Molecular signatures of transgenerational response to ocean acidification in a species of reef fish

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The impact of ocean acidification on marine ecosystems will depend on species capacity to adapt^{1,2}. Recent studies show that the behaviour of reef fishes is impaired at projected CO₂ levels^{3,4}; however, individual variation exists that might promote adaptation. Here, we show a clear signature of parental sensitivity to high CO₂ in the brain molecular phenotype of juvenile spiny damselfish, Acanthochromis polyacanthus, primarily driven by circadian rhythm genes. Offspring of CO₂-tolerant and CO₂-sensitive parents were reared at near-future CO₂ (754 µatm) or present-day control levels (414 uatm). By integrating 33 brain transcriptomes and proteomes with a de novo assembled genome we investigate the molecular responses of the fish brain to increased CO₂ and the expression of parental tolerance to high CO₂ in the offspring molecular phenotype. Exposure to high CO₂ resulted in differential regulation of 173 and 62 genes and 109 and 68 proteins in the tolerant and sensitive groups, respectively. Importantly, the majority of differences between offspring of tolerant and sensitive parents occurred in high CO₂ conditions. This transgenerational molecular signature suggests that individual variation in CO₂ sensitivity could facilitate adaptation of fish populations to ocean acidification.

The uptake of additional carbon dioxide from the atmosphere is changing ocean chemistry, with potentially far-reaching impacts on marine life⁵. Recent studies show that the behaviour of marine fishes and some invertebrates can be impaired by projected nearfuture CO₂ levels, with implications for key ecological processes such as navigation, habitat selection, recruitment, competition and predator-prey interactions^{6,7}. Impaired behaviour at high CO₂ levels have been found in a variety of fish taxa, including sharks⁸, stickleback9 and salmon10. Fish use chemical cues from injured conspecifics (chemical alarm cues (CAC)) to detect the threat of predation and respond by moving away from CAC and decreasing activity¹¹. However, in high CO₂ conditions juvenile fish exhibit a decreased avoidance of CAC and do not learn to associate an increased risk of predation with the presence of CAC^{12-14} . The failure to react to predation threat can have immediate consequences for individual survival and may affect population replenishment^{4,13,14}.

Furthermore, there appears to be limited capacity for within- or between-generation acclimation of impaired behavioural responses to high CO_2 (refs 3,12). Consequently, species will need to adapt to avoid adverse effects of ocean acidification on behaviours that are critical to individual performance and population success.

The adaptive potential of a population depends on the presence of genetic variation upon which selection can act¹. Previous studies have observed variable levels of behavioural impairment among individuals exposed to near-future CO₂ levels^{4,13,15}. However, whether this variation could be transmitted between generations is unknown. One previous study has shown that the average response of juvenile fish to CAC in high CO₂ does not change with parental exposure to high CO₂ (ref. 12), but no studies have yet explored the relationship between individual variation in response to high CO₂ between parents and their offspring. We used a transgenerational rearing experiment to investigate the potential heritability of variation in behavioural sensitivity to ocean acidification in the spiny damselfish, Acanthochromis polyacanthus. The underlying cause of behavioural changes in reef fish exposed to high CO₂ appears to be an effect of ionic change from acidbase regulation on the function of the GABA_A receptor, the major inhibitory neurotransmitter receptor in the vertebrate brain^{14,16,17}. Therefore, we focused on transgenerational molecular signatures of CO₂ tolerance and sensitivity in the fish brain. We first tested the behavioural sensitivity of adult fish to elevated CO₂ (a projected near-future level of 754 µatm). Fish that retained an innate avoidance of CAC in high-CO₂ water were considered 'tolerant' to high CO₂, whereas fish that became attracted by CAC in high CO2 were termed 'sensitive'. Adult males and females of similar tolerance or sensitivity to high CO₂ were paired for breeding, with half of the pairs breeding in control conditions and half of the pairs breeding in high CO₂ conditions. Offspring of these parental pairs were then reared in the same CO_2 conditions as their parents. Full brain transcriptomes were sequenced and proteomes were obtained for a total of 33 offspring from the four parental-sensitivity by CO₂ rearing conditions: nine offspring from tolerant parents reared in control conditions; six offspring from tolerant parents reared in high CO₂ conditions; nine offspring from sensitive parents

