RESEARCH ARTICLE

Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish

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ABSTRACT

Research has recently demonstrated that larval zebrafish show similar molecular responses to nociception to those of adults. Our study explored whether unprotected larval zebrafish exhibited altered behaviour after exposure to noxious chemicals and screened a range of analgesic drugs to determine their efficacy to reduce these responses. This approach aimed to validate larval zebrafish as a reliable replacement for adults as well as providing a high-throughput means of analysing behavioural responses. Zebrafish at 5 days post-fertilization were exposed to known noxious stimuli: acetic acid (0.01%, 0.1% and 0.25%) and citric acid (0.1%, 1% and 5%). The behavioural response of each was recorded and analysed using novel tracking software that measures time spent active in 25 larvae at one time. Subsequently, the efficacy of aspirin, lidocaine, morphine and flunixin as analgesics after exposure to 0.1% acetic acid was tested. Larvae exposed to 0.1% and 0.25% acetic acid spent less time active, whereas those exposed to 0.01% acetic acid and 0.1–5% citric acid showed an increase in swimming activity. Administration of 2.5 mg l^{-1} aspirin, 5 mg l^{-1} lidocaine and 48 mg l^{-1} morphine prevented the behavioural changes induced by acetic acid. These results suggest that larvae respond to a noxious challenge in a similar way to adult zebrafish and other vertebrates and that the effect of nociception on activity can be ameliorated by using analgesics. Therefore, adopting larval zebrafish could represent a direct replacement of a protected adult fish with a non-protected form in pain- and nociception-related research.

KEY WORDS: Nociception, Pain, Behaviour, Zebrafish larvae, Analgesia

INTRODUCTION

Recent investigations have demonstrated that teleost fish have nociceptors, receptors that detect potentially painful stimuli, which are very similar to those found in mammals (Ashley et al., 2007; Roques et al., 2010; Sneddon, 2002). A variety of species also exhibit adverse behavioural and physiological responses to a potentially painful event (Dunlop and Laming, 2005; Reilly et al., 2008). Thus, the use of fish in nociception and pain studies has dramatically increased over the last few years (Eckroth et al., 2014; Prober et al., 2008; Sneddon et al., 2003a). Recent evidence suggests that the development and organization of both peripheral and central nociceptive processing systems is similar between

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teleost fish, including zebrafish, and other vertebrates (Sneddon, 2002, 2003), even at early larval stages (Curtright et al., 2015). In addition, previous studies have identified multiple subtypes of nociceptors in zebrafish, suggesting a similar organization of molecular nociceptive circuits between mammals and zebrafish even as early as 1–3 days post-fertilization (dpf) (Caron et al., 2008; Gau et al., 2013). Thus, it seems prudent to replace adult fish with these young forms, if they still perform distinctive behavioural responses to nociceptive stimuli (Budick and O'Malley, 2000; Ingebretson and Masino, 2013).

It has been demonstrated that fish respond to a wide range of noxious stimuli, including thermal (Malafoglia et al., 2014), electrical (Roques et al., 2012) and chemical (Mettam et al., 2012) stimuli. Specifically, exposure to acidic compounds can cause stress in fish if this falls below normal tolerance levels through gill and epidermal damage. This may lead to problems with osmoregulation and oxygen uptake (Branson, 1992) and changes in behaviour (Brown et al., 2012; Leduc et al., 2004). Reilly et al. (2008) have previously reported that both zebrafish and rainbow trout show behavioural responses after subcutaneous injection of dilute acetic acid. Indeed, the acetic acid test has been used as the reference model for pain or nociception in fish (Ashley et al., 2007; Newby and Stevens, 2008) and specifically in zebrafish (Correia et al., 2011; Maximino, 2011; Nordgreen et al., 2014). Most studies in adult fish use injection of dilute acid; however, Mettam et al. (2012) demonstrated topical application of this chemical stimulated nociceptors on the face of trout, suggesting that any acidic compounds may excite skin nociceptors without any skin damage (below 2% acetic acid). The effect of different concentrations of acetic acid dissolved in water has recently been investigated in 5 dpf larval zebrafish (Steenbergen and Bardine, 2014) but this immersion approach is yet to be developed for other chemicals. Given the strong evidence that nociceptors respond to low pH using a variety of chemicals in both mammals (Carstens et al., 1998) and fish (Ashley et al., 2007; Sneddon, 2003), we hypothesized that the agents used in the present study may evoke a behavioural response that can be quantified.

Appropriate analgesia should be applied to minimize the impact on animals undergoing experimental procedures that cause tissue damage (Sneddon, 2015). In addition, behavioural alterations elicited by any noxious stimulation should be reduced by the use of analgesics or painkillers (Sneddon et al., 2014). Considering the increase in the use of fish as a model for scientific studies including those on pain and nociception, it is imperative that we understand how they react to painful procedures to assess their welfare but also to refine our experimental protocols, providing appropriate analgesia to reduce pain. Finding an alternative to the use of adult fish in these studies is a priority as is finding a rapid means of testing the efficacy of analgesics if we are to adopt an ethical 3Rs (Replacement, Reduction and Refinement) approach to research (Russell and Burch, 1959). However, a very limited number of studies have investigated the effects of analgesics in fish

and most of these have explored intramuscular injection, which is impractical for small species such as zebrafish (Harms et al., 2005; Mettam et al., 2011; Sneddon, 2003). Therefore, a range of doses of these analgesic agents administered via immersion (dissolved in tank water) need to be investigated to determine their efficacy as a high-throughput means of testing drugs in a large number of unprotected 5 dpf zebrafish.

