The Importance of pH: How Aquarium Water is Affecting Behavioural Responses to Drug Exposure in Larval Zebrafish.

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Abstract

There has been rapid growth in the use of larval zebrafish as a complementary vertebrate model for drug discovery, abuse liability and pharmacological toxicology, resulting in a huge increase in zebrafish facilities worldwide. However, many research groups working with zebrafish do not typically report the pH of husbandry conditions in methodologies, nor are the pH of drug treatments reported in many research articles. This unknown factor can be a major contributor in the differential effects of drug treatments. Therefore, as a case study, we tested the impact of altering pH of several drugs of abuse and assessed locomotor changes associated with a single drug concentration delivered at different pHs. We found that a change of a single pH unit, within the pH ranges commonly used in zebrafish husbandry, was enough to alter locomotor activity at a fixed drug concentration. Many pharmacological agents are dependent on environmental factors, such as pH, to determine bioavailability. Efficaciousness for many classes of drug is dependent on their ionization state in which shifts towards uncharged species can influence the ease of a drug crossing biological membranes. Thus, we urge users to report pH in husbandry methods and drug treatments to improve replicability and inter-study comparisons.

Introduction

Zebrafish have been rapidly growing in popularity as a model organism (Kalueff et al., 2014; Parker et al., 2013; A M Stewart et al., 2015), lending themselves exceptionally well to studies of environmental toxicity, teratogenesis, neurotoxicity, and preclinical drug discovery (Chapela et al., 2019; Gehrig et al., 2018; Kalueff et al., 2014; Lovely et al., 2016). The high numbers of offspring, external fertilisation, transparent eggs, cost-effective maintenance and ease of genetic and pharmacological manipulation have resulted in what can now be considered as one of the most versatile and well characterised vertebrate models (Kalueff, 2017). Currently used in over 1,200 labs worldwide (zfin.org), there is growing use of zebrafish as an alternative model for both environmental and translational models. Aided by advancements in technology, zebrafish embryos and larvae have been gaining ground as a model for drug discovery due to the ease of drug administration and high-throughput whole-animal assays (Ali et al., 2011; Gehrig et al., 2018; Lieschke & Currie, 2007; Strähle et al., 2012). Despite the success of mammalian pharmacokinetic and efficacy models, they are often expensive, time consuming, require large quantities of compounds and can be extremely invasive, particularly for dosing

(Zon & Peterson, 2005). Zebrafish, on the other hand, offer a cost-effective alternative which has low compound requirement and an easy method for drug delivery which is facilitated through the aqueous environment (Vaz et al., 2018; Zon & Peterson, 2005). Thus, zebrafish are an invaluable species for screening the pre-regulatory and preclinical phases of drug discovery and pharmacological toxicology (Ali et al., 2011; Basnet et al., 2019; Chapela et al., 2019; Gehrig et al., 2018; Norton, 2013; Adam Michael Stewart et al., 2014).

Understanding the pharmacology of compounds is crucial to interpreting the interaction between chemicals and organisms. This is of particular importance when screening novel drugs for therapeutic treatments or for abuse liability, as biochemical function can be altered by temperature and pH, critically influencing pharmacokinetics (Dowd, 2017; Mitra & Kesisoglou, 2013; E. D. Stevens & Balahura, 2007). Currently, 'Materials and Methods' sections in publications report general husbandry parameters, including a description of water temperature, light-dark cycles and feeding routines; however, it is not mandatory to report pH. Subsequently, many studies do not report either environmental aquarium water (the main system water used for the majority of fish husbandry) or pH values of drug containing solutions. An article by Aleström, et al, outlines updated recommendations for the husbandry and care of zebrafish in line with the EU Commission Recommendation 2007/526/EC and the EU Directive 2010/63 concerning care and well-being of experimental animals (Aleström et al., 2019). This article recommends pH levels between 6.5-8.0. This range is inclusive of weakly acid to weakly basic conditions. Of the 154 articles examined here, only 34.4% of them reported pH, with a range of 5.8-9.0, representing a maximal change of 2.2 units. These changes in pH may be accounted for by the water source for recirculating systems. This can either be from local water supplies (e.g. tap water), which is subject to effects of the local environment, such as hard versus soft water, or RO systems which rely on pre-filtration. The initial water source may influence overall pH levels. For example, hard water areas have increased concentrations of multivalent cations, most commonly calcium or magnesium ions, which consequently increase water pH. Thus, with the global distribution of zebrafish facilities, fed off a vast range of water sources, it is reasonable to expect that baseline pH levels of aquarium water will vary from lab to lab. Although many facilities that have reported pH fall within the recommended pH levels, we demonstrate that some facilities operate outside of these parameters, thus, extending the pH range at which drug studies are conducted.

Here, we varied the pH of the test environment by a single unit, within the pH range recommended by Aleström, et al. We selected pH 7.0 and 8.0 to identify if this small change can influence survival or locomotor responses of larvae exposed to fixed concentrations of drug treatments. The drugs tested have been regularly used to investigate mechanisms underlying neuropsychiatric disorders, such as substance abuse, and have been selected from the same family of organic bases, to identify if pH is a significant factor which can alter behavioural responses to compounds that share similar chemical structures. Our findings suggest that at the concentrations selected there were no detrimental effects on survival. However, slight structural differences between compounds, resulting in variable pK_a values, are significant predictors of altered organism-drug interactions that potentially result in modified locomotor responses to the same dose of drug when exposed at different pH conditions. Our study suggests that, when using locomotor activity as a measure of behavioural response to a drug, pH is a critical factor. The growing use of zebrafish as a model for screening compounds or testing novel therapeutics and many studies using locomotor activity as a behavioural end point, further supports the need to accurately report environmental conditions in which test compounds are introduced.

