Science of the Total Environment 987 (2025) 179822

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Acidification alters anxiety-like behaviour and brain gene expression in zebrafish

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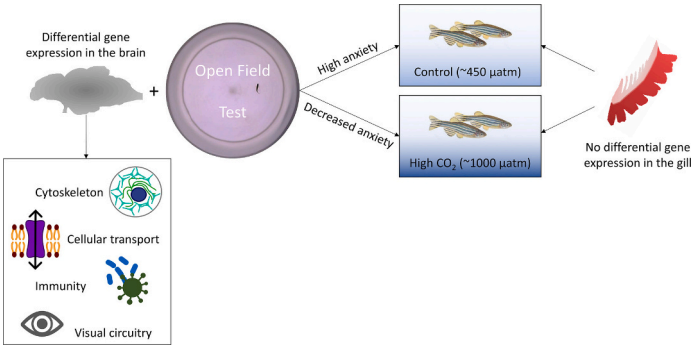
HIGHLIGHTS

- RNASeq of brain and gills identified molecular responses during CO_2-driven anxiety changes
- Gill co-expression of immunity and OxRed genes with acidification point to physiological tolerance
- Acidification altered anxiety levels and gene expression involved in brain cell rearrangements
- Freshwater fishes may be affected by predicted acidification via brain molecular alterations

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Susanne Brander

Keywords:
RNA
Neuro-molecular
Carbon dioxide
Novelty
Stress
Behavioural assay

ABSTRACT

CO_2-driven acidification of freshwater ecosystems is an increasing problem that could impact aquatic life in the future. Despite their physiological tolerance to naturally fluctuating pH, freshwater fishes exhibit behavioural and neurological changes in response to acidification. To determine the molecular responses associated with these anticipated impairments for the near-future, we examined the behavioural and transcriptomic responses of zebrafish (*Danio rerio*) to acidification, focusing on the brain and gills, which mediate behaviour and acid-base regulation. Adult zebrafish were exposed to control (~ 500 µatm) and elevated CO_2 (~1000 µatm) for five days and submitted to Open Field and Novel Object Approach tests, revealing a decrease in anxiety-like behaviour under elevated CO_2. Acidification caused differential expression of genes involved in cytoskeletal organization, cellular transport, immunity, and the visual system in the brain, indicative of brain cell rearrangements. Conversely, there was no differential gene expression observed in the gills. However, the co-expression of genes involved in immune response and oxidoreduction, which are negatively correlated with elevated pCO_2, along with a reduction in anxiety-like behaviour indicate a lower level of oxidative stress. Our findings indicate that zebrafish can perform acid-base regulation despite acidity changes predicted for the end of the century, but reveal that physiological tolerance to acidification does not confer resistance to neurological and behavioural impairments caused by rapid climate change.

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https://doi.org/10.1016/j.scitotenv.2025.179822
Received 28 January 2025; Received in revised form 6 May 2025; Accepted 1 June 2025
Available online 4 June 2025

1. Introduction

Anthropogenic activity increases carbon dioxide (CO_2) levels, driving ocean but also freshwater acidification that affect aquatic ecosystems on many levels (Dupont and Pörtner, 2013; Hasler et al., 2018; Thomas et al., 2022). Sensory, neurological, and behavioural effects of ocean acidification have been intensively reported for marine fish (Hamilton et al., 2023; Porteus et al., 2021; Tsang et al., 2020) with notable findings related to anxiety (Hamilton et al., 2014). However, freshwater fishes also show behavioural alterations (Hasler et al., 2016; Tix et al., 2017), and changes in anxiety-like behaviours such as increased or reduced thigmotaxis (Hamilton et al., 2021; Ou et al., 2015) with CO_2-induced acidification. Such behavioural impairments occur despite freshwater fish being considered "tolerant" to acidification, as fish can buffer their internal pH in acidic waters through acid-base regulation in the gills (Freda and Mcdonald, 1988; Heisler, 1986). It is freshwater fish that more regularly experience wider pH ranges and higher partial pressures of CO_2 (pCO_2) than marine fishes in the wild (Cole et al., 1994; Raymond et al., 2013). Since freshwater fishes are behaviourally susceptible to CO_2-driven acidification (Munday et al., 2019), it is critical to identify the underlying mechanisms altering the brain and behaviour of freshwater fishes under predicted CO_2 conditions.

Yet, we still lack understanding of the processes that impair behaviours of freshwater fishes when they experience elevated CO_2 conditions. Freshwater ecological studies focused on behavioural changes caused by stronger acidification driven by acid rain or acid pollution (Jones et al., 1985; Patrick et al., 1981; Schindler, 1988). Chemical disruption of external cues coupled with damage to sensory organs was pointed as likely mechanisms underlying observed behavioural changes caused by such environmental conditions (Kitamuira and Ikuta, 2001; Leduc et al., 2009). On the other hand, potential mechanisms underlying behavioural impairments caused by milder, CO_2-driven acidification have been investigated in marine fishes mostly (Hamilton et al., 2014). One hypothesis to explain how behaviour can be altered by ocean acidification is the GABA model, referring to the functional reversal of inhibitory γ-aminobutyric acid (GABA) neurotransmission in the brain (Nilsson et al., 2012) that may self-amplify through changes in gene expression (Schunter et al., 2019). On the one hand, the GABA model may extend to freshwater systems, as reversal of acidification-caused changes in anxiety was observed following treatment with the GABA antagonist gabazine in the pink salmon during its freshwater stage (Ou et al., 2015). On the contrary, it is argued that marine and freshwater fish may be behaviourally sensitive to acidification through distinct molecular mechanisms (Leduc et al., 2013) such as alterations in the functioning of glycine receptors, in the modulation of potassium channels in brain cells, or changes of sensitivity in peripheral neurons (Tresguerres and Hamilton, 2017). Finally, as increasing acidification impairs the behaviour in a non-linear manner as found in zebrafish (Hamilton et al., 2021), it is likely that a variety of molecular processes are affected when fish experience elevated CO_2 conditions (Heuer et al., 2019), stressing the need to understand what molecular mechanisms triggered by CO_2-driven acidification in freshwater systems modify fish behaviour.

