© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 3191-3194

CORRESPONDENCE

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

B. K. Diggles[1,*], R. Arlinghaus[2], H. I. Browman[3], S. J. Cooke[4], I. G. Cowx[5], A. O. Kasumyan[6], B. Key[7], J. D. Rose[8], W. Sawynok[9], A. Schwab[10], A. B. Skiftesvik[3], E. D. Stevens[11], C. A. Watson[12] and C. D. L. Wynne[13]

[1]DigsFish Services, Banksia Beach, QLD 4507, Australia.
[2]Department of Biology and Ecology of Fishes, Leibniz-Institute of Freshwater Ecology and Inland Fisheries & Humboldt-Universität zu Berlin, Berlin 12587, Germany.
[3]Institute of Marine Research, Austevoll Research Station, Marine Ecosystem Acoustics Group, Sauganeset 16, 5392 Storebø, Norway.
[4]Department of Biology, Carleton University, Ottawa, K1S 5B6, Canada.
[5]Hull International Fisheries Institute, University of Hull, Hull HU6 7RX, UK.
[6]Department of Ichthyology, Faculty of Biology, Moscow State University, Moscow 119991, Russian Federation.
[7]School of Biomedical Sciences, University of Queensland, St Lucia, QLD 4072, Australia.
[8]Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071, USA.
[9]Infofish Australia, Frenchville, QLD 4701, Australia.
[10]Biglen, Bern 3507, Switzerland.
[11]Biomedical Sciences, Atlantic Veterinary College, Charlottetown, PE, C1A 4P3, Canada.
[12]Tropical Aquaculture Laboratory, University of Florida, Gainesville, FL 33547, USA.
[13]Department of Psychology, Arizona State University, Tempe, AZ 85287, USA.

*Author for correspondence (ben@digsfish.com)

Lopez-Luna et al. (2017) observed behavioral responses of larval zebrafish (*Danio rerio*) exposed for 10 min to pH 2.6–3.6 when acetic acid (0.01–0.25%) or citric acid (0.1–5%) was added to the tank water in the presence or absence of aspirin (1–2.5 mg l^{-1}), morphine sulfate (1–48 mg l^{-1}), lidocaine (1–5 mg l^{-1}) and flunixin (8–20 mg l^{-1}). Fish exposed to 0.1–0.25% acetic acid were less active than controls while those exposed to citric acid and 0.01% acetic acid were more active. Administration of high doses of aspirin, morphine and lidocaine for 30 min before exposure prevented the reduction in activity induced by 0.1–0.25% acetic acid.

These behavioral responses were interpreted as evidence that acetic acid immersion provided a noxious stimulus (i.e. activated nociceptors) that was reliable for use as a model system for the study of analgesic substances. We identify methodological weaknesses and inconsistencies in the interpretation of results, and emphasize that activation of nociceptors was assumed, not demonstrated. As a result of several processes and interactions that were not accounted for or discussed, we warn their conclusions are unfounded.

A critical omission was the failure to report water conductivity, hardness and alkalinity data. These determine the magnitude of acute osmoregulatory effects that occur in fish exposed to highly acidic water (Wood, 1989). Trials by other researchers using water with different conductivity, hardness or alkalinity profiles could, therefore, generate significantly different results. Immersion of fish in low pH water also introduces several unavoidable and uncontrolled interactions that prevent unequivocal interpretation of the behavioral changes observed.

For example, sudden exposure of fish to water of pH <4 results in gill dysfunction, iono-regulatory failure and pathological lesions of the gill epithelium (Wood, 1989). These reduce respiratory efficiency, initiating compensatory behavioral responses such as surface respiration (Kramer, 1987), which appears synonymous with ‘top-dwelling behavior’ reported by Currie (2014) in adult zebrafish immersed in 0.03% acetic acid (pH 3.9–4.0). Notably, aquatic surface respiration can occur in a variety of natural circumstances in the absence of nociception (Kramer, 1987), so this behavior is insufficient evidence that nociception is occurring.