The objective of the present study was to explore the use of these young unprotected zebrafish, *Danio rerio* (Hamilton 1822), as a valid replacement for adult fish through quantifiable behavioural measurements and to test potential analgesics to inform the development of analgesic protocols. Drugs with analgesic properties dissolved in the tank water should ameliorate these responses, which would present an important refinement in juvenile and adult zebrafish experimentation.

MATERIALS AND METHODS
Experimental animals
All experiments were conducted according to the guidelines of research ethics as approved by the Ethics Committee at the University of Liverpool. AB wild-type zebrafish larvae at 5 dpf were used for the purposes of this experiment. Eggs were provided by the in-house breeding programme. Briefly, adult zebrafish were held in breeding pairs and eggs were collected the morning after. Eggs were then kept in 3 l plastic tanks (Pentair Aquatic Habitat, Apopka, FL, USA) in a closed aerated recirculation system supplied with filtered, aerated freshwater at a temperature of 28.0 ± 0.5°C and on a 12 h:12 h light:dark cycle until 5 dpf, at which point fish were selected at random for experiments. Water quality parameters were kept ideal for this species (pH 7.2; nitrite <0.1 mg l^{-1}, nitrate <20 mg l^{-1}, ammonia <0.1 mg l^{-1}). Any animals not used in the present study were either held as stock for other experiments or humanely killed before reaching 6 dpf.

Apparatus
Experiments were carried out in a room maintained at 27 ± 0.5°C, with lights on at 08:00 h and lights off at 20:00 h, similar to rearing conditions. Larvae movements were analysed in 25 square wells (length 16.5 mm, width 16.5 mm, depth 8 mm) on a custom-built plastic plate mounted to the side of a 3 l plastic tank (Pentair Aquatic Habitat) and secured with clear silicon (AquaMate, Everbuild, Leeds, UK). The plastic plate had a 53 μm mesh bottom (Zebrafish Management Ltd, Twyford, UK) which allows chemicals and water to be rapidly flushed in and out. The tank was positioned on top of an infrared light stage (illumination area 450×210 mm; 850 nm, Loligo Systems, Viborg, Denmark) to maximize contrast and facilitate tracking of dark targets on a light background (Fig. 1). The experimental tank was supplied with filtered water (total ammonia nitrogen ≤0.01 mg l^{-1}, nitrite ≤0.01 mg l^{-1}, nitrate ≤5 mg l^{-1}),

maintained at a constant temperature of 28.0 ± 0.5°C and with aeration provided by a compressed air supply.

Video acquisition
Video of spontaneous free swimming was recorded at 2 frames s^{-1} using a digital monochrome infrared-sensitive camera (IDS UI-1240LE-NIR-GL, Stemmer Imaging, Surrey, UK) with an attached lens (Space-Com JHF25M-5MP, Space Inc., Tokyo, Japan) placed above the 25-well plate. The camera was mounted to a tripod at a height of 1.8 m and videos were acquired and saved without compression, via IDS software (uEye Cockpit, IDS Imaging Development Systems GmbH, Obersulm, Germany). The camera was connected to a computer (HP, DSC HM87, Palo Alto, CA, USA). For video analysis, novel tracking software based on an object automated detection, tracking and monitoring algorithm was developed for this project. Briefly, the algorithm can be divided into four different stages, namely pre-processing, object detection, post-processing and monitoring of the object physical activity. In order to monitor the individual behaviour of the larvae, a user-friendly Graphical User Interface (GUI) was designed and developed using open-source MATLAB functions (MathWorks, Inc., Natick, MA, USA). Data files generated by the tracking software were then processed with the bespoke algorithm in MATLAB, which can detect various behavioural larvae patterns based upon standard motion features including average velocity (mm s^{-1}), average acceleration or increase in speed (mm s^{-2}), time active (% total time) and total distance moved (mm). For the analysis of thigmotaxis (the avoidance of a stimulus by moving towards the edge of a well), the well area was divided into two compartments, as displayed in Fig. 1. Thigmotaxis was presented as the percentage of time spent active in the outer zone divided by the time spent in both the outer and inner zones and as the percentage of the distance swum in the outer zone divided by the distance swum in both the outer and inner zones.

Experimental procedure
Testing occurred between 09:00 h and 16:00 h using a randomized trial design to eliminate systematic effects due to time of day. In all experiments, larvae were gently pipetted at random from the rearing tank and then placed individually to acquire video recordings of free-swimming behaviour and to eliminate group interactions in the wells of the experimental apparatus. Animals were then allowed 30 min to acclimate to the experimental arena. Larvae were gently removed from the apparatus and humanely killed once the experiment was finished.