One suitable model to accurately depict the molecular state of freshwater fish as they perform specific behaviours is the zebrafish. As a popular neurological model species ever since the 1960s (Mrinalini et al., 2023), standardized protocols to measure behaviour in zebrafish are easy to use in the context of environmental research (Stegeman et al., 2010), allowing the observation of behavioural responses under predicted CO_2 conditions experimentally. Additionally, its annotated genome allows the identification of genes expressed in neural circuits and involved in behaviour (Norton and Bally-Cuif, 2010), which expression may be sensitive to acidification.

In our study, we aimed to investigate the molecular mechanisms underlying behaviours impacted by near-future predicted aquatic acidification in the zebrafish *Danio rerio* as they underwent standard behavioural assays of anxiety-like behaviours, exploring an open field arena and approaching a novel object. We examined the behavioural responses and gene expression changes caused by acidification in two key tissues of the organism: the gills, where acid-base regulation is mainly performed, and the brain that controls behaviour. We hypothesized that, as previously reported by Hamilton et al. (2021), acidification alters anxiety-like behaviours in zebrafish. We also expected to see differences in gene expression in the brain as the behaviour is altered, but not in the gills which perform acid-base regulation at variable levels of CO_2, already experienced by freshwater fishes naturally. By characterizing tissue-specific transcriptomic profiles in addition to behavioural changes in response to acidification, we aimed to determine how future predicted levels of CO_2 will affect brain and gill molecular states of zebrafish, which could ultimately lead to behavioural alterations.

2. Methods

2.1. Animals housing and exposure to elevated CO_2

Wild-type zebrafish (*Danio rerio*; AB strain) were reared in the School of Biological Sciences (The University of Hong Kong) aquarium facilities until the age of four months. Fish (8 to 10 per tank) were kept in recirculating aerated tanks (80 × 37 × 32 cm) under a 14/10 h light-dark cycle. The water was maintained at 28 °C with temperature record and adjustment every 60 s with heaters (Schego) and a STC-1000 Thermostat (Elitech). Oxygen levels were measured every two days using a WP91 dissolved oxygen-mV meter (TPS) and kept above 90 % levels of saturation throughout the experiment. Fish were fed with TetraMin food daily. Nitrate levels were measured every five days using a HI97728 nitrate photometer (Hanna Instruments). At four months old, control groups of zebrafish ($n = 13$ fish in total) were reared in tanks (80 × 37 × 32 cm, $n = 3$) bubbled with air while treatment groups ($n = 14$ fish in total) were reared in tanks (80 × 37 × 32 cm, n = 3) bubbled with elevated CO_2 gas from two CO_2 cylinders, simulating projections for atmospheric CO_2 levels by the end of the century at approximately 1000 μatm (Pörtner et al., 2019). The fish were kept in experimental conditions for five days, a necessary period to induce CO_2-driven behavioural changes (Chivers et al., 2014; Lai et al., 2017; Nilsson et al., 2012). The pH (NBS) was measured daily with a Seven2Go pH meter (Meter Toledo), and alkalinity was measured every two days using a G20S Compact potentiometric titrator (Mettler Toledo). The realized partial pressure of CO_2 (pCO_2) was calculated from the pH and alkalinity measurements using CO2SYS v3.0 (Pierrot et al., 2011), using the freshwater set of constants K1, K2 from Millero (1979) and the NBS pH scale. The pCO_2 in control conditions was on average 502.87 ± 33.92 μatm, whereas in treatment conditions it was 1077.37 ± 101 μatm (Table S1, Fig. S1). The pH in control conditions was on average 8.27 ± 0.04 whereas in treatment conditions it was 7.95 ± 0.01.

This study was carried out in approval of the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong (#6084–22), all methods were performed in accordance with the CULATR guidelines and regulations as well as the ARRIVE guidelines.

2.2. Behavioural assays and analyses

To assess the effect of acidification on zebrafish behaviours, a total of 27 fish were behaviourally tested in white, opaque circular testing arenas (ø = 24.5 cm; h = 5 cm of water) illuminated from below, following the methodology of a previous study (Hamilton et al., 2021). The first behavioural test was the Open Field test: it is used to measure anxiety levels, which are higher if more time is spent near the wall of the new arena, and are known to be modulated by environmental factors (Prut and Belzung, 2003). The test started immediately after the animal's introduction into the arena: each fish was initially placed in the

arena centre and left to explore the arena for 10 min, during which locomotion was recorded with a tripod mounted Canon EOS M50 camera placed above the testing arena. Directly following the first test, the second behavioural test called Novel Object Approach test started. It is also used to assess animals' response to novel situations notably in fish, which is interpreted as higher curiosity or boldness with increasing time spent near a never-seen-before object and higher anxiety with decreasing time near the object. This response can also be influenced by extrinsic factors (Dean et al., 2021; Hamilton et al., 2017). A multi-coloured Lego figurine was placed in the arena centre and the response was filmed for another 10 min (Fig. 1a). Every three assays, water was changed in the arena to prevent cortisol build-up (Fontana et al., 2021). Control water was used, as previous research demonstrated that high pCO_2 acclimatized fish would not change behaviour following short-term exposure to control pCO_2 (Munday et al., 2016). Details of experimental conditions of behavioural experiments are summarized in Table S2.

