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The dose makes the poison: Non-linear behavioural response to CO_2-induced aquatic acidification in zebrafish (*Danio rerio*)

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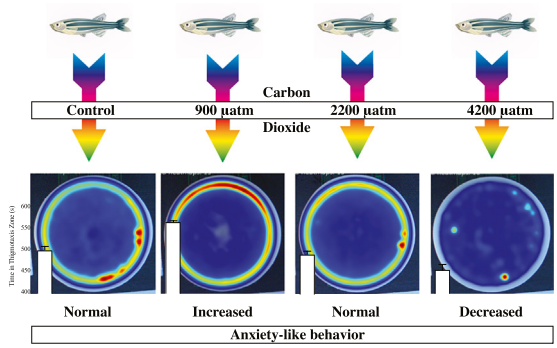
HIGHLIGHTS

- Elevated CO_2 altered behaviour in zebrafish but not in an additive manner.
- Acclimations to ~900, 2200, and 4200 μatm cause increased, normal, and decreased anxiety-like behaviour.
- Exploratory behaviour was not affected by any CO_2 treatment.
- Elevated CO_2 to ~4200 μatm decreased locomotion.

GRAPHICAL ABSTRACT



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ABSTRACT

CO_2-induced aquatic acidification is predicted to affect fish neuronal $GABA_A$ receptors leading to widespread behavioural alterations. However, the large variability in the magnitude and direction of behavioural responses suggests substantial species-specific CO_2 threshold differences, life history and parental acclimation effects, experimental artifacts, or a combination of these factors. As an established model organism, zebrafish (*Danio rerio*) can be reared under stable conditions for multiple generations, which may help control for some of the variability observed in wild-caught fishes. Here, we used two standardized tests to investigate the effect of 1-week acclimatization to four pCO_2 levels on zebrafish anxiety-like behaviour, exploratory behaviour, and locomotion. Fish acclimatized to 900 μatm CO_2 demonstrated increased anxiety-like behaviour compared to control fish (~480 μatm), however, the behaviour of fish exposed to 2200 μatm CO_2 was indistinguishable from that of controls. In addition, fish acclimatized to 4200 μatm CO_2 had decreased anxiety-like behaviour; i.e. the opposite response than the 900 μatm CO_2 treatment. On the other hand, exploratory behaviour did not differ among any of the pCO_2 exposures that were tested. Thus, zebrafish behavioural responses to elevated pCO_2 are not linear; with potential important implications for physiological, environmental, and aquatic acidification studies.
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1. Introduction

The increasing levels of atmospheric CO_2 will invariably result in elevated pCO_2 levels in aquatic environments. Because CO_2 dissolves in water producing H^+ that lower the pH, this process is known as "ocean acidification" and "freshwater acidification"; here, we will use "aquatic acidification" to refer to both. When exposed to aquatic

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acidification conditions in the laboratory, fish have been reported to suffer significant behavioural alterations that have raised concerns about the fate of fish in future aquatic environments. Some of the alterations include impaired olfactory and auditory discrimination, behavioural lateralization, anxiety, locomotion, and boldness (reviewed in Munday et al., 2019). The proposed underlying cause stems from the mechanism fish use to maintain blood acid-base homeostasis: to counteract the acidosis induced by exposure to elevated pCO_2, fish excrete H^+ across their gills and accumulate HCO_3^- (reviewed in Heuer et al., 2019). Together with putative equimolar reductions in the concentration of chloride ions ($[Cl^-]$), the altered blood chemistry is theorized to affect the functioning of $GABA_A$ receptors resulting in neuronal depolarization and altered behaviour (reviewed in Tresguerres and Hamilton, 2017). However, the direction and magnitude of behavioural responses to aquatic acidification that have been reported in different studies are highly variable. Perhaps the highest profile example is the contrasting results in olfactory discrimination reported for coral reef fishes (Munday et al., 2009; Clark et al., 2020a) that has recently sparked what we consider to be an essential debate (Munday et al., 2020; Clark et al., 2020b). Similarly, our group has reported divergent behavioural responses by two congener fish species that inhabit Californian kelp forests: while the splitnose rockfish (*Sebastes diploproa*) demonstrated increased anxiety-like behaviour upon acclimatization to ~1100 μatm CO_2 (Hamilton et al., 2014), the behaviour of blacksmith (*Chromis punctipinnis*) was not affected (Kwan et al., 2017).