Experiment 1: impact of noxious chemicals on larval behaviour
After acclimation, a 10 min period was recorded to assess the pre-stimulation behaviour of the fish. To determine the effect of several
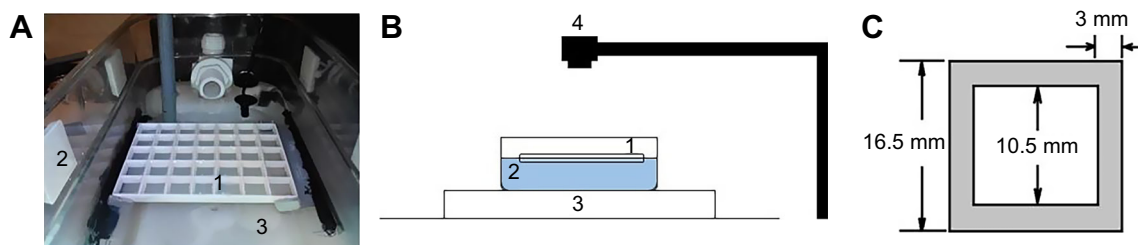

Fig. 1. Experimental set-up. Picture (A) and schematic diagram (B) of the experimental apparatus and schematic diagram of the well arena (C). The 25-well plastic plate (1), 3 l experimental tank (2), infrared (IR) light stage (3) and IR-sensitive camera (4) are displayed in A and B. The inner and outer zones for the analysis of thigmotaxis are displayed in C. The width of the outer zone was set at 3 mm relative to the border of the well.

potentially noxious irritant agents known to typically excite nociceptors in adult fish (Mettam et al., 2012), acetic acid at 0.01%, 0.1% and 0.25% (APC Pure, Manchester, UK) and citric acid at 0.1%, 1% and 5% (citric acid anhydrous, APC Pure) were added to the tank water. To avoid disturbing the larvae, the chemicals were delivered via a 5 mm diameter aquarium tubing attached to the experimental tank, with both ends of the tube hidden from the fish behind the tank. A syringe (BD, Oxford, UK) filled with the appropriate volume of the chemical was connected to the plastic tubing and the content of the syringe was slowly injected into the experimental tank. A pilot study using blue food dye demonstrated this was effective in quickly delivering the chemical as well as ensuring complete mixing. As soon as exposure to agents started, a second 10 min period was video-recorded to assess the post-stimulation behaviour. The larvae were exposed to these agents during the entire period. The pH and osmolarity (measured 20–50 mOsm in all treatments except 432–482 mOsm for 5% citric acid) of the water were measured after the addition of each agent (Table 1). The resulting pH values were considered lethal for larval zebrafish based upon 2 h and longer exposure (Zahangir et al., 2015). For each concentration of irritant agent tested, 18 groups of 25 larvae per group were used (n=450 per treatment). To determine the effect of any potential handling stress, a control 'disturbed' group, which underwent the same experimental procedure but with addition of tank water instead of any irritant agent, and a control 'undisturbed' group, which was left undisturbed for the duration of the experiment, were included.

Experiment 2: efficacy of analgesic drugs

The analgesic efficacy of 1 and 2.5 mg l^{-1} aspirin (aspirin powder, Sparhawk Labs, Lenexa, KS, USA), 1 and 48 mg l^{-1} morphine (morphine sulphate, Martindale Pharmaceuticals, Romford, UK), 1 and 5 mg l^{-1} lidocaine (lidocaine hydrochloride monohydrate, Sigma-Aldrich Co., Gillingham, Dorset, UK) and 8 and 20 mg l^{-1} flunixin (flunixin meglumine, MSD, Milton Keynes, UK) was tested by dissolving different concentrations of these substances in the tank water immediately before the larvae were placed in the apparatus. The addition of these chemicals did not alter the pH or osmolarity of the tank water after treatment (see Table 1). Fish were then allowed to acclimate to the experimental arena for 30 min and, after this, a 10 min period was video recorded to assess the pre-stimulation behaviour of the fish. Subsequently, a solution containing 0.1% of acetic acid (percentage chosen as the threshold for behavioural change) was added to the tank water and immediately after that, the post-stimulation behaviour of the larvae was video recorded for an additional period of 10 min. For each analgesic tested, 18 groups of 25 larvae per group were used (n=450 per treatment).

Statistical analysis

Statistical analyses were performed using SPSS version 22.0.0.1 software. Behavioural data, namely average velocity (mm s^{-1}),

Table 1. Final concentration and pH in the tank water after addition of the different chemical agents in experiment 1

Agent	Final concentration (%)	pH
Control	–	7.2
Acetic acid	0.01	3.6
	0.1	3.3
	0.25	3.1
Citric acid	0.1	3.1
	1	2.8
	5	2.6

average acceleration (mm s^{-2}), time active (%), total distance moved (mm) and thigmotaxis (percentage of time spent active and distance moved in the outer zone) did not fulfil the requirements of a normally distributed population (Kolmogorov–Smirnov, P<0.001) and of the homogeneity of variance (Levene's test, P<0.001). Arcsine and other transformations were used but these did not normalize the data; therefore, non-parametric tests were used. Initial data analysis showed that only the time spent active (%) was affected by noxious treatment. Therefore, for the purposes of experiments 1 and 2, only the time spent active (%) was used as an indicator of the larval behaviour response. In addition, the preliminary results on thigmotaxis (%) are also presented in the Results. Observations where larvae showed no movements at all both before and after stimulation and those in which the tracking process could not successfully track both before and after exposure were excluded from the data set (30 larvae per group on average, which represents 6.66% of the total number of fish: for more details see Figs 2 and 3). We found no effect of the time of day on the behavioural response (time spent active, %) of the control groups, so this was removed from subsequent analysis. A Wilcoxon signed-rank test (P<0.05) was performed to assess any difference between the pre- (baseline) and post-stimulation amount of time spent active on the same larvae exposed to each of the irritant agents (experiment 1) and analgesic substances (experiment 2). The change in activity (or increment) from pre-stimulation to post-stimulation states with each irritant agent and analgesic substance and both the disturbed and undisturbed control groups was determined using a Kruskal–Wallis test (P<0.05). When the Kruskal–Wallis test revealed any significant difference, planned *post hoc* Mann–Whitney U comparisons were made to compare each treatment group with both the undisturbed and the disturbed control groups using a Bonferroni correction, resulting in a significance level set at P<0.0039 per test in experiment 1 (0.05/13) and at P<0.0026 per test in experiment 2 (0.05/19).