Zebrafish movement was tracked and quantified in each video using the ToxTrac software (Rodriguez et al., 2018). Trajectories in which the fish visibility rate dropped to <95 % for the Open Field test and 81 % for the Novel Object Approach test of all frames were discarded. In each behavioural test, average speed, exploration rate and total distance travelled was measured (Tables S3 & S4). The arena was divided into three zones: the inner zone ranging from the centre (0 mm) to 1/3rd of the arena diameter (radius = 40.8 mm), the transition zone ranging from 1/3rd to 2/3rd (radius = 81.6 mm) of the diameter and the outer zone, also called thigmotaxis zone, ranging from 2/3rd to the outer arena limit (radius = 122.5 mm; Fig. 1b). For each tracked fish, the proportion of the total time (%) spent in each zone was measured (Tables S3 & S4). Regarding the Novel Object Approach test, each fish's distance from the object was measured throughout the video (ranging from 0 to 120 mm) and proportions of the total time (%) spent in each 5 mm interval of the distance to the object's distribution were calculated as well.

Mean proportions of time spent in each of the three zones and mean frequencies of time spent in distance intervals to the Novel Object were compared between control and treatment groups in R v 4.3.2 (R Core Team, 2018) using the *glmmTMB* package (Brooks et al., 2017) to create generalized linear mixed-effects models (Fig. S2). All models included experimental date as a random effect to account for repeated experiments and the total number of frames was used to weight models. The significance of the CO_2 effect on the model was estimated with the Anova function from the *car* package, that performs Type II Wald χ^2 tests (Fox et al., 2012). Whenever statistical differences were found between control and treatment groups, effect sizes were estimated by calculating Cohen's d index.

2.3. RNA sequencing and gene expression analyses

Immediately following the behavioural assays, fish were euthanized by quick severing of the spinal cord. Physical euthanasia was chosen to ensure that RNA profiles in the brain and gill tissues would not be altered by exposure to any chemical agent. Dissection was carried out using sterile tools to extract brain and gills tissues. Those were immediately snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA from brain and gill tissues was extracted using the RNeasy Micro Kit (Qiagen) and the highest quality RNA extracts were sequenced ($n = 7$ per group for brain, $n = 6$ per group for gills) at 150 bp paired end on an Illumina NovaSeq at the Centre for PanorOmic Sciences (CPOS) of the University of Hong Kong.

After sequencing, raw sequence data (on average 32,113,222 ± 1,996,990; Table S3) were trimmed for adapters and filtered based on read quality using Trimmomatic (Bolger et al., 2014) with the following parameters: "ILLUMINACLIP: all_adapters.fa:2:30:10:8:TRUE SLIDINGWINDOW:4:20 MINLEN:32". High quality reads (on average 30,887,846 ± 1,962,277; Table S3) were then mapped against the reference genome (Genome Reference Consortium z11) from the RefSeq database (Pruitt et al., 2007). To obtain gene expression levels we performed mapping to the RefSeq annotation using the program HISAT2 with default settings (Kim et al., 2019) and counting sequence reads mapped to genes with featureCounts (Liao et al., 2014).

