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Response to: Responses of larval zebrafish to low pH immersion assay. Comment on Lopez-Luna et al.

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Lopez-Luna et al. (2017a) investigated the utility of using larval zebrafish as a replacement for adults in nociceptive testing. Five days post-fertilisation larvae were held in a 25-well plate and monitored using a video tracking system. Either larvae were undisturbed or system water was added as control to compare with the addition of known noxious substances. In response to concentrations of 0.1% and 0.25% acetic acid, larvae gave the characteristic adult reaction to acetic acid: a reduction in activity. A number of drugs with analgesic properties were investigated to determine their utility in preventing the behavioural alteration to 0.1% acetic acid. Three of the four drugs normalised behaviour. Therefore, larval zebrafish could replace current protocols employing adults.

The criticism of these results by Diggles and colleagues appears to be based on a major misinterpretation or misconception of the study.

Firstly, it is clear Diggles et al. have misunderstood the methods employed. The study only tested the pain-relieving drugs in conjunction with 0.1% acetic acid. The study was successful in demonstrating that immersion in three drugs prevented the reduction in activity after acid exposure.

Secondly, Diggles et al. suggest that data on conductivity, hardness and alkalinity was not reported and this precludes the authors from interpreting the results of acid exposure in larvae. We present data below (Table 1) to demonstrate that none of the analgesics alone affected water quality.

The conductivity of the water was within the range for zebrafish husbandry, 300–1500 µs cm^{-1} (Avdesh et al., 2012), except for 1% and 5% citric acid. Alkalinity remained stable after addition of the analgesic drugs and within recommended limits (50–150 mg $CaCO_3$ l^{-1}; Avdesh et al., 2012). However, adding acids naturally means reducing alkalinity, as evidenced by the reported pH values. Topical application of acid excites nociceptors on the skin of fish (Sneddon, 2015) and also amphibians and humans (e.g. Hamamoto and Simone, 2003; Keele and Armstrong, 1964), justifying the belief that exposure to acid excites nociceptors in larval zebrafish.

Water hardness was unaffected by both acetic acid and the analgesic drugs (recommended range 80–300 mg $CaCO_3$; Avdesh et al., 2012). Only 1% citric acid affected hardness below this lowest recommended value but behaviour did not differ in response to this concentration. Citric acid is a water-softening agent (e.g. Altundoğan et al., 2016). In soft water, fish need to use osmo-regulatory mechanisms; however, these effects are only a cause for concern in chronic situations (Wood, 1989) as opposed to the 10 min exposure in this study, ruling out iono-regulatory failure. Even if acetic acid induced iono-regulatory dysfunction, adding analgesics would not resolve this.

Diggles et al. also fail to cite a similar study that clearly undermines their position that altered water quality explains the behavioural changes (Lopez-Luna et al., 2017b). This study used heat as a noxious stimulus rather than acid. When heat was applied to fully oxygenated water, no changes to the water chemistry occurred, yet the larval zebrafish reduced activity at high temperatures and again this was ameliorated by the use of the same analgesic agents. Therefore, the observed changes in behaviour are a response to noxious stimulation.

Thirdly, Diggles et al. allege we make unfounded assumptions, yet the effects of acetic acid are published. Their alternative explanation, that the response to acetic acid occurs through an olfactory mechanism, is not supported by citations. Further, they state that the analgesics may affect olfaction. However, there are no studies supporting this and it is not reported on public websites detailing side-effects of these drugs on humans (e.g. WebMD: http://www.webmd.com/a-to-z-guides/drug-side-effects-explained#1).

Fourthly, Diggles et al. classified the concentrations used in Lopez-Luna as ‘high’. We find this unsubstantiated for all concentrations except the highest dose of morphine (48 mg l^{-1}). All doses were determined from published studies using fish models (Schroeder and Sneddon, 2017). The higher morphine dose was selected based upon the published research of Stevens (e.g. Newby et al., 2009: at least 40 mg kg^{-1} morphine via injection). A recent study demonstrated that morphine injected intramuscularly at 2.5 and 5 mg kg^{-1} in adult zebrafish is effective at preventing the reduced activity associated with acetic acid treatment (Taylor et al., 2017). This suggests that our dose of 1 mg l^{-1} was too low but morphine is known to increase activity in adult fish (Sneddon et al., 2003) providing a plausible explanation of why morphine alone increased activity in zebrafish larvae.