RESULTS

Experiment 1: impact of noxious chemicals on larval behaviour

There was a significant decrease in the time spent active shown by the larvae exposed to 0.1% acetic acid (Z=−9.152, P<0.001, n=416) and 0.25% acetic acid (Z=−4.657, P<0.001, n=424). However, 10 min of exposure to 0.1% citric acid (Z=−6.629, P<0.001, n=425), 1% citric acid (Z=−2.170, P=0.030, n=374) and 5% citric acid (Z=−10.324, P<0.001, n=419) and 0.01% acetic acid (Z=−7.335, P<0.001, n=427) evoked a significant increase in the time spent active compared with the pre-stimulation period (Fig. 2). The undisturbed and disturbed control groups did not show a change in activity over the two recording periods (undisturbed: Z=−0.787, P=0.431, n=429; disturbed: Z=−1.578, P=0.115, n=437).

There was a significant change in the percentage time spent active (Z=−13.079, P<0.002, n=403) and the percentage total distance swum (Z=−4.443, P<0.001, n=403) in the outer zone between the two recording periods in the undisturbed control group. The fish spent less time moving (median±IQR) in the outer zone during the first period (73±28%) compared with the second period (75±26%). Similarly, they swam a greater distance (median±IQR) in the outer zone during the second 10 min video recording (95±13%) relative to the first period (92±17%). The median distance swum by the larvae during the pre-stimulation period was 184±16 mm in the inner zone and 1619±537 mm in the outer zone. The median distance swum during the post-stimulation period was 208±19 mm in the inner zone and 2103±640 mm in the outer zone.
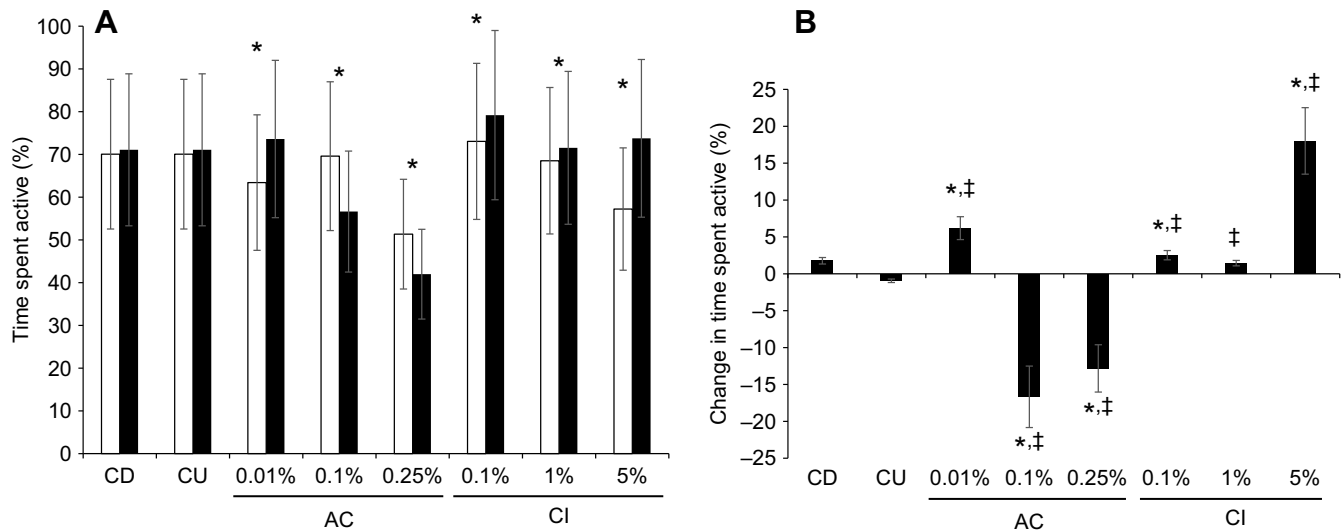
Fig. 2. Experiment 1: effect of noxious chemicals. (A) Median percentage (±IQR) of time spent active shown by 5 days post-fertilization (dpf) zebrafish during a 10 min period before (white bars) and after (black bars) stimulation with different irritant agents. (B) Median percentage (±IQR) change from the pre-stimulation (baseline) values in the time spent active shown by 5 dpf zebrafish exposed to different irritant agents. CD, control disturbed; CU, control undisturbed; AC, acetic acid; CI, citric acid. In A: *significant difference between pre- and post-stimulation periods (Wilcoxon signed-rank test, $P<0.05$). In B: ‡significant difference from the undisturbed control group; *significant difference from the disturbed control group (Mann–Whitney U-test with Bonferroni correction applied, $P<0.0039$). Sample sizes: CD, $n=437$; CU, $n=429$; AC 0.01%, $n=427$; AC 0.1%, $n=416$; AC 0.25%, $n=424$; CI 0.1%, $n=425$; CI 1%, $n=374$; and CI 5%, $n=419$.