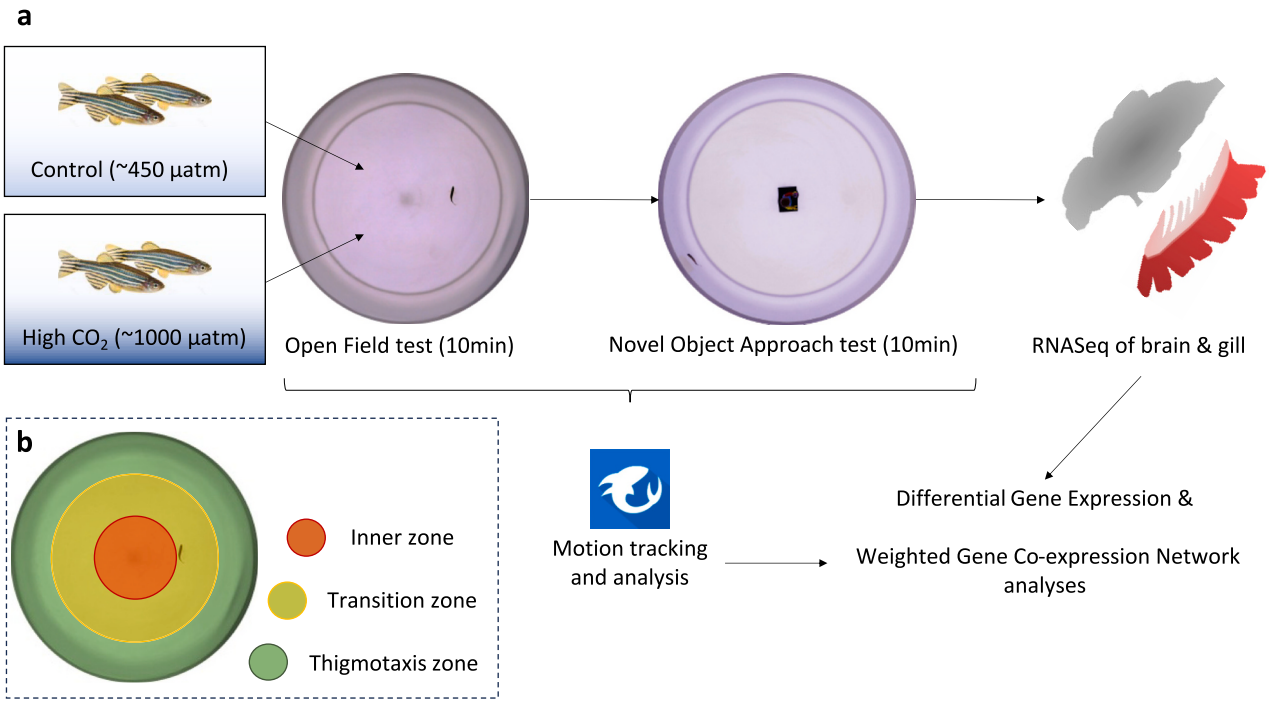

Fig. 1. (a) Experimental design for the study of zebrafish behavioural and molecular responses under elevated CO_2 conditions. Anxiety-related behaviours were studied by submitting zebrafish to an Open Field test followed by a Novel Object Approach test. Movement was tracked using ToxTrac. After behavioural assays, brain and gill tissues were collected to study the gene expression response to elevated CO_2. (b) Zonation of the arena used for behavioural assays with the radius of each zone corresponding to one third of the total arena.

Differential expression analyses were conducted using DESeq2 v1.38 (Love et al., 2014) to investigate which genes are differentially expressed (DE) in the brain and in the gills between control and elevated CO_2 groups. No variable among experimental date, arena number or type of figurine was found to be an important factor influencing the differential expression of genes (Likelihood Ratio Test, factors accounted for <1 % of DE genes), resulting in the statistical model in the design formula with only one factor ("~ CO_2 treatment").

Additionally, to identify which co-expressed sets of genes may correlate with the behaviour and therefore play a role in the behavioural change, a weighted gene co-expression network analysis (WGCNA) was conducted using WGCNA v1.72.1 (Langfelder and Horvath, 2008). This analysis is based on pairwise correlations between expression levels of all genes and other biological variables across samples. It defines consensus modules of genes which expression patterns are clustered together and then tests the statistical significance of correlations between gene expression patterns within a module and other quantitative traits. Here, the proportion of time spent in the thigmotaxis zone, the transition zone and the proportion of time spent at 1000 mm of distance to the novel object (the most frequented distance interval) were provided as trait data, as well as binary encoded information regarding their pCO_2 treatment ("0" = control, "1" = elevated CO_2; Fig. 1a). Two networks were built, one for the brain and one for the gill. The following parameters were used to build the brain network: power = 10 (with $R^2 > 0.80$), TOMType = "signed", minModuleSize = 30, reassignThreshold = 0, mergeCutHeight = 0.25, verbose = 3. For the gill network, the following parameters were used: power = 7 (with $R^2 > 0.90$), TOMType = "signed", minModuleSize = 500, reassignThreshold = 0, mergeCutHeight = 0.25, verbose = 3.

Clusters of genes whose expression patterns were significantly correlated with at least one of the traits of interest were identified. Then, functional enrichment analyses were performed on each module list of genes using OmicsBox v 1.4.11 (Fisher's Exact Test). The GO annotations used for the enrichment analysis were retrieved from BioMart in OmicsBox, using the "Ensembl Genes 111" dataset, the whole genome from the "Zebrafish genes (GRCz11)" database and "Gene Names" type of gene identification. The Gene Ontology (GO) terms with an FDR adjusted *p*-value below the 0.05 threshold were considered enriched, and the list of GO terms was reduced to its most specific.

3. Results

3.1. Behavioural responses to acidification

We hypothesized that acidification would alter anxiety-related behaviours in zebrafish, as found in a previous study (Hamilton et al., 2021). During the Open Field test, fish spent a majority of their time in the thigmotaxis zone over the two other ones, consistent with anxiety triggered by the test as they discover a new area (Prut and Belzung, 2003). There was a subtle effect of elevated CO_2 exposure on the time spent in the thigmotaxis zone (χ^2 test, *p*-value = 2.2×10^{-16}; Cohen's d = 1.04, Table S4; Fig. 2a): fish exposed to control conditions spent 93.96 ± 2.53 % of their time in the thigmotaxis zone ($n = 12$) whereas those who experienced elevated CO_2 spent 91.33 ± 5.94 % of the time in it ($n = 13$), suggesting slightly reduced anxiety under acidification compared to control. Similarly, exposure to elevated CO_2 also influenced occupation of the transition zone (χ^2 test, *p*-value = 2.2×10^{-16}; Cohen's d = 1.06; Table S4; Fig. 2b): individuals who experienced elevated CO_2 spent a higher proportion of time (7.82 ± 2.28 %) in the transition zone (n = 13) than control individuals (5.4 ± 2.28 % of the total time; n = 12). Finally, acidification influenced the proportion of time spent by fish in the inner zone (χ^2 test, p-value = 2.2×10^{-16}; Cohen's d = 0.89), but also its variability (Bartlett's test, p-value = 0.0384; Table S4; Fig. 2c). Fish exposed to control conditions spent 0.64 ± 0.36 % of their time in the inner zone (n = 12) whereas those who experienced elevated CO_2 spent a slightly longer and more variable time in it (0.96 ± 0.7 %; n = 13).

In the Novel Object Approach test, there was no effect of acidification on the distribution of time proportions spent in each distance interval to the novel object (χ^2 test, p-value = 0.961; Table S5). Both groups stayed for most of the test duration between 95 and 100 mm away from the object, 45.66 ± 17.97 % of the time (Fig. 3). However, consistent with the Open Field test, fish exposed to elevated CO_2 spent more time in distance intervals corresponding to the inner zone (radius = 0–40.8 mm; χ^2 test, p-value = 2.2×10^{-16}; Table S5) and to the transition zone (radius = 40.8–81.6 mm; χ^2 test, p-value = 2.2×10^{-16}; Table S5) than control fish, whereas they spent less time in distance intervals corresponding to the Outer zone (radius = 81.6–125 mm; χ^2 test, p-value = 1.029×10^{-16}; Table S5) compared to control fish (Fig. 3).