Materials and Methods

Systematic Review

We carried out a systematic analysis of the reporting of pH in zebrafish behavioural experiments over the past 20 years. Using PubMed, we inserted the search terms “zebrafish” AND “behavio*” AND “locomoto*” AND “drug”. This returned 465 results, of which 154 had full free text available. **Table 1** displays a summary of the data from these studies relating to reporting of pH (full details are included in **Supplementary data_Table 1**).

Breeding and husbandry

Adult, AB wild-type zebrafish (*Danio rerio*) were retained as breeders in the University of Portsmouth Fish Facility. Housing conditions were maintained on a re-circulating system (Aquaneering Inc., San Diego, CA, USA), with aquarium water at pH 8.4 (± 0.4), temperature 25-27 °C, on a 14H: 10H light/dark cycle. The day prior to embryo collection male and female breeders were housed in separate tanks overnight. On the day of embryo collection, 15 mins prior to light onset, all adults were placed in a breeding tank, with males and females separated by a transparent divider. Upon light onset, the divider was removed. One hour after light onset, the adults were returned to the colony, and embryos were collected from the breeder tank. The embryos were pooled, and placed in petri dishes of aquarium water, in groups of ~50 embryos per petri dish and reared at 28 °C in aquarium water at pH 8.4, to mimic adult conditions, in a clear incubator for three days (toxicity assessment) or seven days (behavioural assessment) on a 14H:10H light/dark cycle. All studies were conducted following the guidelines of the University of Portsmouth Animal Welfare and Ethical Review Board and under licence from the UK Home Office.

Pharmacological agents

To study the effect of pH on different drug conditions, four commonly used drugs of abuse were selected for this study. Nicotine (Sigma-Aldrich) was administered at a concentration of 5 μ M (García-González et al., 2020; Petzold et al., 2009; Yoo et al., 2018), morphine sulphate (Sigma-Aldrich) was administered at 2.6 μ M (Lopez-Luna et al., 2017), *d-amphetamine* (Sigma-Aldrich) was administered at 22 μ M (Irons et al., 2010), and caffeine (Tocris) was administered at 200 μ M (Gutiérrez et al., 2020). Concentrations were based on a combination of larval and adult zebrafish studies investigating effects on locomotor activity.

Toxicity assessment

For control groups, aquarium water was buffered with citric acid to either pH 7.0 or 8.0. For treatment groups, respective concentrations of drug were added to aquarium water and then buffered using either NaHCO₃ or citric acid to bring the final pH to either pH 7.0 or 8.0. [The final solution pH values were reported at 26°C](#). From the final buffered solution, 0.9 mL was added to each well of a 48-well plate. Individual, 3 dpf larvae were transferred into each well for 24H. Tail-touch-evoked motor (TEM) responses were used to assess survival at 0.5H, 1H,

1.5H, 2H, 3H, 4H, 5H and 24H intervals. Plates were maintained at room temperature, 26°C. Each drug challenge/control was conducted in separate plates with $n = 8$ larvae/dose/pH. 48-well plates were used due to the well sizes minimising volume of drug required per test and providing enough space for larvae to swim and for TEM to be conducted with ease. Pilot work was conducted demonstrating that TEM was a suitable and effective method for assessing survival with the drugs tested as part of this study. However, it is worth noting here that when testing drugs with analgesic properties or causing extreme hypolocomotion, TEM may be an unsuitable method for assessing survival. Under these circumstances it would be recommended that heartbeat is used as an alternative method for assessing survival. During the transfer of larvae to individual wells and the use of TEM have the potential to inflict a low level of mortality due to regular handling of larvae. To account for this, a significant effect of drug on survival was determined by treatments that caused more than 50% mortality.

Larval locomotor response

Following toxicity assessments, drugs that did not cause a significant impact on survival were used for subsequent detailed behavioural analysis. The same method of buffering drug treated, and control groups were applied as above. 7 dpf larvae show spontaneous movement and were used to assess locomotor changes in response to drug and pH challenges. 7dpf larvae were placed in individual wells of a 48-well plate. Movement was tracked by placing plates immediately into the Zantiks MWP larval behavioural unit. Each larva was tracked individually using automated tracking software (Zantiks MWP, Cambridge, UK). Locomotor assays were run in the dark as previous studies have demonstrated that larvae move more in dark than light environments (Burgess & Granato, 2007). Movement data were logged after 0.5H (acute) and 4H (prolonged) exposure, larvae were recorded for 30 mins. Any larvae that displayed severe immobility behaviour, defined as less than 10 mm travelled during each assessment, were not included in any data analyses or figures. Variance in immobility was due to individual larval differences (i.e. not overrepresented in any drug or pH condition), and from the total animals tested represented less than 2% of the total population.

Statistical analyses

All data were analysed using GraphPad Prism (version 8.4.2). Toxicity was assessed using Kaplan-Mayer survival curves, proportion of survival was compared to pre-drug exposure viability. Outliers from behavioural groups were identified using boxplot with Tukey analysis and excluded from subsequent analysis. Comparison of each pH condition was tested for normality using the Shapiro-Wilk test. Groups that were normally distributed were analysed using the unpaired t -test on data after outliers were excluded. Groups with non-normally distributed data were analysed using the Mann Whitney test, after the exclusion of outliers. Within each treatment group, an interaction was considered significant when $p \leq 0.05$. All data are presented as mean $\pm$ standard error of the mean (SEM).