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Figure 1 | **Sampling design of juvenile fish for molecular analysis of brain transcriptomes and proteomes.** Offspring were sampled from breeding pairs of spiny damselfish characterized as tolerant or sensitive to the behavioural effects of elevated CO_2 (see grid). T are 'tolerant' parents, whose behaviour is not impeded, and S are 'sensitive' parents, whose behaviour changed when exposed to high CO_2 . Three offspring (biological replicates) from different parental pairs each were sampled from four parental-sensitivity by CO_2 rearing conditions. The colour of the fish indicates different family lines. Fish brains were dissected and processed for transcript and protein differential expression analysis by using a *de novo* assembled genome as the reference.

reared in control conditions; and nine offspring from sensitive parents in high CO_2 conditions (Fig. 1). The *de novo* genome of *A. polyacanthus* was sequenced and assembled to facilitate the assembly and annotation of the transcriptome and proteome data sets (Supplementary Methods).

The expression of brain messenger ribonucleic acid (mRNA) and proteins differed markedly between offspring reared in elevated CO_2 compared with control conditions, as well as between offspring of tolerant and sensitive parents (Fig. 2a). We identified 173 and 62 mRNA transcripts (Fig. 2b) and 109 and 68 proteins (Fig. 2c) showing differential expression between control and CO_2 conditions for offspring of tolerant and sensitive parents, respectively. Only seven transcripts and eighteen proteins were commonly differentially expressed in response to high CO_2 in both parental groups (Fig. 2b,c), revealing a distinct parental influence in responses to CO_2 exposure. Importantly, the majority of differences between these two groups of offspring occurred in fish reared at high CO_2 , with 152 transcripts and 46 proteins in control conditions (Fig. 2 and Supplementary Fig. 1).

The general response to high CO₂, irrespective of parental sensitivity, involved genes and proteins associated with the

brain's glucose, serine and glycine metabolism. pck1, cytosolic phosphoenolpyruvate, is the main control gene for gluconeogenesis and the most upregulated gene of the seven found differentially expressed between high CO2 and control conditions regardless of parental phenotype (Supplementary Table 1). Thirty-three per cent of these differentially expressed genes matched to differentially expressed proteins directly, but multiple glycolytic proteins involved in similar pathways, such as fructose-bisphosphate aldolase or glyceraldehyde-3-phosphate dehydrogenase, also showed increased expression at the high CO₂ level (Supplementary Table 2). Several other fish species exhibit increased blood glucose levels when exposed to stress and environmental perturbations such as pH changes¹⁸. Furthermore, upregulation in pck1 has also been shown to promote a glucose side-branch metabolism: the serine and glycerol-3-phosphate pathways¹⁹. *Phgdh*, phosphoglycerate dehydrogenase, catalyses the early step of the L-serine synthesis from 3-P-glycerate and is upregulated at the transcript and protein level here. Shmt2 (serine hydroxymethyltransferase 2) then converts serine into glycine. Serine and glycine are involved in a wide range of processes, such as the biosynthesis of lipids and proteins, and are necessary for cell proliferation. Upregulation of these metabolic pathways in the brain has also been seen



Figure 2 | Differential expression of transcripts and proteins for the four different comparisons of parental-sensitivity by CO₂ rearing conditions. **a**, Heatmap of all differentially expressed transcripts with hierarchical gene clustering. Expression level is indicated by the *z*-score. **b**,**c**, Venn diagram of differentially expressed transcripts (**b**) and differentially expressed proteins (**c**). Square brackets in **b** represent the overall factorial comparisons, for example, 41 transcripts are differentially expressed for control versus CO₂, regardless of parental phenotype. Values enclosed in rectangles show the total amount of differential expression. Upward arrows represent the number of transcripts/proteins that are upregulated in the respective condition. T, tolerant parents; S, sensitive parents; Control, control condition; CO₂, high CO₂ condition.

in zebrafish after the exposure to chemicals²⁰. All high- CO_2 individuals, regardless of parental phenotype, exhibited an increased expression of genes related to serine biosynthesis (Supplementary Table 3), revealing a common cost in the stress response to high CO_2 exposure.