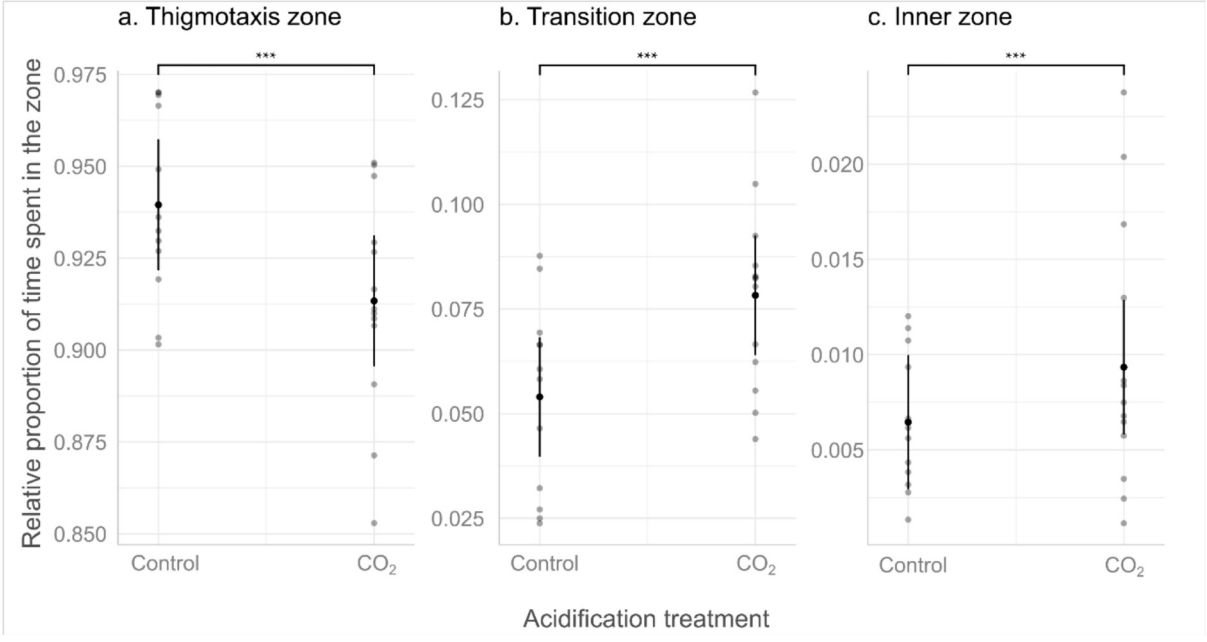

Fig. 2. Predicted (black) and observed (blue) proportions of time (%) spent by zebrafish in the thigmotaxis zone (a), the transition zone (b) and the inner Zone (c) of the Open Field Arena according to their CO_2 exposure; error bars (black) represent standard deviation; stars (***) indicate the significant difference between values.

3.2. Transcriptomic responses to acidification

In the brain, ten genes were significantly differentially expressed, with two being downregulated in the elevated CO_2 treatment (Table S6). One gene, *si:rp71-39b20.4*, is predicted to be a voltage-dependent potassium channel and the other, *guca1c,* is a guanylate cyclase activator involved in cGMP signalling and is expressed in cranial nerves, a part of the vision circuitry. Among the upregulated genes in elevated CO_2, *ictacalcin 2* is involved in vision as it participates in glial cell and retina development. Three upregulated genes code for GTPases that have a role in immunity: two of them (*LOC100149234* and *LOC101882166*) are predicted to code for interferon-induced very large GTPase 1-like proteins and the last one *LOC101885874* codes for a GTPase of the immunity-associated protein (IMAP) family, member 8-like. Another upregulated gene, *si:ch211-51c14.1,* is a paralogue to the mammalian *pacsin3* gene and therefore likely involved in cytoskeleton organization and regulation of endocytosis (Fig. 4). Finally, the upregulated gene *abcb5* codes for a member of the ATP-binding cassette (ABC) transporter family. Two upregulated genes (*LOC103911639* and *LOC110439059*) code for uncharacterized proteins.

Two co-expressed networks of genes in the brain were both positively correlated with the proportion of time spent in the thigmotaxis zone and negatively correlated with the proportion of time spent in the transition zone (Fig. 5; Fig. S3). Among functions performed by those genes, organelle organization, plasma membrane bounded cell projection organization and cytoskeletal protein binding were enriched (Fig. 5; Table S7), meaning that anxiety-like behavioural responses are linked to cellular reorganization in the brain. Changes occurring go beyond the cellular level in the zebrafish brain as indicated by enrichment of functions involved in anatomical development (Fig. 5; Table S7). Finally, enrichment of "carboxylic acid metabolic process", "thiolester hydrolase activity" and "proteolysis" (Fig. 5; Table S7) may illustrate regulation processes in the brain through lysis of certain proteins or fatty acids during anxiety-like behaviours. The production of new proteins also occurs in the brain during anxiety since regulation of transcription and translation were also enriched (Fig. 5; Table S7). On the other hand, one cluster of genes was negatively correlated with the proportion of time spent in the thigmotaxis zone (Fig. 5; Fig. S3), where cellular response to stimulus, peptidyl-amino acid modification and negative regulation of transcription were enriched functions (Fig. 5; Table S7): the less zebrafish spent time in the thigmotaxis zone, the more cellular