Results

Systematic Review

To identify the extent to which pH of either husbandry or drug treatment conditions were reported in research articles relating to zebrafish behaviour, we conducted a systematic analysis of a population of published research articles in the fields of toxicology, neuroscience, drug discovery, drug testing, genetics or other. **Table 1** provides a summary of our findings. We found that for each research field, 50% or less studies reported pH, and for toxicology, neuroscience, drug discovery and drug testing, the pH range was greater than one unit between studies.

Table 1.

Research field	Behavioural endpoint (e.g. locomotion)	N. articles with pH reported/Total articles	pH value (or range)
Toxicology	Locomotion, aggression, exploration, predator interactions, social interactions, shoaling	10/21	6.5-7.7
Neuroscience	Locomotion, anxiety, aggression, exploration, escape, predator interactions, social interactions, shoaling, cognition	23/80	6-9
Drug discovery	Locomotion	7/14	7-9
Drug testing	Locomotion, anxiety, freezing, exploration, predator interactions, shoaling, stress	9/26	6.5-9
Genetics	Locomotion, anxiety, shoaling	3/9	7.2-7.7
Other	Locomotion	1/4	7.9-8.3

Water challenge

The survival curve for larvae exposed to aquarium water, buffered with citric acid to either pH 7.0 or 8.0, is shown below in Fig. 1A. 3 dpf larvae showed no adverse effects on survival when exposed to altered pH conditions for 24H. Fig. 1B shows the effect of pH on the total activity levels of 7 dpf larvae during the first 0.5H and after a further 4H of exposure to their respective pH conditions. In the absence of drug, changes of ± 1.0 pH was not sufficient to evoke changes in locomotor activity (0.5H: $t = 1.719$, $df = 84$, $p = 0.089$; 4H: $t = 1.213$, $df = 84$, $p = 0.229$).

Figure 1. The effect of pH controlled aquarium water on A) survival of 3 dpf larvae over 24 hours, assessed using tail-touch-evoked (TEM) motor response at 0.5H, 1H, 1.5H, 2H, 3H, 4H, 5H and 24H intervals. Analysis was carried out using the Kaplan-Meier survival curve, with proportion of survival compared to pre-drug exposure viability. Curves show 100% survival for both pH conditions. pH7, n=8; pH8, n=8 B) The effect of pH controlled aquarium water on locomotor behaviour in 7 dpf larvae during the first 0.5H of exposure, and at 4H of exposure was analysed for outliers using boxplot with Tukey analysis. Following removal of outliers, Shapiro-Wilk normality test was conducted. Samples that were not normally distributed were analysed using Mann Whitney test. pH7 n=46; pH8 n=46. There was no significant difference in distance travelled between the two groups. Bars represent mean, error bars are SEM; ns – not significant.

Drug challenge

Larvae at 3 dpf were treated with four commonly used drugs of abuse; nicotine, morphine sulphate, *d*-amphetamine and caffeine. Treatment water was buffered to either pH 7.0 or 8.0 and the effect on survival was monitored over 24H of exposure (Fig. 2). Like control animals, nearly all treatment groups at both pH 7.0 and 8.0 had 100% survival after 1 day of continuous treatment. The only exception was in the nicotine treated group at pH 7.0, in which a single larva was reported to have died at the 24H time point, which, as an isolated death, it is unlikely to be the result of the treatment on survival. The survival curve analyses, demonstrated that at each of the tested concentrations, the drugs and pH conditions used were safe to conduct behavioural analysis on older larvae.

Following survival curve analysis, acute (0.5H) and prolonged (4H) exposures to a single concentration of drug under two pH conditions were conducted using 7 dpf larvae. Fig. 3A shows the response of larvae exposed to 5 μ M of nicotine, which caused an increase in locomotor activity in larvae treated at pH 8.0 compared to those treated at pH 7.0. This increased activity level was evident after acute and prolonged exposure (0.5H: $t = 2.175$, $df = 13$, $p = 0.0487$; 4H: $t = 2.446$, $df = 13$, $p = 0.0294$). Treatment with 2.6 μ M of morphine sulphate, showed no locomotor difference after an acute exposure (0.5H: $t = 0.314$, $df = 14$, $p = 0.758$). However, differences in activity levels were evident after prolonged treatment, resulting in a significant increase in the distance travelled by larvae in the group buffered to pH 8.0 (4H: $t = 2.990$, $df = 14$, $p = 0.0098$) (Fig. 3B). Larvae treated with 22 μ M of *d*-amphetamine showed no differences between pH groups after acute exposure (0.5H: $t = 1.984$, $df = 13$, $p = 0.0688$). However, after prolonged exposure there was a significant effect of pH 8.0 causing a decrease in locomotor activity compared to larvae treated at pH 7.0 (4H: $t = 2.883$, $df = 13$, $p = 0.0128$) (Fig. 3C). Caffeine caused no detectable differences between pH groups either at the acute or prolonged treatment stages (0.5H: $t = 1.183$, $df = 13$, $p = 0.258$; 4H: $t = 0.835$, $df = 14$, $p = 0.418$) (Fig. 3D).

Figure 2. The effect of pH controlled drug treatment on the survival of 3 dpf larvae over 24 hours, assessed using tail flick at 0.5H, 1H, 1.5H, 2H, 3H, 4H, 5H and 24H intervals for **A)** 5 μ M nicotine, **B)** 2.6 μ M morphine sulphate, **C)** 22 μ M of d-amphetamine and **D)** 200 μ M caffeine. Analysis was carried out using the Kaplan-Mayer survival curve, with proportion of survival compared to pre-drug exposure viability. Survival curves show 100% survival for both pH conditions for all drug exposures, except pH7 nicotine, which reported a single death. pH7 n=8; pH8 n=8 for each drug condition.