The molecular phenotype of offspring from CO₂-sensitive parents was substantially different to that of offspring from CO₂-tolerant parents (Fig. 2). At the protein level, offspring of sensitive parents exhibited a ninefold overexpression of histone 1 (H1), possibly compacting the chromatin and regulating gene expression by inaccessibility to transcription factors²¹. On the transcript level, gene ontology analysis showed that genes involved in transfer ribonucleic acid (tRNA) aminoacylation were uniquely enriched in the sensitive-parents group (Supplementary Table 3). These included several tRNA synthetases, such as aars, dars and kars. tRNA synthetases are necessary for the translation from mRNA into proteins, as they bind the proper amino acid to tRNA. Until recently, tRNA synthetases were thought to be housekeepers, but new evidence links differential expression and mutation in these synthetase genes to human diseases²², stress response and rapid adaptation to environmental stressors in yeast and Escherichia coli²³. tRNA synthetases are also responsible for adaptive translation²³ and, although rarely studied in fish, seem to be involved in temperature acclimation²⁴. Thus, the elevated expression of tRNA synthetases in offspring of sensitive parents may be triggered by an unsuccessful attempt to acclimate. It is possible that this may even become maladaptive in offspring of sensitive parents.

Not only were tRNA synthetases more highly expressed in the transcriptomes of offspring from CO₂-sensitive parents, but there were also differences between the sensitive and tolerant offspring group in the genomic sequence of two tRNA-related genes. We measured the fixation index (F_{ST}) of all single nucleotide polymorphisms (SNPs) found across the transcriptomes of all tolerant-parent offspring against all sensitive-parent offspring to evaluate a potential difference due to a fixed genetic variation. Four outliers were found with different genotypes for offspring of tolerant and sensitive parents within the sequenced coding regions (Supplementary Fig. 2). One SNP was found in the coro1a gene involved in immune deficiency and another just upstream of the igdcc3 (immunoglobulin superfamily) coding region. For both SNPs, the sensitive-parents offspring revealed homozygosity (both copies of the same allele), possibly indicating less adaptive potential for these genes. The other two outlier SNPs were located

in *trnt1* and *iars*, tRNA-synthetase-related genes of which *iars* was also differentially expressed for sensitive-parent offspring at high CO_2 . This is consistent with a role of tRNA synthetase in the cellular response to environmental stressors²³. In this study we focused on coding regions of the genome, but additional important genetic variants might be located in upstream regulatory regions of the differentially expressed genes.

The main inhibitory neurotransmitter receptor in vertebrate brains, the gamma-aminobutyric acid receptor A (GABA_A), is an ion channel with conductance for Cl⁻ and HCO₃⁻, and its function has been shown to be affected by the exposure to near-future CO₂ levels^{16,17}. Fish with impaired behaviour regain normal behaviour after treatment with gabazine, a GABA_A receptor antagonist, and the underlying mechanism is thought to be related to pH regulatory processes altering the neuronal gradients for Cl⁻ and HCO₃⁻ (refs 9,14,17). The GABA receptor genes were highly expressed in the transcriptomes of all our tested fish, but at the same level across treatments. However, on the protein level we found aldehyde dehydrogenase 9 member A1 (Al9a1), a protein involved in the dehydrogenation of gamma-aminobutyraldehyde to GABA, to be 1.7-fold overexpressed at high CO_2 in offspring of tolerant parents. Altered GABA receptor function with high CO₂ exposure could be expected to affect the expression of transporter genes and proteins such as the solute carrier family (slc). However, only one non-GABA-related neurotransmitter transporter gene (slc6a15) and the glycine neurotransmitter transporter protein (Sc6a5) were differentially expressed, but again were upregulated in offspring of CO₂-tolerant parents in high CO₂ conditions (Supplementary Table 4). This upregulation might help fish deal with the interference of high CO₂ with the function of the GABA_A receptor and at least partly explain individual variation in CO₂ tolerance.