The observed variability of behavioural responses is often ascribed to yet unidentified species-specific and life stage-dependent differences in the mechanisms responsible for acid-base regulation and neuronal function. In addition, it has been suggested that behavioural responses to elevated pCO_2 might not be linear, resulting from complex neuronal responses to intra- and extra-cellular acid-base parameters (Heuer et al., 2016). Finally, many of the discrepancies in the literature may be due to differences in the life history of wild-caught fish and that of their parents, the levels of experimental CO_2 to which the fish are exposed, and inherent variability and limitations of the behavioural tests that are employed.

With this in mind, we conducted a series of experiments using zebrafish (*Danio rerio*). This fish species is commonly bred under standardized conditions for generations, and therefore provide a set of experimental organisms with a more homogenous life history compared to wild-caught fish. To explore the possibility of a non-linear relation between pCO_2 levels and behavioural responses, we examined the effects of four increasing pCO_2: ~480 μatm as the control condition, ~900 μatm to simulate aquatic acidification predicted for the end of the century in the surface of large water bodies including the ocean, ~2200 μatm as a more pronounced yet still ocean-acidification relevant scenario, and ~4200 μatm as a more extreme scenario. To address the potential issue of behavioural repeatability, we chose to employ the "open field" and "novel object approach" tests. These are standardized, robust, and relatively simple tests proven to reflect anxiety-like and exploratory behaviours that depend, at least in part, to the functioning of the $GABA_A$ receptor ($GABA_AR$). This has been validated in rodents (Lowery-Gionta et al., 2018), and in zebrafish by our group (Hamilton et al., 2017) and others (Ferreira et al., 2019). Unconscious bias was eliminated by recording and analysing fish behaviour using a camera system and motion tracking software.

2. Methods

2.1. Animals and housing

Adult wild-type (short-fin) zebrafish (*Danio rerio*) were obtained from Aquatic Imports (Calgary, AB) ($n = 150$) and were held in the lab for a minimum of 60 days prior to experimentation. All fish were 9–12 months old, approximately equal male:female ratio, and were housed in an Aquatic Habitats (AHAB, Aquatic Ecosystems, Inc. Apopka, FL, USA) three-shelf bench top system with controlled filtration and aeration, in clear 3 L polypropylene tanks. Density was seven to eight fish per tank and fish were fed once per day. Temperature was maintained at 29.8 ± 1.4 °C and water was buffered with NaCl, $NaHCO_3$, and $C_2H_4O_2$ to achieve an average pH of 7.1 ± 0.03 over the experimental period (see Supplementary data). Weekly water quality measurements included nitrates, nitrites, alkalinity and conductivity (Hamilton et al., 2017). Zebrafish were fed Gemma Micro 300 pellets (Skretting/BioOregon, ME, USA) daily between 10 am and 2 pm. All experiments were approved by the Grant MacEwan University Animal Research Ethics Board (AREB) under protocol number 05-12-14, in compliance with the Canadian Council for Animal Care (CCAC) guidelines for the care and use of experimental animals.

2.2. CO_2 acclimatization

Randomly selected zebrafish were moved from the main habitat to experimental tanks (9 L) fitted with an individual filter (Marineland, Blacksburg, VA, USA) and heater (Hydro Aquarium) in one of four CO_2 conditions (Table 1) (15 zebrafish in each experimental tank) (Supplementary Material) for 7 days of experimentation. Pure compressed CO_2 (Praxair, Edmonton, AB) was injected to experimental tanks using an Apex control system (Neptune Systems, San Francisco, CA, USA) allowing for two CO_2 tanks and one control tank to be run in parallel. Fish were placed into tanks then CO_2 levels were ramped up to their experimental levels over a 24 h period. Experiments were repeated on four occasions, each time containing at least one control tank. Actual pCO_2 levels were monitored with a Vaisala GMP 251 CO_2 probe (Finland) connected to a closed-loop system made up of a CO_2-impermeable plastic PVC tubing attached to medical grade CO_2 permeable silicon tubing (0.8 mm thickness) submerged in the tank with the enclosed air pumped through the system (Watson et al., 2017). To allow for stable and consistent measurements, the pCO_2 readings were made 50 min after immersing the probe (Apex double junction lab grade pH probe, Neptune) in the tank. In addition, pH (NBS) was measured using the Apex system; these readings were performed twice a day, once in the morning and once in the afternoon. 15% water changes were done on days 3 and 6. On the day of behavioural testing zebrafish were not fed until after recording.