Figure 3. The effect of pH controlled drug treatments on locomotor activity of 7 dpf larvae assessed using automated tracking software for the first 0.5H of exposure (acute) and after 4H of exposure (prolonged). Treatments were buffered to either pH 7.0 or pH 8.0 (± 0.1) using citric acid. **A)** 5 μ M nicotine, **B)** 2.6 μ M morphine sulphate, **C)** 22 μ M of d-amphetamine and **D)** 200 μ M caffeine. Analysis for outliers was carried out using boxplot with Tukey analysis. Following removal of outliers, Shapiro-Wilk normality test was conducted. Samples that were normally distributed were analysed by unpaired t-test, not normally distributed samples were analysed using Mann-Whitney test. Nicotine, 0.5H: pH7 n=7, pH8 n=8, 4H: pH7 n=7, pH8 n=8. Morphine, 0.5H: pH7 n=8, pH8 n=8, 4H: pH7 n=8, pH8 n=8. Amphetamine, 0.5H: pH7 n=8, pH8 n=7, 4H: pH7 n=8, pH8 n=7. Caffeine, 0.5H: pH7 n=8, pH8 n=8, 4H: pH7 n=8, pH8 n=8. Bars represent mean, error bars are SEM; ns – not significant, * $p < 0.05$, ** $p < 0.01$.

Discussion

The aim of this study was to assess if a change of environmental pH was substantive enough to cause a change in behavioural activity when larvae were exposed to the same concentration of drug at different pH's. We found that a change in pH of ± 1.0 unit significantly impacted locomotor activity of larvae for three of the four test drugs. Nicotine was found to have a pH-dependent effect that immediately impacted locomotor activity. This effect was sustained over both acute and prolonged exposure. Morphine and *d*-amphetamine both showed altered locomotor responses based on pH, but only after prolonged exposure. Caffeine was the only drug tested that did not cause different activity levels between pH groups. Our findings suggest that the chemical composition of a drug can be a strong determinant in the effect of pH on behavioural response, potentially due to altered bioavailability. Our results thus highlight the differential effects that small changes in pH can have on behavioural output in response to drug exposure.

Zebrafish have been found to live in bodies of water with pH varying from 6 to 10, demonstrating suitability to a wide range of environments (Aleström et al., 2019; Arunachalam et al., 2013). However, in laboratory settings, the aim has been to provide stable husbandry conditions, and thus zebrafish housing systems have been recommended at pH 6.5-8.0 (Aleström et al., 2019). Analysis of 154 studies assessing effects of drugs on zebrafish behaviour showed the most widely used pH levels fell between pH 7.0-8.0, found in 77% of studies reporting pH (**Supplementary data_Table 1**). We assessed if aquarium water buffered to either pH 7.0 or 8.0, reducing our normal aquarium water levels by 1.4 or 0.4 respectively, caused differences in toxicity to 3 dpf larvae or baseline locomotor activity of 7 dpf larvae. We found that there was no difference in the survival rate over 24H, in which both pH conditions had 100% survival. There was also no evidence of pH related changes in locomotion, as both groups showed similar distances moved after acute and prolonged exposure to pH altered aquarium water. Similar to control groups, toxicity studies of test drugs at pH 7.0 and 8.0, revealed that over 24H of exposure, the selected concentrations did not cause any toxic effects, thus resulting in 100% survival in all conditions. However, behavioural assessment of locomotion at 7 dpf revealed significant pH-dependent differences in response to a fixed dose of drug with variable pH conditions.

Here, we demonstrated that within the first 0.5H of exposure of 7 dpf larvae to 5 μ M nicotine, there was an immediate difference in the locomotor activity of larvae at pH 7.0 compared to those treated at pH 8.0. Nicotine is a weakly basic drug and can exist in three forms in aqueous solution: the univalent (pyrrolidinium) cation, divalent cation and the unionized 'free base' (Barlow & Hamilton, 1962). At physiological pH (ca. 7.4), 69% of nicotine is present in the ionized form (predominantly the univalent cation) (Pogocki et al., 2007). The rest remains unionized (uncharged) and it is in this form that molecules are easily absorbed, as the free base can readily pass across biological membranes (Benowitz et al., 2009; Pogocki et al., 2007). Absorption in this state is rapid, and in conjunction with moderate hydrophobicity and low polarity (due to the presence of the pyridine and pyrrolidine rings) nicotine can easily cross the blood-brain barrier to access the brain (W. H. Oldendorf, 1974; William H. Oldendorf et al., 1993; Spector & Goldberg, 1982; Tega et al., 2018). When nicotine is present in aqueous solution at 25 °C, it has two pK_a values; 8.02 and 3.12. If the environmental pH, that the drug is dissolved in, is equal to the pK_a value, then there are equal quantities of drug in the ionized

and nonionized forms (50:50) (Dowd, 2017). Therefore, when nicotine is solubilized in pH 8.0, equal to the pK_a value, ~50% of the nicotine must be in the unionized state. Many studies have reported that at physiological pH, 31% of nicotine is the free base species. Therefore, at pH 7.0, it would be expected that an even higher degree of ionization would occur, thus reducing the total amount of free base available to less than 30% (Pogocki et al., 2007). This represents a difference of greater than 20% nonionized, biologically available nicotine between pH 7.0 and 8.0. Zebrafish larvae treated at pH 8.0 had a mean of 572.4 mm travelled during acute exposure to nicotine, while larvae treated at pH 7.0 on average only travelled 294.2 mm, almost half the distance travelled by the pH 8.0 treated group. These findings of increasing locomotor activity with increasing nicotine concentration is supported by a previous study from Petzold, et al, investigating the effect of nicotine concentration on zebrafish larvae locomotion. They demonstrated that increasing concentrations of nicotine from 2.5 μ M to 50 μ M resulted in increases in movement (Petzold et al., 2009). Therefore, it is likely that hyperlocomotion of larvae treated at pH 8.0 are the result of increased nicotine availability (effectively increasing brain concentration of nicotine) by altering the amount of unionized nicotine in solution.