The Kruskal–Wallis test revealed that there was a statistically significant difference in the percentage change in the pre- and the post-stimulation behaviour between the different irritant agents tested ($H=381.216$, d.f.=7, $P<0.001$). Larvae exposed to 0.1% and 0.25% acetic acid showed a reduction in activity compared with both the disturbed and undisturbed control groups (Fig. 3). However, fish exposed to the lowest concentration of acetic acid and to 0.1% and 5% citric acid displayed significantly higher increments in the duration of activity (%) in comparison with the control groups (Table 2). No differences were observed between larvae exposed to 1% citric acid and the disturbed control group or between the disturbed and undisturbed control groups.

Experiment 2: efficacy of analgesic drugs
When larvae were exposed for 40 min (30 min prior to video recording plus 10 min of video recording) to 2.5 mg l⁻¹ aspirin, 48 mg l⁻¹ morphine and 5 mg l⁻¹ lidocaine prior to exposure to 0.1% acetic acid, there was no difference between the pre- and post-stimulation amount of time spent active ($Z=-1.321$, $P=0.187$, $n=438$; $Z=-0.544$, $P=0.586$, $n=439$; and $Z=-1.803$, $P=0.071$, $n=391$, respectively; see Fig. 3). However, fish were less active when they were treated with 1 mg l⁻¹ aspirin ($Z=-4.497$, $P<0.001$, $n=416$), 1 mg l⁻¹ lidocaine ($Z=-7.339$, $P<0.001$, $n=402$), 8 mg l⁻¹ flunixin ($Z=-14.545$, $P<0.001$, $n=411$) and 20 mg l⁻¹ flunixin ($Z=-9.811$, $P<0.001$, $n=443$). Finally, larvae
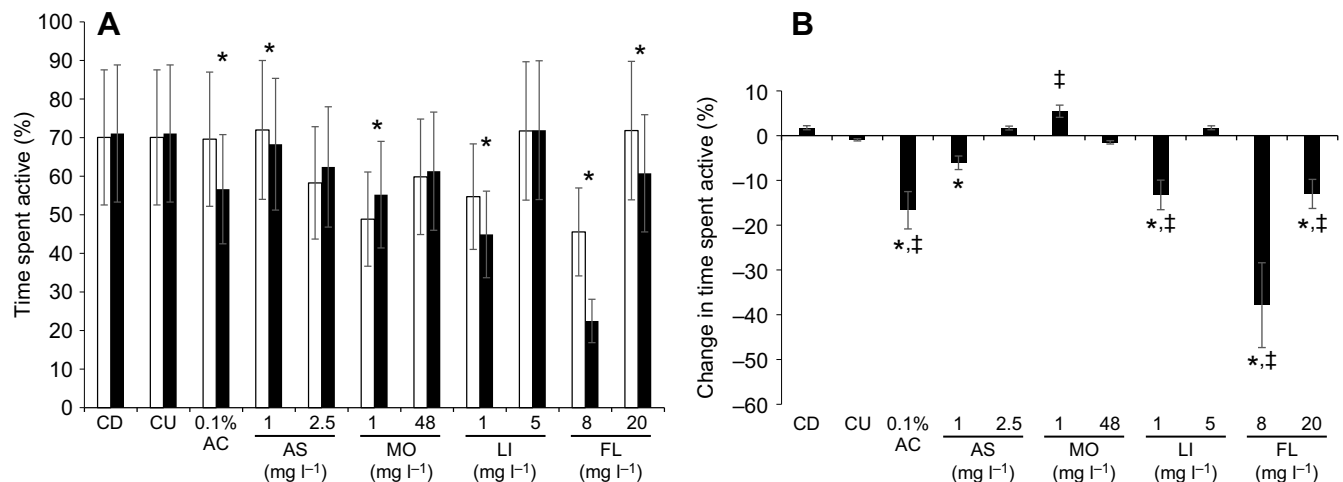

Fig. 3. Experiment 2: effect of analgesics. (A) Median percentage (±IQR) of time spent active shown by 5 dpf zebrafish during a 10 min period before (white bars) and after (black bars) stimulation with 0.1% acetic acid with 30 min prior exposure to different analgesic substances. (B) Median percentage (±IQR) change from the pre-stimulation (baseline) values in the time spent active shown by 5 dpf zebrafish exposed to 0.1% acetic acid with 30 min prior exposure to different analgesic substances. CD, control disturbed; CU, control undisturbed; AC, acetic acid; AS, aspirin; MO, morphine; LI, lidocaine; FL, flunixin. In A: *significant difference between pre- and post-stimulation periods (Wilcoxon signed-rank test, $P<0.05$). In B: ‡significant difference from the undisturbed control group; *significant difference from the disturbed control group (Mann–Whitney U-test with Bonferroni correction applied, $P<0.0026$). Samples sizes: CD, $n=437$; CU, $n=429$; 0.1% AC, $n=416$; 1 mg l⁻¹ AS, $n=416$; 2.5 mg l⁻¹ AS, $n=438$; 1 mg l⁻¹ MO, $n=435$; 48 mg l⁻¹ MO, $n=439$; 1 mg l⁻¹ LI, $n=402$; 5 mg l⁻¹ LI, $n=391$; 8 mg l⁻¹ FL, $n=411$; and 20 mg l⁻¹ FL, $n=443$.