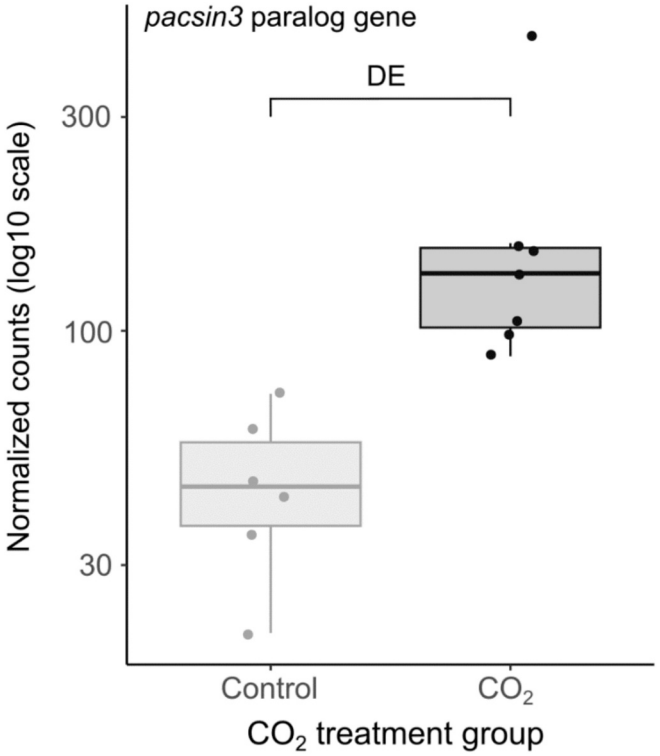

Fig. 4. Normalized counts (log_{10}) for the gene si:ch211-51c14.1, a paralogue of the pacsin3 gene, in the whole brain of zebrafish exposed to two different pCO_2 (control in light grey or treatment, "CO_2" in dark grey); each dot corresponds to one individual; "DE" indicates the significant difference between expression levels.

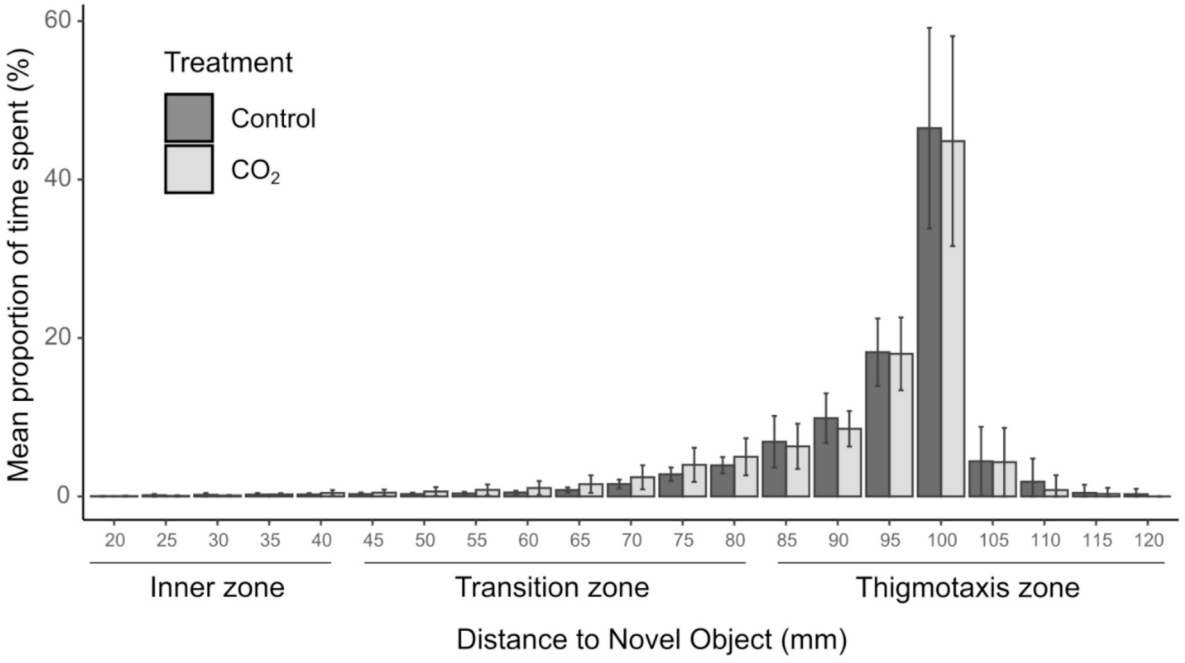

Fig. 3. Mean proportion of time (%) spent by zebrafish depending on their distance to the Novel Object (mm) and according to their acidification exposure (black = control; grey = elevated CO_2); full bars represent mean values while error bars correspond to 95 % confidence intervals.

Fig. 5. Enriched GO biological processes and molecular functions in WGCNA clusters of genes co-expressed with [1]proportion of time spent in the Thigmotaxis zone or [2]pCO_2 treatment, in brain and gill tissues; dot size reflects the number of genes associated with the GO function within the module and colour indicates the correlation value between gene expression levels within the cluster and the behavioural or treatment trait.

response to stimulus, transcription regulation and protein modifications were occurring.

In the gills, no gene was significantly differentially expressed as fish exposed to elevated CO_2 showed more variable transcriptional profiles than fish reared in control conditions (Fig. S4). One cluster of co-expressed genes was negatively correlated with pCO_2 exposure (Fig. 5; Fig. S5) with functions enriched such as protein modification, cell cycle regulation, DNA repair, oxidoreductase activity, immune response and cell signalling functions (Fig. 5; Table S7), suggesting that gills show reduced expression of stress responsive genes when faced with acidification predicted for the end of the century. Another cluster of genes showed its expression levels positively correlated with the proportion of time spent in the thigmotaxis zone (Fig. S5). Among those genes, several functions were enriched, involved in processes such as anatomical structures development, macromolecules transport, cellular localization and DNA damage response (Fig. 5; Table S7). This suggests a subtle reorganization of the gills during anxiety-related responses.