In contrast to Currie (2014) and Steenbergen and Bardine (2014), Lopez-Luna et al. (2017) considered reduced (not increased) activity as evidence of ‘alleged pain behavior’ in zebrafish exposed to 0.1–0.25% acetic acid. Steenbergen and Bardine (2014) interpreted increased activity and cyclooxygenase-2 gene expression as evidence of nociception in larval zebrafish immersed in 0.0025–0.025% acetic acid. However, cyclooxygenase-2 expression is a non-specific marker of several physiological processes (Wang et al., 2016), meaning its expression is also insufficient evidence of nociception. A critical observation is that larval zebrafish in the study by Lopez-Luna et al. (2017) continued to exhibit increased activity when exposed to pH 2.6 in the 5 mg l^{-1} citric acid experiment. Because of the strong likelihood of acute pathological damage to gills, eyes and other tissues at such low pH (Daye and Garside, 1976), the absence of ‘alleged pain behavior’ in the citric acid treatment calls into question whether nociception was occurring at all. Furthermore, the fact that both increased and decreased activity are being interpreted by different researchers as evidence that nociception is occurring in larval zebrafish exposed to acetic acid casts doubt upon the construct validity of the assay.

The authors noted that at pH 3.3, exposure to 0.25% acetic acid had the opposite effect on behavior (less activity) compared with 0.1% citric acid (more activity). They stated this indicated ‘another mechanism affecting the response of the nociceptors other than the pH’, but did not elucidate further. Because of the immersion design, we contend those other mechanisms do not have anything to do with nociception. Rather, an alternative and more parsimonious explanation is the behavioral changes were due to detection by, or interference with, chemosensors (Kasumyan, 2001).

Chemosensory systems are active, and chemosensory cells are fully developed and functional in zebrafish before 5 days post-hatching (Kotrschal et al., 1997). Dose-dependent behavioral responses to different chemicals are common and could explain the behavioral differences found between citric acid, acetic acid and, importantly, also the pharmaceuticals used. Indeed, citric acid was identified as a potent gustatory feeding stimulant in zebrafish (Kasumyan and Doving, 2003). Furthermore, acute exposure to pH <4.0 can cause pathological alteration of the olfactory epithelium

(Daye and Garside, 1976) and low pH interferes with chemoreceptors responsible for both olfaction (Tierney et al., 2010) and gustation (Kasumyan, 2001). Acute exposure to low pH can extinguish or change behavioral responses to odors, including attraction to previously repulsive chemicals (Royce-Malmgren and Watson, 1987). Because the chemicals studied drop pH and activate chemoreceptors, this interaction makes it difficult to determine what mechanism(s) was driving fish behavior.

Currie (2014) reported bottom-seeking behavior consistent with chemosensory avoidance responses in adult zebrafish exposed to 0.5–3 mg l^{-1} morphine or 0.03% acetic acid via the water. Increased locomotor activity in zebrafish was also reported by Lopez-Luna et al. (2017) as a 'side-effect' of morphine administration. They tried to circumvent these behavioral artefacts using a 30 min 'acclimation period' prior to exposure to the acid treatments. The pathological effects of immersion in high concentrations of drugs such as morphine or aspirin are largely unknown, though exposure to anti-inflammatory drugs (e.g. Diclofenac) causes damage to gill epithelia at extremely low concentrations (ca. 1 µg l^{-1}). Immersion in high concentrations of pharmaceuticals for 30 min prior to treatment therefore may have significant unintended effects on chemosensory receptors and gill function, making subsequent behavioral responses and interactions with other chemicals unpredictable and/or hopelessly confounded.

Immersion trials therefore provide no advantage over the injection methods previously used, which, while having their own problems (Rose et al., 2014), are more likely to target specific tissues and induce nociception, all while being more economical with the use of reagents. Injection inflicts fewer negative effects on the welfare of wild fishes whereas chemicals used in tank immersion enter waste water and, ultimately, the environment as organic contaminants (Tierney et al., 2010).