Morphine and *d*-amphetamine have similar chemical properties to nicotine. In aqueous solution, morphine can be found in three forms: two ionized forms (one with a charge on the nitrogen atom and the other lacking a proton on the phenolic hydroxyl group at carbon 3) and an uncharged, free base (E. D. Stevens & Balahura, 2007). *D*-amphetamine is only found in two forms: an ionized cation and the free base (Pubchem). Morphine, like nicotine, has two equilibrium constants (pK_a) of 8.00 and 9.75, whilst *d*-amphetamine has one of 9.9 at 25 °C (Heal et al., 2013). Due to the weakly basic properties of both drugs, like nicotine, there is consequently more of the drug present in the lipid soluble, free base form when in more alkali pH conditions, thus increasing absorption and brain concentration of drug (Dowd, 2017). Acute exposure to both morphine and *d*-amphetamine, had no pH-induced differences in motor activity. However, the effect of altered pH condition became evident after prolonged exposure (4H of treatment), which caused significant differences in the distances travelled in the drug treated groups at pH 7.0 compared to pH 8.0. Morphine treated larvae behaved similarly to nicotine treated larvae, both groups demonstrating hyperlocomotion in pH 8.0 compared to pH 7.0. Almost identical pK_a values of nicotine (8.02) and morphine (8.00) may be a principle factor in the similar behavioural responses of increased locomotion with increasing brain concentrations of available drug (Barlow & Hamilton, 1962; E. D. Stevens & Balahura, 2007; Tomar, Scott & Henningfield, Jack, 1997).

The percentage of the ionized versus nonionized form depends on the number of pH units above or below the pK_a (Bhagavan, 2002). As morphine and nicotine have pK_a values of ~8.0 (± 0.2), the degree of ionization at pH 7.0 would be expected to be similar, ~20% lower than at pH 8.0. Therefore, the increase in bioavailability of morphine would be expected to cause a similar response to nicotine based on the same degree of ionization at their respective pHs. *D*-amphetamine, however, caused the opposite effect in the prolonged treated group, resulting in hypolocomotion of larvae treated at pH 8.0 compared to pH 7.0. Previous studies of zebrafish larvae exposed to *d*-amphetamine reported an “inverted U” dose-response in locomotor activity dependent on drug concentration (Irons et al., 2010). Irons, et al, reported that low doses, up to 2.2 μ M caused hyperlocomotion, whilst doses from 6.6 μ M began to cause increased hypolocomotion, with the maximal dose tested (20 μ M) having the greatest effect on hypoactivity, similarly found here with a dose of 22 μ M (Irons et al., 2010). Thus, in line with this study, we found that the pH with the highest level of *d*-amphetamine in the free base form, pH 8.0, caused the greatest reduction in distance moved in 7 dpf larvae.

Differences in the onset of pH-dependent locomotor changes found between amphetamine and morphine compared to nicotine, could be the result of relative absorption times. Oral administration of nicotine has been reported to have an almost immediate effect, whilst previous studies using amphetamine have reported that peak responses from oral administration was 1-3H post exposure (Schepers et al., 2003). It is therefore possible, that the lack of effect seen in the acute dose of morphine and *d*-amphetamine was a result of incomplete or limited absorption at that stage, thus only after sufficient exposure time is there an effect of the drug on behavioural output. These three drugs each have similar chemical compositions which include an alkaloid amine group which is deprotonated forming the free base (nonionized) species (Völgyi et al., 2010). The weak alkalinity and the similar pK_a values result in drugs that are readily absorbed under more basic conditions (Bhagavan, 2002).

The only drug tested that did not follow this trend of pH-dependent locomotor activity was caffeine. Although, like the other test drugs, caffeine also has an alkaloid amine group and is weakly basic, it has two pK_a values of 0.7 and 14.0. Subsequently, caffeine is found in a neutral state at pH 5-9 (National Center for Biotechnology Information, 2020; Švorc, 2013). Consequently, as the test environments both fall within this range, there is no difference in the percentage of ionized/nonionized caffeine in solution, thus behavioural changes as a result of exposure to 200 µM caffeine, would be expected to affect larvae in both pH groups equally. Therefore, as expected, there were no detectable changes in locomotor activity between pH groups treated with caffeine after acute or prolonged exposure. Previous studies investigating the effect of different concentrations of caffeine on locomotion have also reported no difference between doses (Gutiérrez et al., 2020).