2.3. Behavioural testing

The testing arena was a circular, opaque white cylinder 34 cm in diameter and 15 cm deep (Supplementary Figure 1), which was filled with water to a height of 6 cm. Because constantly switching water pCO_2 between trials was unfeasible, all testing took place in control water. In any case, previous research has indicated that short term exposure of high pCO_2 acclimatized fish to control pCO_2 does not affect fish behaviour (Munday et al., 2016). An enclosure of white corrugated plastic was mounted around the arena to minimize external visual stimuli. An individual fish was transferred in a small net from the CO_2-dosing tank and placed into the centre of the testing arena; transfer took a maximum of 5 s. The "open field test" started immediately after the fish was placed in the center of the arena and lasted 10 min (Dean et al., 2020). Shortly after the open field test ended, a Lego® figurine was placed in the center of the arena and the "novel object approach test" commenced (Hamilton et al., 2017; Johnson and Hamilton, 2017; Leighton et al., 2018). To prevent zebrafish preference to single colours (Avdesh et al., 2012), the figurine was multi-coloured (Supplementary Figure 1). Fish movement was tracked for 10 min. Water was changed after every five fish to maintain temperature between 26 and 29 °C and prevent the build-up of waste products. Fish behavioural responses were not significantly different among fish tested in the same arena water (see Supplementary Figure 2), ruling out potentially confounding effects of conspecific or 'alarm cues'. Light levels in the testing room were 28 cd/m^2 (measured with a cal Spot photometer; Cooke Corp. CA, USA) lighting was provided with incandescent lamps above and beside the arena.

Table 1
Experimental groups and parameters. Mean ± 1 s.e.m.

	pCO_2 (μatm)	pH_{NBS}	Number of replicates (15 fish each)	Number of fish (total)
Control	481 ± 14	7.86 ± 0.03	4	60
900 μatm CO_2	874 ± 29	6.48 ± 0.01	2	30
2200 μatm CO_2	2238 ± 99	6.24 ± 0.01	2	30
4200 μatm CO_2	4220 ± 168	6.07 ± 0.02	2	30

2.4. Quantification of behaviour

Zebrafish movement was recorded using the differencing method in EthoVision XT motion tracking software (version 11, Noldus, Leesburg, VA, USA) (Hamilton et al., 2017; Pham et al., 2009). For both the open field and novel object approach test, the arena was divided into three zones: inner (the centremost zone 0–12 cm in diameter), transition (in between the other two zones, 12–23 cm in diameter), and thigmotaxis (the outermost zone, 23–34 cm in diameter). Time in inner, transition, and thigmotaxis zones, average velocity, and immobility were quantified for all fish using EthoVision (Hamilton et al., 2017). Heatmaps represent a coloured representation of the location of the fish ($n = 15$) during the full trial and were generated by combining all trials from an experimental tank in the EthoVision program.

2.5. Statistical analysis

Data were analyzed using Graphpad Prism 9.0 (CA, USA). An alpha level of $p < 0.05$ and 95% confidence intervals were used for assessing statistical significance in all tests. All datasets were assessed for normality using the D'Agostino and Pearson omnibus normality test. Data sets that failed the normality test were normalized. Datasets with normal distribution were analyzed using a nested one-way ANOVA with Tukey's multiple comparison post-hoc test. If the nested ANOVA indicated that subgroups were significantly different the data sets were shown graphically but removed from the analysis (McDonald, 2014; Riley and Edwards, 1998) (only one subgroup fit this criteria, for two parameters; velocity in the open field test and time immobile in the open field test 4200A vs. 4200B). Data for all subgroups is in Supplementary data.