The strong possibility that the authors measured behavioral changes due to factors other than nociception cannot be excluded. It is, therefore, premature for Lopez-Luna et al. (2017) and others (Steenbergen and Bardine, 2014) to claim zebrafish larval immersion models have utility for nociception research.

References

Currie, A. D. (2014). Toward a novel model of pain in zebrafish: exposure to water containing dilute concentrations of acetic acid. Psychology Honors Projects, paper 33, Macalester College. http://digitalcommons.macalester.edu/psychology_honors/3.

Daye, P. G. and Garside, E. T. (1976). Histopathologic changes in surficial tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. *Can. J. Zool.* **54**, 2140-2155.

Kasumyan, A. O. (2001). Effects of chemical pollutants on foraging behavior and sensitivity of fish to food stimuli. *J. Ichthyol.* **41**, 76-87.

Kasumyan, A. O. and Doving, K. B. (2003). Taste preferences in fish. *Fish Fish.* **4**, 289-347.

Kotrschal, K., Krautgartner, W. and Hansen, A. (1997). Ontogeny of the solitary chemosensory cells in the zebrafish, *Danio rerio*. *Chem. Senses* **22**, 111-118.

Kramer, D. L. (1987). Dissolved oxygen and fish behavior. *Environ. Biol. Fishes* **18**, 81-92.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. and Sneddon, L. U. (2017). Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. *J. Exp. Biol.* **220**, 1451-1458.

Rose, J. D., Arlinghaus, R., Cooke, S. J., Diggles, B. K., Sawynok, W., Stevens, E. D. and Wynne, C. D. (2014). Can fish really feel pain? *Fish Fish.* **15**, 97-133.

Royce-Malmgren, C. H. and Watson, W. H. (1987). Modification of olfactory-related behavior in juvenile Atlantic salmon by changes in pH. *J Chem Ecol* **13**, 533-546.

Steenbergen, P. J. and Bardine, N. (2014). Antinociceptive effects of buprenorphine in zebrafish larvae: An alternative for rodent models to study pain and nociception? *Appl. Anim. Beh. Sci.* **152**, 92-99.

Tierney, K. B., Baldwin, D. H., Hara, T. J., Ross, P. S., Scholz, N. L. and Kennedy, C. J. (2010). Olfactory toxicity in fishes. *Aquat. Toxicol.* **96**, 2-26.

Wang, T., Mai, K. and Ai, Q. (2016). A review of cyclooxygenase-2 role in fish. *Austin J. Nutr. Metab.* **3**, 1037.

Wood, C. M. (1989). The physiological problems of fish in acid waters. In *Acid Toxicity and Aquatic Animals* (ed. R. Morris, E. W. Taylor, D. J. Brown and J. A. Brown), pp. 125-152. Society for Experimental Biology Seminar Series 34. Cambridge: Cambridge University Press.

10.1242/jeb.162834

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

Lynne U. Sneddon*, Javier Lopez-Luna[1], David C. C. Wolfenden[2], Matthew C. Leach[3], Ana M. Valentim[4], Peter J. Steenbergen[5], Nabila Bardine[6], Amanda D. Currie[7], Donald M. Broom[8] and Culum Brown[9]

[1]University of Liverpool, Institute of Integrative Biology, The BioScience Building, Liverpool L69 7ZB, UK.
[2]Blue Planet Aquarium, Cheshire Oaks, Cheshire CH65 9LF, UK.
[3]School of Agriculture, Food & Rural Development, Agriculture Building, Newcastle University, Newcastle upon Tyne NE1 7RU, UK.
[4]Institute for Research and Innovation in Health (i3S), Institute of Molecular and Cell Biology (IBMC), University of Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal.
[5]Department of Pediatrics I, University Children's Hospital, University of Heidelberg, 69126 Heidelberg, Germany.
[6]Holistic Life Coach, 69126 Heidelberg, Germany.
[7]Macalester College, Psychology, 1600 Grand Avenue, Saint Paul, MN 55105-1899, USA.
[8]Centre for Animal Welfare and Anthrozoology, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK.
[9]Macquarie University, Department of Biological Sciences, Level 2, Building E8B, NSW 2109, Australia.