4. Discussion

Acidification slightly reduced the time spent by fish in the thigmotaxis zone and increased time spent in the transition zone and the inner zone, suggesting mildly reduced anxiety under acidification (Võikar and Stanford, 2023). Despite the potential anaesthetic effect of CO_2 on fish (Fish, 1943; Wagner et al., 2002), it is unlikely that our observations are a result of anaesthesia, since the minimum CO_2 concentrations required to produce such an effect on freshwater fish would be 10^2 higher than our treatment (Bernier and Randall, 1998; Gelwicks et al., 1998). Alteration of an anxiety-like behaviour due to CO_2-driven acidification has also been seen in other fishes, such as rockfish and salmon (Hamilton et al., 2014; Ou et al., 2015), yet with contrasting trends. Our observation of slightly decreased anxiety between control and CO_2-treated individuals is consistent with a previous report on zebrafish, suggesting that the acidification influence on anxiety during the Open Field test is non-linear (Hamilton et al., 2021). In that study, control individuals were exposed to pH levels which were similar to our CO_2-treated individuals – due to Hong Kong's high freshwater alkalinity – and spent similar proportions of time in the thigmotaxis zone (85–90 %). On the other hand, our control individuals, which displayed higher anxiety, experienced higher pH conditions than zebrafish in the Hamilton and collaborators' study. Taken together, CO_2-driven acidity levels influence anxiety in a non-linear manner, whereby less anxiety behaviour is observed at intermediate levels of basicity (pH between 7.8 and 8) and high anxiety is observed at intermediate levels of acidity (pH ~ 6.5) in zebrafish. Nonetheless, with overall contrasting reports of CO_2-driven acidification induced-anxiety in other freshwater fish species, this suggests that CO_2 effects are also context-specific and may depend on freshwater conditions. Therefore, our results further support that behavioural responses to CO_2 exposure are complex even at the species level due to interactions between dissolved CO_2 and other elements in the water acting on its acidity.

Elevated CO_2 did not affect the spatial distribution of zebrafish around the object during the Novel Object Approach test, which suggests that acidification does not affect boldness or curiosity when facing a novel object, as previously reported in zebrafish (Hamilton et al., 2021) but contrary to other marine and freshwater species that spent more time near the novel object with elevated CO_2 (Jutfelt et al., 2013; Ou et al., 2015). Nevertheless, increased boldness while facing novelty in other species is indicative of reduced anxiety levels (Maximino et al., 2010) and consistent with our observations in the Open Field test. Overall, our findings therefore support that zebrafish curiosity while discovering a new object may not be affected by acidification, unlike

anxiety-related behaviours.

Reduced anxiety due to acidification may be caused by a change in the efficacy of neurotransmission, as zebrafish that experienced acidification exhibited downregulated expression of a voltage-dependent potassium channel gene (*si:rp271-39b20.4*) in the brain. This could decrease the overall activity performed by potassium channels in fish brain cells, on top of the activity decrease caused by changes in intracellular bicarbonate (HCO_3^-) concentrations as previously suggested (Tresguerres and Hamilton, 2017), with potentially less potassium channels in the brain of zebrafish experiencing elevated CO_2. Acidification may also reduce anxiety via changes in cytoskeleton organization and intraneuronal transport across the zebrafish brain, as gene networks linked to cellular reorganization were associated with strong anxiety-like behavioural responses. Furthermore, upregulation of a paralogue to the *pacsin3* gene (*si:ch211-51c14.1;* Morgan et al., 2022) in the brain while in elevated CO_2 suggests changes of cytoskeletal conformations,. Such mechanisms have already been described in humans, where the expression of anxiety-related behaviours is often correlated with neuromorphological plasticity through changes at the level of dendritic branches (Leuner and Shors, 2013). Therefore, one way through which exposure to acidification may have a reducing effect on anxiety could be through intracellular rearrangements of brain cells, notably at the cytoskeletal level.

Expression of genes involved with carboxylic acid metabolism in the brain was positively correlated with time spent in the thigmotaxis zone, suggesting increased expression in fish displaying high levels of anxiety. This is consistent with previous studies linking fatty acid metabolism with anxiety-like behaviour (Liśkiewicz et al., 2020; Moon et al., 2014). Therefore, changes in anxiety-related behaviour provoked by acidification may alter protein and fatty acid metabolism. Furthermore, acidification, which was found here to decrease anxiety levels, is also known to cause changes in lipid metabolism in fish (Díaz-Gil et al., 2015; Frommel et al., 2012), notably through differential gene expression of genes involved in fatty acid synthesis (Frommel et al., 2020). Acidification may therefore act on anxiety levels by disrupting fatty acid homeostasis. Furthermore, in elevated CO_2 that caused reduced anxiety-like behaviour, proteolysis-related genes were expressed at lower levels. Reduced anxiety was also paralleled with downregulation of genes or proteins taking part in proteolysis in the brain of other species (Asano et al., 2022; Szego et al., 2010). Overall, acidification may either directly act on fatty acid and protein metabolism, ultimately causing changes in anxiety levels, or the influence of acidification on those functions could also be indirect, by causing anxiety levels changes that in turn alter lipid and protein metabolism.