3. Results and discussion

3.1. Open field test

The open field test is used to assay general locomotion activity and anxiety levels (Prut and Belzung, 2003). Under water quality parameters common in zebrafish husbandry (see Supplementary Data) and at ambient pCO_2, zebrafish in the open field test spend the majority of the time near the wall of the arena (i.e. the thigmotaxic zone) and are considered to have intrinsically high anxiety levels, but are still amenable to modulation in both directions by extrinsic factors (Hamilton et al., 2017). In the current study, zebrafish acclimatized to control pCO_2 (~480 μatm CO_2) demonstrate the expected high anxiety level (Fig. 1a, and note the different y-axes range in 1b-d). However, zebrafish acclimatized to ~900 μatm CO_2 spent significantly more time in the thigmotaxis zone compared to controls (Fig. 1b; $F(3,6) = 10.40$, $P = 009$) which indicates increased anxiety-like behaviour. Although the time in the transition zone and inner zone were not significantly different from control (Fig. 1c; $F(3, 6) = 8.35$, $P = 0.015$, and Fig. 1d; $F(3, 6) = 4.87$, $P = 0.048$, respectively) the trend was consistent with decreased anxiety. According to the $GABA_AR$ hypothesis, acclimatization to further elevated CO_2 levels should further alter the reversal potential of $GABA_A$ receptors, giving these receptors less of a hyperpolarizing influence on neuronal circuits (Heuer et al., 2016; Tresguerres and Hamilton, 2017). The behavioural outcome on anxiety would be a further increase. However, zebrafish acclimatized to ~2200 μatm CO_2 spent the same amount of time in the inner, transition, and thigmotaxic zones (Fig. 1a–d), which implies similar anxiety-like behaviour across groups. Moreover, zebrafish acclimatized to the highest pCO_2 of ~4200 μatm spent the most time in the transition and inner zone and the least time in the thigmotaxic zone among all pCO_2 levels (Fig. 1a–d), and therefore had the lowest levels of anxiety-like behaviour. These responses are readily evident in the heat maps shown in Fig. 1a, which denote the average movement patterns of all fish from a given trial. The continuous movement of fish along the edge of the arena appeared as a bright halo in warm colours, while fish immobility resulted in bright red spots.

The non-linear response whereby anxiety-like behaviour was maximal at ~900 and minimal at ~4200 μatm CO_2 (Fig. 1b–d) suggests the involvement of other mechanisms in addition to $GABA_A$ receptors; for instance, K^+ channels, or dopamine, glycine, or glutamate receptors. It seems possible that neurons from zebrafish acclimatized to ~900 μatm CO_2 experience a combination of $[HCO_3^-]$, $[Cl^-]$, and Vm resulting in an excitatory depolarization reflected as increased anxiety-like behaviour. Alternatively, compensation of altered excitability of $GABA_AR$ during exposure to ~2200 μatm CO_2 could drive the system back to equilibrium, and overcompensation during exposure to ~4200 μatm CO_2 could drive the system beyond equilibrium and into an anxiolytic state.

Velocity was not different across treatment groups (Fig. 1e; $F(2,5) = 1.67$, $P = 0.279$), nor was immobility (Fig. 1f; $F(2,5) = 1.152$, $P = 0.402$). This suggests that the difference in zone preference (ie. more time in the thigmotaxic zone for 900 μatm CO_2 and less for 4200 μatm CO_2) was not due to locomotion-induced causes. Furthermore, fish from the two replicate 4200 μatm tanks displayed statistically significant different velocity and immobility, yet they spent comparable amount of time in the different areas of the arena. In addition, the differences in these parameters disappeared during the novel object test (see next section).

3.2. Novel object approach test

The novel object approach test is used to quantify "boldness behaviour" based on the animal's tendency to explore a never-before-seen object placed into an open field arena (Toms et al., 2010) (i.e. time spent in the inner zone where the object is located, Fig. 2a). This test has been validated for zebrafish with drugs known to increase boldness in mammals, like ethanol (Hamilton et al., 2017; Dean et al., 2021) and nicotine (Dean et al., 2020).

In the current study, CO_2 acclimatization did not induce any significant differences in time spent in the thigmotaxis (Fig. 2b; $F(3,6) = 1.25$, $P = 0.372$), transition (Fig. 2c; $F(3,6) = 1.31$, $P = 0.356$) or inner (Fig. 2d; $F(3,6) = 1.54$, $P = 0.207$) zones, but note that the variability is high in the ~4200 μatm group in Fig. 2d. Interestingly, zebrafish in the novel object approach test moved at slower average velocity and their time spent immobile roughly doubled relative to the open field test, regardless of the CO_2 acclimatizations (Fig. 2e, f). In addition, zebrafish exposed to ~4200 μatm tended to move slower than control zebrafish (Fig. 2e; $F(3,6) = 1.25$, $P = 0.372$), and there was a significantly greater time immobile for the 4200 μatm compared to 900 μatm group (Fig. 2d; $F(3,6) = 5.06$, $P = 0.044$). These trends are qualitatively similar to those from the open field test (compare Fig. 1e,f and Fig. 2e,f). These behaviours during the novel object approach test are readily evident in the heat maps shown in Fig. 2a, especially the bright red spots that result from fish remaining immotile for long periods of time.