To our knowledge this is the first systematic investigation of the effects of varying pH conditions on the biological activity of drugs, using locomotion as a behavioural endpoint. The aim of this study was to highlight the importance of reporting this environmental condition, demonstrating that changes of a single pH unit can be sufficient to influence locomotor responses to a fixed concentration of drug. We appreciate that the controlling factor of pH conditions in aquatic facilities is the water source used to feed the system. Changing this system is impractical and in many cases can be cost prohibitive. We are therefore not suggesting that facilities attempted to alter husbandry conditions to fit within the range recommended by (Aleström et al., 2019). Zebrafish can be found living under a broad range of conditions in the wild, and as part of the literature review, we found no study that conducted work outside of the natural range that zebrafish have been found to live in. Although it has not been systematically assessed how long-term exposure to varying pH conditions affects behaviour in a laboratory setting, it is our belief that as long as fish are kept within the natural environmental range this should be acceptable. However, future investigations would be of great use as a resource to the zebrafish community. Based on our findings here, we suggest carrying out drug treatments at a similar pH to the housing conditions to prevent any shock responses from rapidly changing environmental conditions, which could require additional habituation periods or cause behavioural artefacts. Instead we propose that when carrying out drug treatments the pH value is taken into consideration as part of the experimental design. To prevent the addition of drug induced fluctuations to test conditions, we recommend either neutralising or matching drug solutions to aquarium water pH. Reporting of pH conditions and awareness of pK_a values of test drugs when designing experiments and drawing conclusions will greatly aid in improving replicability between laboratories and provide a potential mechanism for interlab differences when testing the same drug at what appears to be the same concentration. Our understanding of how environmental conditions can influence

experimental design, particularly in light of toxicology studies, drug discovery and drug testing using aquatic organisms is in its infancy. Future work investigating the effects of altered environmental conditions in the presence of a drug on behaviour and bioavailability and how drug x pH interactions apply to adult zebrafish behaviour would provide invaluable information for those testing zebrafish with drugs or toxins. Understanding how environmental conditions can influence experiments may prove a valuable tool in optimising zebrafish as a model organism.

Conclusion

With the increasing use of high-throughput behavioural screening of zebrafish larvae for assessing pharmacological agents, the need to improve inter-lab reproducibility is of critical importance. This study demonstrates that four drugs from the same family of organic bases can all exert different effects on locomotor responses by altering environmental conditions by a single pH unit. We have reported that even small changes can cause significant effects and could result in substantial differences in the way larvae, and potentially adult zebrafish respond to drug exposure. It is currently not mandatory to report the pH of aquarium water or of drug treated water in which fish are immersed. We demonstrated that from a sample of studies less than 35% reported pH. We therefore propose that the zebrafish community start to report pH values of drug conditions and general husbandry so that results can be more accurately replicated and compared between studies.

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Supplementary data

Table 1. Systematic review of zebrafish behaviour publications in the fields of drug testing, drug discovery, toxicology, genetics, neuroscience and other on the status and detail of reporting pH values of husbandry or drug treatment conditions.

Area (Toxicology, drug discovery, etc.)	Behavioural endpoint (locomotion, freezing, etc.)	pH reported	pH value (or range)	Reference
Drug testing	Aggression	No		Gutiérrez et al., 2020
Neuroscience	Locomotion	Yes	8.2	Weuring et al., 2020
Neuroscience	Brain structure	No		Kedra et al., 2020
Drug testing	Locomotion	No		Zhao et al., 2020
Toxicology	Locomotion	No		Morbiato et al., 2020
Drug testing	Open Field	Yes	7-7.5	Pandelides et al., 2020
Toxicology	Locomotion	No		B. Wang et al., 2020
Drug discovery	Locomotion	No		Sourbron et al., 2019
Genetics	Locomotion	No		Nakamachi, Tanigawa, Konno, Shioda, & Matsuda, 2019
Neuroscience	Locomotion	No		Pant et al., 2019
Genetics	Locomotion	Yes	7.2-7.7	Annunziato, Jantzen, Gronske, & Cooper, 2019
Drug discovery	Locomotion	Yes	7.2	Ko et al., 2019
Drug testing	Locomotion, Freezing	Yes	7.2	Macrì, Clément, Spinello, & Porfiri, 2019
Drug testing	Locomotion	Yes	7.2 ± 1	Souders et al., 2019
Neuroscience	Locomotion, novel tank exploration, aggression, predator	No		Sarasamma et al., 2019

	avoidance test, shoaling, conspecific social interaction, circadian rhythm, colour preferences, and short-term memory tests			
Drug discovery	Locomotion, Light/dark preference	Yes	9	Chhabria et al., 2019
Neuroscience	Locomotion	No		Luchtenburg, Schaaf, & Richardson, 2019
Development	Locomotion	Yes	8.1 ± 0.2	Fitzgerald, Kirla, Zinner, & Vom Berg, 2019
Toxicology	Locomotion	No		Brun et al., 2019
Development	Locomotion	No		Anthony N. Oliveri & Levin, 2019
Neuroscience	Locomotion	No		Chen, Tu, Schirch, Safo, & Fu, 2019
Neuroscience	Locomotion, Predatory response	Yes	7.0 ± 0.2	Frank et al., 2019
Neuroscience	Locomotion	No		Xie et al., 2019
Genetics	Light/Dark, Open field, Shoaling	No		C.-X. Liu et al., 2018
Genetics	Locomotion	Yes	7.4	Jensen, Korbut, Kania, & Buchmann, 2018
Neuroscience	Locomotion, dark flash, sociability	No		Baronio et al., 2018
Neuroscience, Drug testing	Locomotion	No		S. Zhang et al., 2018
Air pollution	Locomotion	No		J. S. Stevens et al., 2018
Neuroscience	Locomotion	No		Crilly et al., 2018
Neuroscience	Locomotion	No		Zheng, Chen, Jiang, & Zhang, 2018
Neuroscience	Locomotion	No		Lange, Froc, Grunwald, Norton, & Bally-Cuif, 2018
Neuroscience	Locomotion	Yes	7.2 ± 1	Shontz, Souders, Schmidt, & Martyniuk, 2018
Drug discovery	Locomotion	Yes	7.3	Bugel & Tanguay, 2018
Drug discovery	Locomotion	Yes	7.6	Gauthier & Vijayan, 2018
Neuroscience	Locomotion	Yes	7.2	Du et al., 2018
Drug testing	Locomotion	Yes	7.2	Lobao-Soares et al., 2018