The brain molecular response of zebrafish suggests that acidification notably affects the visual system, as indicated by the downregulation of a guanylate cyclase activator involved in cGMP signalling in the optic neural circuitry (Fries et al., 2013; Rätscho et al., 2010; Scholten and Koch, 2011), and the upregulation of *ictacalcin 2* that participates in Müller glial cells development in the retina (Tworig and Feller, 2022; Vöcking and Famulski, 2023). CO_2-driven ocean acidification was also previously reported to affect the visual system of marine fishes, leading to impairments (Chung et al., 2014; Ferrari et al., 2012) and related changes in gene expression (Ramírez-Calero et al., 2023). Consistently, changes in expression of genes involved in vision here further indicate visual impairments may be expected under predicted CO_2 conditions, with potential consequences on visually mediated behaviours such as exploration of new environments or predator avoidance.

Changes in anxiety-like behaviours and brain gene expression were not paralleled with molecular changes in zebrafish gills, which suggests that acidification can act on the brain and behaviour of fish without impairing acid-base regulation performed by the gills (Heuer and Grosell, 2014). Functions such as structure development, macromolecules transport, cellular localization and DNA damage response co-expressed with anxiety-like behaviour, suggesting a subtle reorganization of the gills during anxiety-related responses, were not enriched with acidification. Moreover, elevated CO_2 did not provoke gene differential expression in the zebrafish gills, although their transcriptomic profiles tended to be more variable in fish exposed to acidification. Here, the absence of a large transcriptomic response to our levels of acidification ($\sim$ 1000 μatm, pH $\sim$ 7.9) further indicates that the zebrafish is physiologically well adapted to variable and sometimes acidified conditions (pCO_2 $\sim$ 4000 μatm; pH $\sim$ 6.6) encountered in its natural habitat (Kwong et al., 2014; McClure et al., 2006; Sundin et al., 2019). It is possible that zebrafish may effectively cope with smaller increases in acidification via a more subtle molecular response not resulting in any significant differential gene expression. By simultaneously observing behavioural changes as well as transcriptomic changes in the brain, but not in the gills under acidification, our findings support the hypothesis that the compensatory response during successful acid-base regulation, and not a physiological impairment of the gill, may be responsible for brain and behavioural changes observed with elevated CO_2 (Heuer and Grosell, 2014).

Zebrafish gills were shown to strongly respond to higher levels of acidification, with differential expression of genes involved in paracellular uptake regulation, oxidoreduction and cellular stress response (pH $\sim$ 4; Kumai et al., 2011; Tiedke et al., 2013). Consistently, we found co-expression of genes involved in DNA repair, immune response, oxidoreduction and cell cycle regulation, but negatively correlated with acidification. These pathways have also been implicated in studies on other fish species which demonstrate reduced antioxidant capacity, oxidative stress induced damage and differential expression of immune response genes under acidified conditions in the gills (Copatti et al., 2019; Enzor and Place, 2014; Machado et al., 2020). Here, reduced expression of such genes with acidification instead could be due to our acidity levels not being high enough to trigger cellular stress. This hypothesis is supported by the fact that our pH levels in elevated pCO_2 were still more alkaline than control conditions of other acidification studies led on zebrafish (Kumai et al., 2011; Tiedke et al., 2013). However, increased variability of the zebrafish gill transcriptomic profile under acidification indicates that not all individuals had the same response to elevated CO_2. This could be interpreted as a plasticity at the population level, with different molecular strategies employed from one individual to another to cope with acidification. Such transcriptomic heterogeneity was observed across eukaryotes (Raj and van Oudenaarden, 2008) and proposed to confer advantages at the level of the population, especially in naturally fluctuating environments (De Jong et al., 2019). Nevertheless, the absence of a gill response indicative of physiological impairment, in contrast to observed behavioural changes in a relatively pH tolerant species, does not necessarily confer an overall tolerance to the many effects of CO_2-driven acidification across the organism. In the case of the brain, trade-offs or downstream consequences to acid-base regulation may still occur (Heuer and Grosell, 2014) and result in the observed gene expression changes as well as behavioural alterations. For example, in marine fishes the GABA hypothesis presents one such consequence to acid-base regulation: changes in chloride (Cl^-) and HCO_3^- ion concentrations in blood plasma and neurons alter the function of the ionotropic $GABA_A$ receptor and in turn provoke behavioural impairments (Nilsson et al., 2012). Because of such alterations in neurosensory systems, fishes may display behavioural impairments with near-future predicted acidification despite their ability to successfully maintain their internal pH levels, even in freshwater habitats where species are adapted to more acidic waters.

In summary, we found zebrafish exposed to near-future predicted levels of pCO_2 had slightly reduced anxiety likely through modified neurotransmission, brain cells intracellular rearrangements notably at the cytoskeletal level, fatty acid and protein metabolism, as well as altered visual circuitry. This is further supporting that acidification can impair teleost fish behaviour by acting on the brain at the molecular level on different targets. Despite gills not showing a molecular stress response to acidification, indicative of physiological tolerance to reduced pH, molecular responses and subtle behavioural changes may

reveal freshwater fishes to be more affected than anticipated by rapid climate change.

CRediT authorship contribution statement

Jade M. Sourisse: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Munisa Tabarova:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Daniele Romeo:** Writing – review & editing, Methodology, Formal analysis. **Yan Chit Kam:** Writing – review & editing, Methodology, Data curation. **Celia Schunter:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

JMS was funded by the start-up of CS and this study was financed by General Research Fund from the research Grants Council Hong Kong #17300721 and the NSFC Excellent Young Scientist Award (AR225205) to CS. We thank all the members of the lab for support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179822.

Data availability

The raw sequencing data can be found in BioProject PRJNA1102381.

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