In summary, introduction of the novel object significantly affected zebrafish immobility compared to the open field test, but location preference in the arena was not altered by the CO_2 acclimations. Interestingly, the mere action of placing a novel object in the centre of the

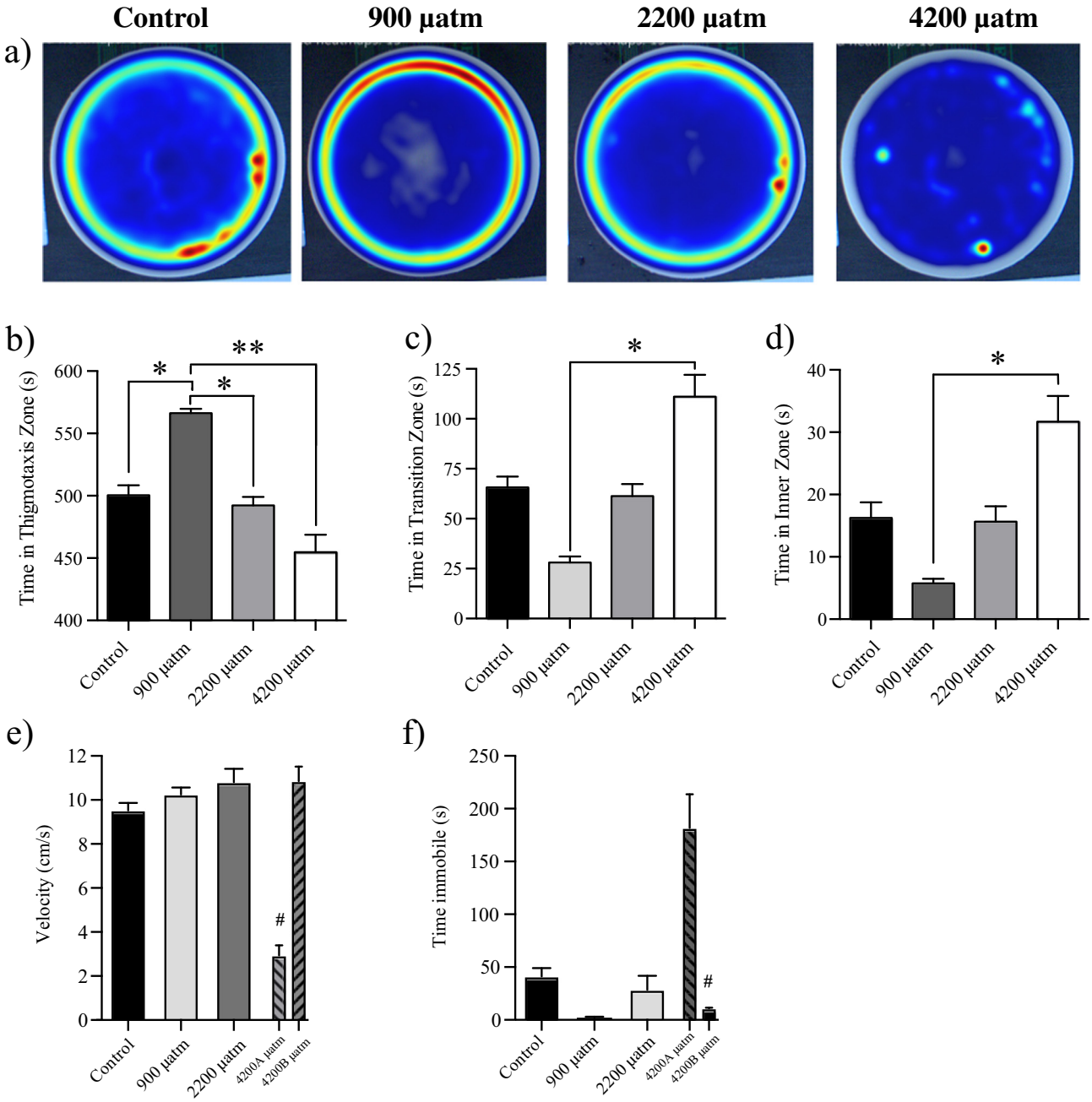

Fig. 1. Acclimatization to increasing pCO_2 causes non-linear behavioural alterations in the open field test. (*a*) Group heatmaps showing a coloured representation of the location of the average of the group of fish ($n = 15$) over the trial (proportional to the time spent in each pixel). Average time the fish spent in the thigmotaxis (*b*), transition (*c*), and inner (*d*) zones during the open field test. Mobility was quantified by measuring velocity (*e*) and time spent immobile (*f*). Data are mean ± 1 s.e.m. Significant differences between controls and aquatic acidification groups are indicated by *($P < 0.05$), **($P < 0.01$). # denotes a significant difference between subgroups, identified by nested one-way ANOVA.

arena reversed all CO_2 acclimatization-dependent effects on zebrafish anxiety-like behaviour (based on the open field test, Fig. 1a–d). Thus, we highlight how one change in a behavioural test can dramatically alter the inferred outcome, and are therefore cautious about predicting how and if aquatic acidification will affect the behaviour of fish in their natural environment, which is infinitely more complex than a testing arena with a novel object in it.

The novel object approach test has been used to examine the effects of aquatic acidification on fish boldness in only a few previous studies, which have all reported different outcomes: decreased boldness (Jutfelt et al., 2013), no effect (Tix et al., 2017), or increased boldness (Ou et al., 2015). These differences might be due to the specific pCO_2 levels that were tested, the relation between the preferred pCO_2 level of each fish species and the experimental pCO_2 levels, or unknown stochastic variables. Placing our results in the broader field of aquatic acidification is even less straightforward because most other previous studies have used diverse tests to estimate boldness. These include the tendency of fish to swim away from their home reef (Munday et al., 2010), the time spent in the inner circle within an open field test (Jarrold and Munday, 2018, Laubenstein et al., 2018), and the time that takes to leave a shelter (Jutfelt et al., 2013). In addition to changes to boldness behaviour, these methods and parameters may reflect interactive and confounding effects of changes in locomotion, anxiety, and other unknown variables.

There has yet to be a consensus on the ecological consequences that will result from aquatic acidification. Many studies on freshwater fish species have shown negative effects including impaired growth, altered olfaction and feeding (reviewed in Hasler et al.,

