Responses of larval zebrafish to low pH immersion assay generate multiple interpretations, questions and problems. Comment on Lopez-Luna et al. (2017) Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish.

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Lopez-Luna et al. (2017) observed the behavioral responses of larval (5 days post hatch(dph)) zebrafish (*Danio rerio*) exposed for 10 minutes to low environmental pH (pH 2.6-3.6) when either acetic acid (0.01-0.25%) or citric acid (0.1-5%) was added to tank water in the presence or absence of pharmaceutical compounds, including aspirin (1-2.5 mg/L), morphine sulfate (1-48 mg/L), lidocaine (1-5 mg/L) and flunixin (8-20 mg/L). Fish exposed to 0.1 or 0.25% acetic acid (pH 3.3 or 3.1) were less active than control fish while those exposed to citric acid and 0.01% acetic acid (pH 3.6-2.6) were more active than controls. Administration of the highest doses tested of aspirin, morphine and lidocaine for 30 minutes before exposure prevented the reduction in activity induced by 0.1% or 0.25% acetic acid, but did not change the activity of fish exposed to citric acid or 0.01% acetic acid.

These observations were interpreted as evidence that the acetic acid immersion protocol provided a noxious stimulus (i.e. activated nociceptors). It was also suggested that the behavioral responses elicited in larval zebrafish were reliable enough to be used as a model system for the study of analgesic substances, allowing replacement of adult zebrafish with larvae. We identify methodological weaknesses, inconsistencies in interpretation of results, and emphasize that activation of nociceptors was assumed, not demonstrated. Indeed, due to several physiological processes and interactions that were not accounted for or discussed, we warn their conclusions are unfounded.

A critical omission was failure to report water conductivity, hardness and alkalinity data. These play a pivotal role in determining the magnitude of the acute osmoregulatory effects (particularly ionic and acid-base disturbances) that occur within seconds of exposure of fish to highly acidic water (Wood 1989; Kwong et al. 2014). Trials using water with different conductivity, hardness or alkalinity profiles could, therefore, generate significantly different results. The immersion assay design also introduces several other unavoidable and uncontrolled interactions inherent with exposure of fish to low pH water (see below). These interactions prevent unequivocal interpretation of the behavioral changes observed.

Exposure of fish acclimated to neutral pH to water of pH <4.0 results in near immediate gill dysfunction, iono-regulatory failure (Wood 1989), and pathological lesions of the gill epithelium, including epithelial lifting, necrosis, edema or hypertrophy, as well as chloride cell hyperplasia, all of which are particularly evident in soft water (Daye and Garside 1976; Mallat 1985; Wood 1989). This disruption of the gill epithelium reduces respiratory efficiency which typically initiates compensatory behavioral responses such as surface respiration or “piping” (Kramer 1987). This behavior appears synonymous with the respiratory distress described by Zahangir et al. (2015) and “top dwelling behavior” reported by Currie (2014) in adult zebrafish exposed to 0.03% acetic acid via immersion (pH 3.9-4.0). It is notable that respiratory distress and aquatic surface respiration can occur in a wide variety of natural circumstances in the absence of nociception (Kramer 1987), so even if this behavior

is observed in fish exposed to acidic water, the behavior alone is insufficient evidence that nociception is occurring (Currie 2014).

In contrast to Currie (2014) and Steenbergen and Bardine (2014), the zebrafish larvae immersion model used by Lopez-Luna et al. (2017) considered reduced (not increased) activity as evidence of “alleged pain behavior” in fish exposed to 0.1 and 0.25% acetic acid. Steenbergen and Bardine (2014), interpreted increases in activity and cyclooxygenase-2 (cox-2) gene expression as evidence of nociception in larval zebrafish immersed in 0.0025-0.025% acetic acid. However, cox-2 gene expression is a non-specific marker that is induced by several physiological processes (Wang et al. 2016), meaning its expression is also insufficient evidence that nociception is occurring. The fact that larval zebrafish in the present experiment continued to exhibit increased activity when exposed to pH as low as 2.6 in the 5 mg/L citric acid experiment is a critical observation. Due to the strong likelihood of acute pathological damage to gills, eyes and other tissues at such low pH (Daye and Garside 1976; Mallat 1985), 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 calls into question the construct validity of the assay.