Diggles et al. also cite an Honours thesis (Currie, 2014) which they claim contrasts with our findings. However, they misinterpret the results as they state that activity increased in

Table 1. Conductivity, alkalinity and water hardness

Sample	Dose (per 3 l)	Exposure (min)	Added after first time period of 30 min (per 3 l)	Exposure (min)	Conductivity (µS cm^{-1})	Hardness (mg $CaCO_3$ l^{-1})	Alkalinity (mg $CaCO_3$ l^{-1})
Water only		30			300	120	80
Lidocaine	3 mg	30			305	120	80
	15 mg	30			305	120	80
Aspirin	3 mg	30			306	120	80
	7.5 mg	30			300	120	80
Morphine	3 ml	30			304	120	80
	144 ml	30			301	120	80
Flunixin	24 mg	30			304	120	80
	60 mg	30			300	120	80
Acetic acid	0.3 ml	10			302	120	80
	3 ml	10			382	120	0
	7.5 ml	10			469	120	0
Citric acid	3 g	10			679	95	0
	30 g	10			2180	35	0
	150 g	10			4820	120	0
Lidocaine	3 mg	30	Acetic acid (3 ml)	10	367	120	0
	15 mg	30	Acetic acid (3 ml)	10	374	120	0
Aspirin	3 mg	30	Acetic acid (3 ml)	10	372	120	0
	7.5 mg	30	Acetic acid (3 ml)	10	370	120	0
Morphine	3 ml	30	Acetic acid (3 ml)	10	375	120	0
	144 ml	30	Acetic acid (3 ml)	10	368	120	0
Flunixin	24 ml	30	Acetic acid (3 ml)	10	369	120	0
	60 ml	30	Acetic acid (3 ml)	10	374	120	0

Measurements were taken from the study by Lopez-Luna et al. (2017a), where zebrafish larvae at 5 days post-fertilisation were held in 3 l of normal water from the aquarium facility or exposed to a range of drugs, and also following exposure to acetic acid and citric acid. Alkalinity was measured using Methyl Orange. Mean values are shown. Morphine and flunixin were added as 1 mg ml^{-1} solutions. Note: when too much citric acid is added to soften water, it can have no effect; thus, 5% citric acid does not affect hardness.

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Currie's study, but only top-dwelling behaviour was measured and statistically analysed. To quote: 'top-dwelling behavior was the most commonly-observed response to 0.03% acetic acid'. Therefore, there appears to be an increase in top-dwelling behaviour but no quantification of activity. Both the sub-threshold concentration of acetic acid and low dose of morphine explain Currie's results.

A comparable study by Steenbergen and Bardine (2014) using 5-dpf zebrafish is cited, but larvae were exposed to 0.025% acetic acid, which is again sub-threshold to elicit a nociceptive response. In that study, larvae increased activity in response to the low concentrations in a similar manner to that seen in Lopez-Luna et al. (2017a) using 0.01% acetic acid. Therefore, the results of the two studies confirm one another. However, Steenbergen and Bardine (2014) mention that exposure to higher acetic acid concentrations resulted in a decrease in larval locomotor activity and subsequently death. These authors clearly demonstrated the involvement of the opioid pathway in this response and Cox-2 expression. Diggles et al. appear to ignore data and peer-reviewed articles where Cox-2 is strongly linked to pain and nociception in zebrafish (Grosser et al., 2002) as well as in other vertebrates.

Lopez-Luna et al. provide compelling evidence that zebrafish larvae are indeed a useful replacement for adult fish, assessing them in a high-throughput manner rather than one adult per tank. Indeed, another laboratory has demonstrated that larvae exhibit thermonociception (Curtwright et al., 2015), showcasing their utility in studies of nociception and analgesia. Diggles et al. suggest that anaesthetising adults and injecting them with chemicals is a better approach, yet have previously criticised the use of anaesthesia as a confounding factor as well as low sample sizes (Rose et al., 2014). Lopez-Luna et al.'s study circumvents these issues with no anaesthesia and large sample sizes using an immature form that under European legislation is not protected.

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