Table 2. Mann–Whitney signed-rank test of the change in time spent active (%) in experiment 1

	Control disturbed		Control undisturbed	
	U	P	U	P
Control undisturbed	88,016	0.134	–	–
Acetic acid: 0.01%	75,302	<0.001	70,225	<0.001
0.1%	62,631	<0.001	65,328	<0.001
0.25%	77,750	<0.001	78,381	<0.001
Citric acid: 0.1%	79,615	<0.001	72,605	<0.001
1%	75,302	0.319	78,226	0.228
5%	62,323	<0.001	58,526	<0.001

The table shows the U- and P-values of the comparisons between the different irritant agents and the control disturbed/undisturbed groups.

exposed to 1 mg l^{-1} morphine moved more ($Z=-3.503$, $P<0.001$, $n=435$).

Comparisons between groups after treatment with analgesics demonstrated that there was a significant effect of the drug administration on the post-stimulation behaviour ($H=491.475$, d.f.=10, $P<0.001$). Larvae treated with 2.5 mg l^{-1} aspirin, 48 mg l^{-1} morphine and 5 mg l^{-1} lidocaine and then exposed to 0.1% acetic acid had a similar change in activity to that of the undisturbed control or the disturbed control group (Table 3). There was a reduction in activity 10 min after exposure to acetic acid when larvae were treated with 1 mg l^{-1} lidocaine, 8 mg l^{-1} flunixin and 20 mg l^{-1} flunixin compared with both undisturbed and disturbed control groups. Fish treated with 1 mg l^{-1} aspirin exhibited reduced activity (Fig. 3) compared with the disturbed control fish but not with the undisturbed control group. In contrast, larvae treated with 1 mg l^{-1} morphine displayed a greater change in activity after 10 min of exposure to 0.1% acetic acid compared with the undisturbed control group, whereas there was no significant difference from the activity of the disturbed control group.

DISCUSSION

Exposure to 0.1% and 0.25% acetic acid resulted in a reduction in the amount of time spent active in 5 dpf zebrafish larvae. No significant variation was found on the control groups throughout the day, suggesting that the only effect that had a consistent impact on the behavioural response of the larvae was the chemicals themselves. However, an increase in activity was observed when larvae were exposed to any of the three concentrations of citric acid used and to the lowest concentration of acetic acid. After stimulation with the lowest possible effective concentration of acetic acid (0.1%), larvae treated with 2.5 mg l^{-1} aspirin, 48 mg l^{-1} morphine and 5 mg l^{-1} lidocaine did not show a decrease in activity and were

Table 3. Mann–Whitney signed-rank test of the change in time spent active (%) in experiment 2

	Control disturbed		Control undisturbed	
	U	P	U	P
Aspirin: 1 mg l^{-1}	75,320	<0.001	78,959	0.0038
2.5 mg l^{-1}	95,637	0.986	89,439	0.221
Morphine: 1 mg l^{-1}	86,840	0.027	81,753	0.0016
48 mg l^{-1}	90,708	0.148	93,157	0.741
Lidocaine: 1 mg l^{-1}	63,403	<0.001	65,109	<0.001
5 mg l^{-1}	84,279	0.737	77,895	0.078
Flunixin: 8 mg l^{-1}	38,082	<0.001	39,079	<0.001
20 mg l^{-1}	65,055	<0.001	69,723	<0.001

The table shows the U- and P-values of the comparisons between the larvae treated with analgesics and the control disturbed/undisturbed groups.

similar to controls, possibly indicating that these substances have some analgesic action in the larvae. We were able to detect significant differences in behaviour when a potentially painful stimulus was applied and screened four drugs for their efficacy as analgesics; thus, we have provided a novel, high-throughput means of assessing drugs in this paradigm.

Experiment 1: impact of noxious chemicals on larval behaviour

Acetic acid elicited a behavioural response in the larvae, with fish showing reduced activity compared with control groups. This response seems to be partially dose dependent, as fish exposed to a concentration higher than 0.1% (or pH 3.3) spent less time swimming, which did not occur with the lower concentration (pH 3.6). This behavioural response characterized by a reduction of locomotor activity has been observed in other animal groups (Sneddon et al., 2014), including fish, under pain (Reilly et al., 2008) or stress conditions (Kristiansen et al., 2004). Recent studies in rainbow trout and zebrafish have demonstrated a reduction in swimming activity when fish were administered a potentially painful stimulus, injection of acetic acid (Correia et al., 2011; Reilly et al., 2008). Recently, another method to test the noxious effects of acetic acid in larvae has been developed, using dilute concentrations of acetic acid in the tank water (Steenbergen and Bardine, 2014), ranging from 0.0025% to 0.025%.

Despite the differences in the experimental procedures between the work of Steenbergen and Bardine (2014) and the present study [Steenbergen and Bardine (2014) did not report any settling period prior to treatment with acid and the exposure time to the acid was much lower at 3 min versus 10 min in the present study], their results agree with those of our experiment, which confirms that very low concentrations of acetic acid evoke an increase in the locomotor activity of the fish. This may reflect an escape response from a potentially harmful, noxious stimulus and represents a nociceptive reflex (Sneddon, 2015; Sneddon et al., 2014). The minimum concentration required to elicit a significant change in the locomotor activity of the zebrafish has not been determined yet, as different factors could play a role in the threshold that the nociceptors need to respond to the acid via immersion and different concentrations could have different effects. For example, in a study with adult zebrafish, exposure to 0.03% acetic acid in a beaker for 30 min (pH 4.1) resulted in a significant increase in top-dwelling behaviour (Currie, 2014) but this was not observed with 0.01% (pH 4.7) or 0.05% (pH 3.8) acetic acid. Additionally, there is a debate whether fish may have a different response to acetic acid when this is administered via immersion or injection. However, results from this experiment show that larvae display a behavioural response after treatment with acid dissolved in the water, confirming that this approach could be a valid model for the study of pain and nociception. Current legislation (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes; http://eur-lex. europa.eu/eli/dir/2010/63/oj) states that early life stages of zebrafish are not protected as animals until the stage of being capable of independent feeding, which occurs after 5 dpf. According to the principles of the 3Rs (Russell and Burch, 1959), replacement refers to any method that avoids or replaces the use of animals defined as protected under the Animals (Scientific Procedures) Act 1986, amended 2012 (http://www.legislation.gov.uk/uksi/2012/3039/ contents/made). The reduction in the locomotor activity observed in our study has previously been reported in adult zebrafish (Correia et al., 2011; Reilly et al., 2008) and other fish species when exposed