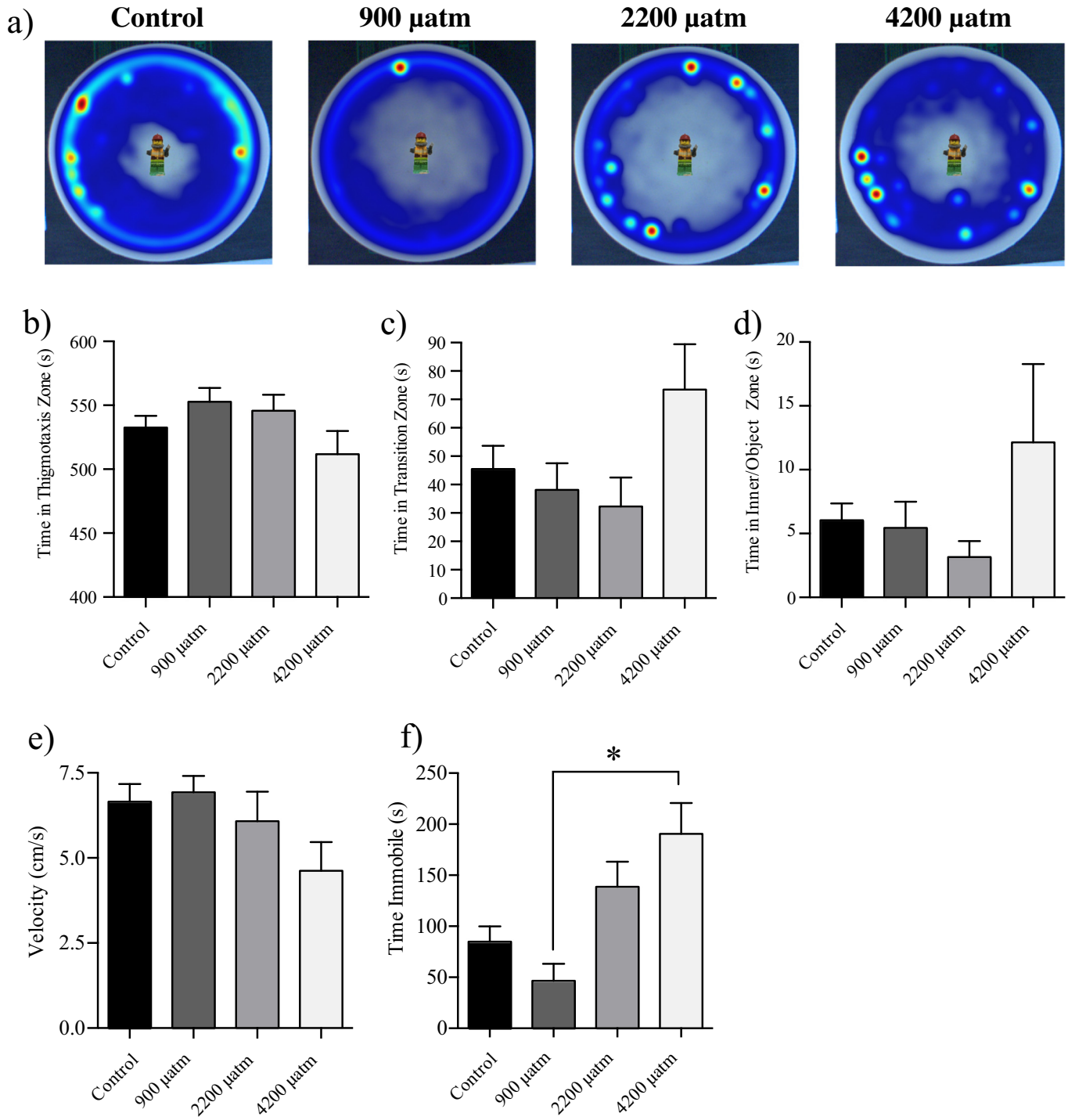

Fig. 2. Acclimatization to increasing pCO_2 only alters mobility in the novel object approach test. (*a*) Group heatmaps showing a coloured representation of the location of the average of the group of fish (n = 15) over the trial (proportional to the time spent in each pixel), with an image of the novel object where it was placed into the centre of the arena (standing vertically). Average time the fish spent in the thigmotaxis (*b*), transition (*c*), and inner/object (*d*) zones during the novel object approach test. Mobility was quantified by measuring velocity (*e*) and time spent immobile (*f*). Data are mean ± 1 s.e.m. Significant differences between controls and aquatic acidification groups are indicated by *($P < 0.05$).

2018), and behaviour (Ou et al., 2015; Ikuta et al., 2003). However, other studies have found minimal or no behavioural alterations (Vossen et al., 2016; Tix et al., 2017; Midway et al., 2017). As pointed out by our study, these discrepancies might be explained by the different pCO_2 levels used in each study and putative ensuing non-linear effects.

It is interesting to note that wild zebrafish inhabit streams with relatively high pCO_2 levels (~4000 µatm; Sundin et al., 2019). Therefore, while our protocol allowed us to identify non-linear effects of pCO_2 acclimation on zebrafish behaviour under controlled laboratory conditions, they might not represent the responses of zebrafish in their natural hypercapnic environment. Importantly, this situation applies to most other studies, and realizing these limitations will lead to better design of experiments and downstream will further our understanding of the ecological consequences of aquatic acidification. In addition, these results cast new questions about the effects of acid-base homeostasis and highlight the need for multiple levels of CO_2 exposures to capture the potential non-linear effects.