Drug testing	Locomotion	No		F. Li et al., 2018
Drug testing	Locomotion, Predatory response	Yes	7.2-7.8	Frank et al., 2018
Neuroscience	Locomotion	No		Kopp, Legler, & Legradi, 2018
Toxicology	Locomotion	No		J. Li et al., 2018
Neuroscience	Locomotion	Yes	7 ± 0.5	Han, Zheng, Zhang, & Hu, 2018
Toxicology	Locomotion	No		Anthony N Oliveri, Ortiz, & Levin, 2018
Toxicology	Locomotion	No		Liao et al., 2018
Drug discovery	Locomotion	Yes	7	Batista et al., 2018
Toxicology	Locomotion, mirror biting, predator avoidance, social interaction, shoaling	No		Sarasamma et al., 2018
Toxicology	Locomotion, predator avoidance, shoaling	No		Glazer et al., 2018
Toxicology	Locomotion	Yes	7.2 ± 0.4	González, Carty, Tran, Cole, & Lein, 2018
Neuroscience	Open Field	Yes	7.5-8	Grone, Qu, & Baraban, 2017
Toxicology	Locomotion, exploration, aggression	Yes	6.5-7.5	Bridi, Altenhofen, Gonzalez, Reolon, & Bonan, 2017
Drug testing	Exploration	Yes	6.5-7.5	Altenhofen et al., 2017
Neuroscience	Locomotion	Yes	8	(Zabinyakov et al., 2017)
Neuroscience	Locomotion	No		(Hu et al., 2017)
Neuroscience	T maze	Yes	6.8-7.1	(Kundap et al., 2017)
Drug discovery	Locomotion	No		(Basnet et al., 2017)
Neuroscience	Locomotion	No		Knecht et al., 2017
Toxicology	Locomotion	Yes	7.5	Jia et al., 2017
Drug discovery	Locomotion	No		Brock et al., 2017
Neuroscience	Locomotion	Yes	7.2	Turrini et al., 2017
Neuroscience	Locomotion	Yes	6	Itskovits, Levine, Cohen, & Zaslaver, 2017
Drug testing	Locomotion, Freezing	Yes	9	Savoldi et al., 2017
Toxicology	Locomotion	Yes	7.4	Kao et al., 2017
Neuroscience	Locomotion	Yes	7.2	Carvan et al., 2017
Drug testing	Locomotion	No		Misztal et al., 2017
Neuroscience	Locomotion	Yes	6.8–7.5	Y. Zhang et al., 2017
Neuroscience	Locomotion	No		Y. Liu et al., 2017
Drug discovery	Locomotion	No		Palmér et al., 2017
Neuroscience	Locomotion	No		Ek et al., 2016

Drug discovery	Locomotion	Yes	7.2	Sun et al., 2016
Drug testing	Locomotion	No		Wilson et al., 2016
Genetics	Open Field, aggression	Yes	7.2-7.7	Jantzen, Annunziato, & Cooper, 2016
Neuroscience	Locomotion	Yes	7.5	Sterling, Chang, Karatayev, Chang, & Leibowitz, 2016
Toxicology	Locomotion	Yes	7.2-7.7	Jantzen, Annunziato, Bugel, & Cooper, 2016
Toxicology	Locomotion	Yes	6.8-7.5	Pham et al., 2016
Toxicology	Locomotion	Yes	7	Kristofco et al., 2016
Toxicology	Locomotion	Yes	7-7.6	Xiaowei He et al., 2016
Neuroscience	Thigmotaxis, Scototaxis	No		Baiamonte, Parker, Vinson, & Brennan, 2016
Toxicology	Locomotion, shoaling	No		Glazer, Hahn, & Aluru, 2016
Drug Testing	Locomotion. Thigmotaxis	No		X. Liu et al., 2016
Toxicology	Locomotion	No		Sternberg et al., 2016
Neuroscience	Locomotion, exploration, predator avoidance	No		Bailey et al., 2016
Neuroscience	Locomotion	No		Benedetti et al., 2016
Toxicology	Locomotion	Yes	7.2	Mora-Zamorano, Svoboda, & Carvan, 2016
Neuroscience	Locomotion	No		Gonzalez, Remick, Creton, & Colwill, 2016
Neuroscience	Locomotion	No		Kasica et al., 2016
Neuroscience	Locomotion	Yes	7.2	Braga et al., 2016
Neuroscience	Light/Dark	No		X. Li et al., 2015
Drug testing	Locomotion, anxiety, exploration	No		Sterling, Karatayev, Chang, Algava, & Leibowitz, 2015
Neuroscience	Locomotion	No		Seebacher, Little, & James, 2015
Drug testing	Locomotion	No		Jarema, Hunter, Shaffer, Behl, & Padilla, 2015
Drug Testing	Locomotion	Yes	6.8-7.2	Nowicki, Tran, Chatterjee, & Gerlai, 2015
Neuroscience	Exploration	No		Facchin, Duboué, & Halpern, 2015
Genetics	Locomotion	No		Radev et al., 2015
Drug discovery	Locomotion	Yes	7	Y. Wang et al., 2015
Neuroscience	Exploration, sociability	No		Bortolotto, de Melo, Cognato, Vianna, & Bonan, 2015
Drug testing	Exploration, sociability, predatory response	No		A N Oliveri, Bailey, & Levin, 2015
Drug testing	Stress test	No		Baiamonte, Brennan, & Vinson, 2015