The authors noted that the pH of the three solutions of citric acid used was equal to or lower than that of all three concentrations of acetic acid. However, exposure to

0.25% acetic acid had the opposite effect on behavior (less activity) compared with 0.1% citric acid (more activity), even though the pH values were the same (pH = 3.3). They stated their results could indicate there is "*another mechanism affecting the response of the nociceptors other than the pH*", but the authors did not elucidate what those mechanisms may be.

Due to the immersion design, we contend that those other mechanisms may not have anything to do with nociception. Rather, an alternate and more parsimonious explanation is that the behavioral changes were due to detection by, or interference with, chemosensory cells. These are highly sensitive receptors located within the olfactory epithelium (olfactory receptors) as well as inside and outside the mouth (in the case of gustatory and chemosensory receptors) that are triggered by a wide range of chemical substances (Kasumyan 2001; Hara 2011a, 2011b). The chemosensory systems are active, and chemosensory cells are fully developed and functional in zebrafish before 5 dph (Kotrschal et al. 1997; Hansen et al. 2002; Lindsay and Vogt 2004). Dose-dependent behavioral responses to different chemicals are common in studies of chemoreception, and this would help explain the behavioral differences found between the chemicals used as putative noxious stimuli (citric acid vs acetic acid) and importantly, also the pharmaceuticals used as treatments. Indeed, citric acid was identified as a potent gustatory feeding stimulant in zebrafish that can increase pellet feed consumption by 250% over unscented pellets (Kasumyan and Doving 2003; A.O. Kasumyan unpublished). 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; Hara 2011a), and gustation (Kasumyan 2001; Hara 2011b). Acute exposure to low pH can extinguish (Moore 1994) or change behavioral responses to odors, including attraction to chemicals that previously elicited avoidance responses (Royce-Malmgren and Watson 1987, Munday et al. 2009), which in some cases is due to neurotransmitter interference (Chivers et al. 2014). Because the chemicals being studied in the larval zebrafish activate chemoreceptors and are also responsible for the drop in pH, this interaction makes it very difficult to determine what mechanism(s) were driving variation in fish behavior amongst treatments.

Currie (2014) reported immediate avoidance (bottom seeking) behavior in adult zebrafish exposed to either 0.5-3 mg/L morphine, or 0.03% acetic acid via the water. This behavior is consistent with a chemosensory avoidance response to the presence of both waterborne chemicals. Increased locomotor activity in zebrafish treated with 1 mg/L morphine was also reported by Lopez-Luna et al. (2017), who considered it a "side-effect" of morphine administration. It may be these "side effects" are simply chemosensory avoidance responses to the pharmaceuticals being studied. Lopez-Luna et al. (2017) tried to circumvent these behavioral artefacts by allowing experimental fish a 30 min "acclimation period" after introduction of pharmaceuticals prior to exposure to the acetic or citric acid treatments. It is not clear what effects these pharmaceuticals have on chemoreceptors during the acclimation period. Application of morphine via the water requires large quantities of the drug (Stevens

2008, Rose et al. 2014), and the pathological effects of immersion in high concentrations of drugs such as morphine or aspirin are unknown. Chronic (28 days) exposure to a non-steroidal anti-inflammatory drug (Diclofenac) caused damage to gill epithelia at extremely low concentrations of 1 µg/L (Triebskorn et al. 2007). This suggests that immersion in high concentrations of pharmaceuticals for 30 minutes prior to treatment may have significant unintended effects on chemosensory receptors and gill function, such that subsequent behavioral responses and interactions with other chemicals (e.g. citric and acetic acid) may become unpredictable and/or hopelessly confounded.

Immersion trials therefore appear to provide no advantage over the injection methods previously used, which while having their own problems (see Rose et al. 2014), at least are more likely to target specific tissues and induce nociception, all while being more economical with use of reagents. Importantly, the latter inflicts fewer negative downstream effects on the welfare of wild fishes as the chemicals used enter the waste water stream and, ultimately, the environment as organic contaminants (Triebskorn et al. 2007; Tierney et al. 2010; Brodin et al. 2013).

The strong possibility that the authors measured behavioral changes that were due to factors other than nociception (e.g. differences in chemoreceptor stimulation, suppression or interference; differences between pharmaceuticals in their sublethal damage to gill epithelia; and/or differences between the putative noxious stimuli in their sublethal damage to tissues) cannot be excluded. It is, therefore, premature for

Lopez-Luna et al. (2017) and others (e.g. Steenbergen and Bardine 2014; Curtright et al. 2015) to claim zebrafish larval immersion models have utility for nociception research.

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