to a chemical stimulus. Thus, we believe that our study provides robust evidence to justify the replacement of adults with 5 dpf zebrafish in pain and nociception studies. This might pose the question of whether 5 dpf zebrafish should be protected if they display adverse changes in behaviour that are ameliorated by pain-relieving drugs. However, more data would need to be collected to inform this decision as these results would need to be supplemented by further studies. There are many other criteria an animal must fulfil to be considered capable of sensing pain (Sneddon et al., 2014). For example, information is needed on brain processing during potentially painful events and on whether these larval zebrafish can learn to avoid noxious stimuli.

Larvae exposed to all three concentrations of citric acid swam more relative to the control groups (except those exposed to 1% citric acid relative to the undisturbed control group), possibly indicating that citric acid did not evoke the same noxious response in the fish compared with the highest concentrations of acetic acid. Increased activity may represent an escape or nociceptive reflex withdrawal response to a potentially noxious stimulus rather than a response to a stimulus that actually does cause harm (Sneddon et al., 2014). Citric acid was chosen to determine the behavioural response of the larvae because it has been shown to stimulate some but not all nociceptor afferents in the head of rainbow trout (Mettam et al., 2012). Although these authors report a threshold for response to citric acid of 1%, there might be some discrepancies with the present study as a result of species-specific differences and the application route of the chemical (direct topical application to nociceptor on the skin). Interestingly, the pH of the three solutions of citric acid used here was equal to or lower than that of all three concentrations of acetic acid. Moreover, exposure to 0.25% acetic acid had an opposite effect on the behaviour compared with 0.1% citric acid, even though the resulting pH values were the same. These results could indicate that there is another mechanism affecting the response of the nociceptors other than the pH. Indeed, the above-mentioned authors (Mettam et al., 2012) suggest that the osmolarity may also play a role in the way afferent receptors respond to a chemical at a sub-threshold pH and it has been reported that the combination of pH and osmolarity is the most important factor evoking a wiping response in frogs exposed to acetic acid (Hamamoto et al., 2000). However, citric acid at a concentration of only 5% significantly altered the osmolarity of the tank water, which represents an almost 10-fold change. This value could be potentially damaging; however, a study by Perez-Camps and Garcia-Ximenez (2008) reported that the survival rate was 100% when zebrafish larvae were kept for 48 h at 315 mOsm and that larvae kept for 1 h at the same osmolarity had a survival rate of 100% after 6 days. Thus, further studies are needed to clarify the impact of osmolarity both in isolation and combined with pH.

Thigmotaxis, a preference for avoiding the centre of a novel environment and remaining close to the wall, is a widely used indicator of fear and anxiety in both adult (Peitsaro et al., 2003) and larval zebrafish (Richendrfer et al., 2012; Schnörr et al., 2012). However, it has not previously been used in the study of nociception in this species. Our results indicate that there was a significant change in the percentage of time that the fish spent active as well as in the distance swum in the outer zone in the control group. Therefore, it was not measured in the rest of the experimental groups, as it should not be considered as a valid indicator of nociception under our experimental conditions. These results may suggest a wall-seeking behaviour, although the reasons are not clear. While the experimental approach used in our study was valid as an indicator of nociception and the assessment of the behaviour (time

active) after exposure to irritant agents, it might not be suitable for observing differences in wall-hugging behaviour. Regardless, more research is needed to investigate other approaches (different size of the arena, area of the outer zone, habituation time to the experimental apparatus, etc.).

Experiment 2: efficacy of analgesic drugs

Fish exposed to 2.5 mg l^{-1} aspirin, 48 mg l^{-1} morphine and 5 mg l^{-1} lidocaine showed similar activity to the control groups, which suggests that these three substances have some analgesic or anti-nociceptive effect.

We found that administration of aspirin had a beneficial effect on activity after exposure to 0.1% acetic acid. However, this effect was limited to the highest concentration (2.5 mg l^{-1}), with no apparent effect at 1 mg l^{-1}, probably because this is too low a dose to be effective in 5 dpf zebrafish. NSAIDs are a group of drugs inhibiting arachidonate cyclo-oxygenase (COX) enzymes. These enzymes are involved in the biosynthesis of specific prostanoids, which in turn control many physiological mechanisms including inflammation. Thus, NSAIDs have an effect through anti-inflammatory and analgesic properties among others (Sneddon, 2012). Moreover, COX-2, a COX isozyme responsible for inflammation and pain, has been found to be expressed in larval zebrafish (Grosser et al., 2002). Thus, it seems prudent to explore the analgesic efficacy of aspirin in 5 dpf zebrafish. However, despite all this evidence, research on the use of NSAIDs as analgesics in fish is very limited (Davis et al., 2006; Harms et al., 2005; Mettam et al., 2011). Although the analgesic effects of aspirin have been investigated in other animals (Jablonski and Howden, 2002; Shyu and Lin, 1985), we found no studies in larval or adult fish. Therefore, further tests on adult zebrafish should be done before its use can be recommended.