*Author for correspondence (lsneddon@liverpool.ac.uk)

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.

Journal of Experimental Biology

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.

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.

References

Altundoğan, H. S., Topdemir, A., Çakmak, M. and Bahar, N. (2016). Hardness removal from waters by using citric acid modified pine cone. *J. Taiwan Inst. Chem. Engineers* **58**, 219-225.

Avdesh, A., Chen, M., Martin-Iverson, M. T., Mondal, L., Ong, D., Rainey-Smith, S., Taddei, K., Lardelli, M., Groth, D. M., Verdile, G. and Martins, R. N. (2012). Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: An introduction. *J. Exp. Vis.* **69**, e4196.

Currie, A. D. (2014). Toward a novel model of pain in zebrafish: exposure to water containing dilute concentrations of acetic acid. Psychology Honors Projects. Paper 33. http://digitalcommons.macalester.edu/psychology_honors/33/

Curtright, A., Rosser, M., Goh, S., Keown, B., Wagner, E., Sharifi, J., Raible, D. W. and Dhaka, A. (2015). Modeling nociception in zebrafish: A way forward for unbiased analgesic discovery. *PLoS One* **10**, e0116766.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M. A. and FitzGerald, G.A. (2002). Developmental expression of functional cyclooxygenases in zebrafish. *Proc. Natl. Acad. Sci. USA* **99**, 8418-8423.

Hamamoto, D. T and Simone, D. A. (2003). Characterization of cutaneous primary afferent fibers excited by acetic acid in a model of nociception in frogs. *J. Neurophysiol.* **90**, 566-577.

Keele, C. A. and Armstrong, D. (ed.) (1964). Pain due to acids and alkalis. In *Substances Producing Pain and Itch*, pp. 73-88. Baltimore, MD: Williams and Wilkins.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. and Sneddon, L. U. (2017a). Activity reduced by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. *J. Exp. Biol.* **220**, 1451-1458.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. and Sneddon, L. U. (2017b). Impact of analgesic drugs on the behavioural responses of larval zebrafish to potentially noxious temperatures. *Appl. Anim. Behav. Sci.* **188**, 97-105.

Newby, N. C., Wilkie, M. P. and Stevens, E. D. (2009). Morphine uptake, disposition, and analgesic efficacy in the common goldfish (*Carassius auratus*). *Can. J. Zool.* **87**, 388-399.

Rose, J. D., Arlinghaus, R., Cooke, S. J., Diggles, B. K., Sawynok, W., Stevens, E. D. and Wynne, C. D. L. (2014). Can fish really feel pain? *Fish and Fisheries* **15**, 97-133.

Schroeder, P. and Sneddon, L. U. (2017). Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach. *Appl. Anim. Behav. Sci.* **187**, 93-102.

Sneddon, L. U. (2015). Pain in aquatic animals. *J. Exp. Biol.* **218**, 967-976.

Sneddon, L. U., Braithwaite, V. A. and Gentle, M. J. (2003). Novel object test: Examining nociception and fear in the rainbow trout. *J. Pain*, **4**, 431-440.

Steenbergen, P. J. and Bardine, N. (2014). Antinociceptive effects of buprenorphine in zebrafish larvae: An alternative for rodent models to study pain and nociception? *Appl. Anim. Beh. Sci.* **152**, 92-99.

Taylor, J. C., Dewberry, L. S., Totsch, S. K., Yessick, L. R., DeBerry, J. J., Watts, S. A. and Sorge, R. E. (2017). A novel zebrafish-based model of nociception. *Physiol. Behav.* **174**, 83-88.

Wood, C. M. (1989). The physiological problems of fish in acid waters. In *Acid Toxicity and Aquatic Animals* (ed. R. Morris, E. W. Taylor, D. J. Brown and J. A. Brown), pp. 125-152. Society for Experimental Biology Seminar Series 34. Cambridge: Cambridge University Press.

10.1242/jeb.163451

Journal of Experimental Biology