Neuroscience	Locomotion, shoaling	No		Dubey, Ganeshpurkar, Bansal, & Dubey, 2015
Neuroscience	Locomotion, anxiety	No		Vignet et al., 2015
Drug discovery	Locomotion	No		Holcombe, Schalomon, & Hamilton, 2014
Neuroscience	Locomotion	No		Makhija & Jagtap, 2014
Neuroscience	Locomotion	Yes	7	Zada, Tovin, Lerer-Goldshtein, Vatine, & Appelbaum, 2014
Neuroscience	Locomotion	Yes	7-7.5	Long et al., 2014
Neuroscience	Locomotion	No		Fernandes, Tran, Abraham, & Gerlai, 2014
Neuroscience	Locomotion	No		Woods et al., 2014
Neuroscience	Locomotion, thigmotaxis	Yes	7-7.2	X. Li et al., 2014
Neuroscience	Locomotion	No		Ward et al., 2014
Neuroscience	Locomotion	No		Knogler & Drapeau, 2014
Neuroscience	Locomotion	No		Menelaou, Udvadia, Tanguay, & Svoboda, 2014
Neuroscience	Locomotion	No		Knogler, Ryan, Saint-Amant, & Drapeau, 2014
Drug testing	Locomotion	No		Puttonen, Sundvik, Rozov, Chen, & Panula, 2013
Toxicology	Locomotion	Yes	7.4	Duan et al., 2013
Neuroscience	Locomotion	No		Kyzar et al., 2013
Neuroscience	Locomotion	No		Irons, Kelly, Hunter, Macphail, & Padilla, 2013
Neuroscience	Locomotion, shoaling	No		Maaswinkel, Zhu, & Weng, 2013
Neuroscience	Locomotion	No		Ikeda et al., 2013
Neuroscience	Locomotion	Yes	7.6	Afrikanova et al., 2013
Drug testing	Locomotion	No		Tran & Gerlai, 2013
Neuroscience	Shoaling, thigmotaxis	No		Maaswinkel, Le, He, Zhu, & Weng, 2013
Neuroscience	Locomotion	No		Mirat, Sternberg, Severi, & Wyart, 2013
Neuroscience	Locomotion	Yes	7	Baldissarelli, Capiotti, Bogo, Ghisleni, & Bonan, 2012
Genetics, drug testing	Locomotion	No		Shimada, Hirano, Nishimura, & Tanaka, 2012
Environmental	Locomotion	No		
Neuroscience	Locomotion	Yes	5.8-7.8	Xiong He, Zhong, & Che, 2012
Neuroscience	Locomotion	No		Formella et al., 2012
Neuroscience	Locomotion	No		Saili et al., 2012
Toxicology	Locomotion	No		Pruvot et al., 2012
Neuroscience	Escape	No		Mu, Li, Zhang, & Du, 2012
Neuroscience	Locomotion	No		Lambert, Bonkowsky, & Masino, 2012

Drug testing	Locomotion	No		Truong, Saili, Miller, Hutchison, & Tanguay, 2012
Drug testing	Locomotion, shoaling	Yes	7.2	Gebauer et al., 2011
Drug testing	Locomotion	No		Klee, Ebbert, Schneider, Hurt, & Ekker, 2011
Neuroscience	Locomotion	No		Cachat et al., 2011
Neuroscience	Light/Dark	Yes	7	Maximino, Lima, Olivera, Picanço-Diniz, & Herculano, 2011
Neuroscience	Locomotion	No		Kyriakatos et al., 2011
Neuroscience	Scototaxis	Yes	7	Maximino, da Silva, Gouveia, & Herculano, 2011
Neuroscience	Locomotion	Yes	8	Int J Comp Psychol. 2010;23(1):82-90.
Drug discovery	Locomotion	No		Rihel et al., 2010
Neuroscience	Locomotion	No		Knogler, Liao, & Drapeau, 2010
Neuroscience	Locomotion	No		Egan et al., 2009
Neuroscience	Locomotion	No		V Sallinen et al., 2009
Neuroscience	Locomotion	No		Ville Sallinen et al., 2009
Genetics	Locomotion	No		Pietri, Manalo, Ryan, Saint-Amant, & Washbourne, 2009
Drug testing	Locomotion	No		Peng et al., 2009
Drug discovery	Locomotion	No		Barros, Alderton, Reynolds, Roach, & Berghmans, 2008
Neuroscience	Locomotion, thigmotaxis	No		Bencan & Levin, 2008
Neuroscience	Locomotion	No		Thirumalai & Cline, 2008
Neuroscience	Locomotion	No		McLean, Masino, Koh, Lindquist, & Fetcho, 2008
Neuroscience	Locomotion	No		Boehmler et al., 2007
Neuroscience	Locomotion	No		Baraban et al., 2007
Neuroscience	Locomotion	No		McDearmid & Drapeau, 2006
Genetics	Locomotion	No		Cui et al., 2005
Neuroscience	Locomotion	No		Brustein & Drapeau, 2005
Neuroscience	Locomotion	No		Anichtchik, Kaslin, Peitsaro, Scheinin, & Panula, 2004
Neuroscience	Locomotion	No		Saint-Amant & Drapeau, 2000