Flunixin is another type of NSAID that has been explored as an analgesic in different mammalian species (Foreman and Ruemmler, 2011; Welsh and Nolan, 1995) but its efficacy in aquatic species is not well known. The precise site and mode of action are still unknown but it may act peripherally in inflamed tissue, probably by inhibiting the enzyme COX to decrease the formation of precursors of prostaglandins and possibly by inhibiting other local mediators of the inflammatory response (Lees and Higgins, 1985). In our study, flunixin meglumine was not an effective drug for the provision of analgesia in 5 dpf zebrafish, as fish spent significantly less time swimming compared with control larvae. It may well be that the concentration used was not sufficient to observe any analgesic effect, as the noxious impact of acetic acid was more pronounced at 8 mg l^{-1} in comparison with 20 mg l^{-1}. Further studies evaluating the analgesic potential of this drug are needed; however, higher doses substantially increase the cost of analgesia, which may be prohibitive in adopting its use.

Administration of 48 mg l^{-1} of morphine had a beneficial effect on the activity of the larvae, thus suggesting an analgesic or anti-nociceptive action. Morphine is the best-known opioid drug. These drugs act on the three opioid receptors located on neuronal cell membranes, inhibiting neurotransmitter release, thereby not only blocking the activity of nociceptors but also centrally blocking transmission (Sneddon, 2012). Recent evidence demonstrates the existence of an endogenous opioid system that is similar to those found in mammals (Gonzalez-Nunez and Rodríguez, 2009), and morphine-induced changes in expression of genes that are specific for zebrafish embryos and other genes that are similar to those found in mammals (Herrero-Turrión et al., 2014). Thus, morphine has proved to be an effective analgesic in fish (Newby et al., 2009; Nordgreen et al., 2009; Sneddon et al., 2003b) and specifically in

adult zebrafish (Correia et al., 2011) but no studies have been conducted in larval zebrafish. Moreover, administration via immersion has been tested in adult zebrafish (Currie, 2014) but no beneficial effects were found after exposure to acetic acid using up to 3 mg l^{-1} of morphine sulphate; the lower concentration of morphine explains why morphine was not effective in that study (Currie, 2014). Interestingly, treatment with the lower dose (1 mg l^{-1}) seemed to have the opposite effect, increasing the time that the fish spent active. A recent study on the conditioned place preference on morphine-exposed zebrafish demonstrated that adults are sensitive to concentrations as low as 1.5–3 mg l^{-1} (Khor et al., 2011), so the higher locomotor activity we found in the group treated with 1 mg l^{-1} may be associated with a side-effect of the morphine present at low concentrations. Our results suggest that the most effective dose of morphine in larval zebrafish is between 1 and 48 mg l^{-1}. As morphine appears to have a slow rate of uptake in fish when administered via ambient water (Newby et al., 2009), high doses may be required to provide effective analgesia. Indeed, there is only one study that has determined a dose–response relationship for morphine in fish after noxious stimulation, with larger doses of morphine associated with an increase in the analgesic index (Jones et al., 2012). However this was injected and not added to the water directly. Thus, although the analgesic properties of this drug have been demonstrated in our study, more research is needed to find the most appropriate dose for larval zebrafish and to understand the mechanisms explaining the effectiveness of morphine dissolved in water in these young forms of fish.

Only the higher dose of lidocaine had a positive effect on the locomotor activity of larval zebrafish, with fish exposed to the lower concentration exhibiting a dramatic reduction in swimming activity. We found that lidocaine at 1 mg l^{-1} was not effective in 5 dpf zebrafish, as the group treated with this concentration showed a similar response to the group exposed to acetic acid. However, larvae exposed to the higher dose, i.e. 5 mg l^{-1}, showed no differences from the control group, thus suggesting that it was effective at preventing the changes observed with acetic acid. Lidocaine is a local anaesthetic that inhibits the propagation of action potentials by blocking sodium channels and by affecting membrane function (Sneddon, 2012). It was first tested as an analgesic agent in fish, with positive results by Mettam et al. (2011) for injection of 1 mg at the site of damage, but its efficacy has never been demonstrated in larval fish. However, it has been successfully examined as an anaesthetic in zebrafish (Collymore et al., 2014). Because of its anaesthetic properties, it may induce a neuromuscular blockage but the doses used in the present study are much lower than the effective dose needed for anaesthesia, with no apparent side-effects observed. Therefore, the effectiveness of the highest concentration tested here has been confirmed. More research is needed to evaluate other concentrations in other fish species.

Conclusions

We have demonstrated that 5 dpf zebrafish respond to a variety of noxious chemical stimuli by reducing activity and that the behavioural response observed can be ameliorated by providing appropriate analgesia with aspirin, lidocaine and morphine at relevant doses. Similar responses have previously been reported in adult fish, which implies that larval zebrafish can be used as a model for the study of pain and nociception and, thus, they represent a direct replacement of a protected adult vertebrate. Our novel system provides a high-throughput means of testing the impact of chemical exposure and could be extrapolated to studies exploring toxicants and pharmaceuticals.

Data availability
Primary data on swimming activity have been deposited in figshare (Lopez-Luna et al., 2016): https://doi.org/10.6084/m9.figshare.4285778.v3.

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