Fish have acid–base and osmo-regulatory mechanisms allowing them to avoid tissue acidosis when exposed to high CO_2 , which is one of the predicted physiological costs of acidified oceans²⁵. The importance of this is demonstrated by the overexpression of the arginine vasotocin protein in our fish at high CO_2 level, which is a key component in the coordination of osmotic challenges²⁶. Most fish closely regulate their acid–base relevant ions (primarily CI^- , HCO_3^- and H^+) in response to environmental fluctuations of CO_2 (ref. 16). Many processes involving osmoregulation are under circadian regulation, such as acid–base regulation when exposed to different levels of pH (refs 27,28). In this study we find the molecular signature of CO_2 tolerance to be defined by

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Figure 3 | Differential regulation of circadian rhythm genes for offspring of tolerant parents at high CO₂ condition. All pathway genes are differentially expressed and mRNA upregulation is represented by different colours and refers to different offspring/treatment groups. Pathways of activation (green arrows) and repression (red lines) of different genes²⁹. Importantly, repressors are upregulated for offspring of tolerant parents reared in high CO₂ conditions, whereas activators are found downregulated for this group. The scissors represent the posttranscriptional activity of *ccrn4l* by degrading the poly-A tails.

the differential regulation of nearly all components of the circadian rhythm system (Fig. 3). Differential expression of most circadian genes and several proteins are found in offspring of tolerant parents in high CO₂, in comparison to offspring of the same parents at control CO₂ and offspring of CO₂-sensitive parents at high CO₂ levels. Circadian rhythm and rhythmic process are also enriched biological functions (Supplementary Table 3). The main circadian rhythm activator genes such as *bmal1* (also known as ARNTL in mammals) or *clock* were downregulated, whereas circadian rhythm repressors such as *per1*, *nr1d1* or the Paraspeckle component protein 1 were upregulated in offspring of tolerant parents in high CO₂ conditions. Altered levels of circadian rhythm genes evoke a phase shift in the circadian clock²⁹ and such phase shifts can provide an adaptive advantage when faced with environmental change³⁰. Opposing this downregulation in circadian rhythm for offspring of tolerant parents, we find asmt (acetylserotonin O-methyltransferase), the enzyme that catalyses the final reaction in the synthesis of melatonin, a key regulator of the circadian rhythm, upregulated in offspring of sensitive parents. Ion-regulatory adjustments in fish are managed by melatonin, concurrent with the

circadian rythm³¹. It is possible, therefore, that offspring of sensitive parents display more pronounced ion-regulatory adjustments in response to elevated CO₂, which in turn leads to more profoundly altered Cl⁻ and HCO₃⁻ gradients that interfere with the GABA_A receptor function. We hypothesize that offspring of tolerant parents inherit the 'flexibility' in ion-regulatory control, and therefore the ability to phase shift the circadian clock and avoid a maladaptive reaction to elevated levels of CO₂. This transgenerational signal suggests the adaptive potential of impaired behaviours from high CO₂ due to existing natural variation.

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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 together with H.Z. protein samples for mass spectrometry. T.Ryu performed the genome assembly and gene annotation and wrote the corresponding part. C.S. analysed transcriptome expression data, and performed quantitative real-time PCR expression validation and variant analysis. C.S. analysed mass spectrometry data and integrated the data sets. G.E.N. assisted in interpreting the expression data. C.S., PLLM., T.Ravasi and G.E.N. wrote the paper and all authors read and approved the final manuscript.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. RNA-seq transcriptome sequences have been deposited in GenBank under BioProject ID PRJNA311159. Correspondence and requests for materials should be addressed to P.L.M. or T.Ravasi.

Competing financial interests

The authors declare no competing financial interests.