4. Conclusion

Acclimatization to increasing pCO_2 levels had a non-linear effect on zebrafish anxiety-like behaviour but not on exploratory behaviour. The implication of this research is at least threefold: (1) increased pCO_2 levels do not necessarily affect fish behaviour in an additive manner. This might help explain some of the reported diverse responses of fish to aquatic acidification, and guide meta-analyses that attempt to identify universal responses. (2) The type and design of behavioural test is paramount to unravelling the impacts of aquatic acidification on fish behaviour, stressing the need for standardizing behavioural tests in the field of aquatic acidification. (3) While behavioural studies might detect effects of aquatic acidification on fish behaviour, establishing their relevance to the real world is greatly complicated by habitat complexity, rate of pCO_2 increase, species-specific pCO_2 adaptations, among multiple other parameters. Thus, we recommend a cautious approach when extrapolating experimental results to predictions of environmental outcomes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions statement

Trevor J. Hamilton: Conceptualization, Methodology, Formal Analysis, Writing, Visualization, Supervision, Resources, Project administration, Funding acquisition. **Nicole Hurst Radke**: Methodology, Investigation, Formal Analysis, Writing – Original draft. **Jasmin Bajwa**: Methodology, Investigation, Formal Analysis. **Shayna Chaput**: Methodology, Investigation, Formal Analysis. **Martin Tresguerres**: Conceptualization, Methodology, Writing, Project administration.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146320.

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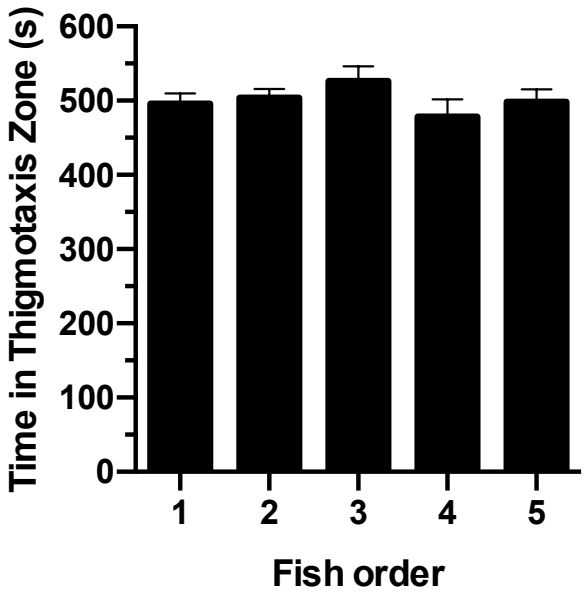
Supplementary Figure 1

a) b)

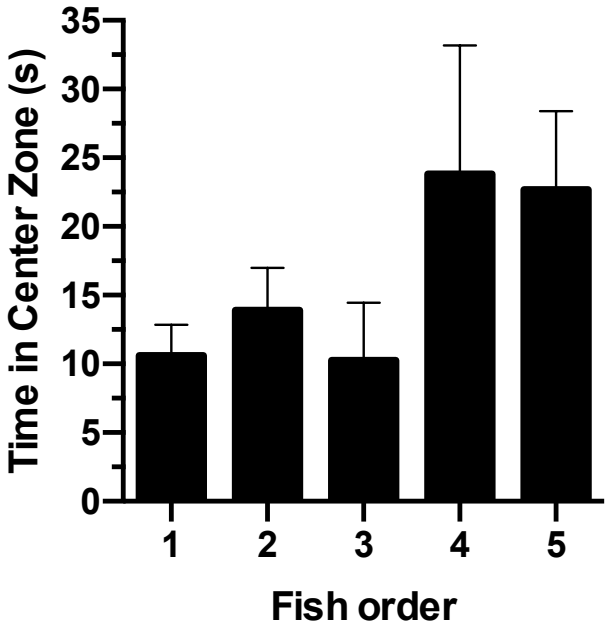
Supplementary Figure 1: Behavioral testing apparatus. a) The testing arena used for both the open field and novel object approach tests was a circular, opaque white cylinder 34 cm in diameter and 15 cm deep. Water from the aquatic habitat was used to fill the arena to a height of 6 cm. b) For the novel object approach test, a Lego ® figurine was inserted into the middle of the arena by the researcher, wearing a rubber glove. The figurine was 5 cm in height and 1.5 cm in width.

Supplementary Figure 2

A



B



Supplementary Figure 2: Comparison of fish control tested in the open field arena based on the order fish were tested. Fish 1 was tested immediately after fresh water was placed in the arena. A) There were no significant differences in time in the center zone for fish order ($H(5,60)=7.133$, $P = 0.129$, Kruskal-Wallis test). B) There were no significant differences for time in the thigmotaxis zone ($F(4,55)=0.938$, $P=0.449$, one-way ANOVA). N=12 for each group. Order of fish testing does not have an impact on behaviour in our study.