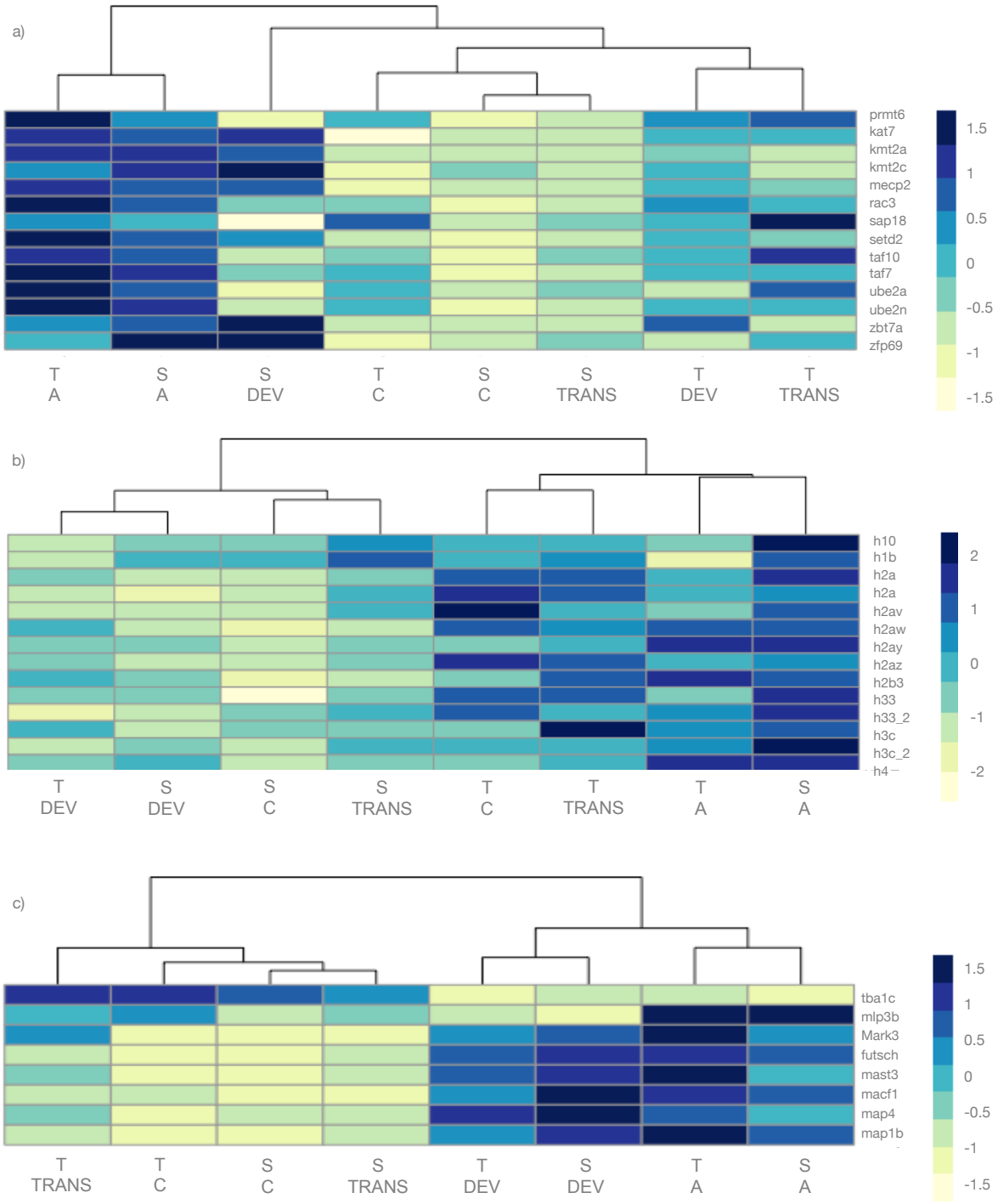


Figure 5. Expression pattern of histone-related transcripts across all CO_2 treatments. Expression levels of a) core histones, b) differential expression of histone-related transcripts between developmentally CO_2 treated fish from tolerant and sensitive

835 offspring and c) microtubule-related transcripts. S=sensitive, T=tolerant, C=control,
836 A=acute, DEV=developmental, TRANS=transgenerational.