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4 Responses of larval zebrafish to low pH immersion assay generate multiple
5 interpretations, questions and problems. Comment on Lopez-Luna et al. (2017)

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1 Lopez-Luna et al. (2017) observed the behavioral responses of larval (5 days post
2 hatch(dph)) zebrafish (*Danio rerio*) exposed for 10 minutes to low environmental pH
3 (pH 2.6-3.6) when either acetic acid (0.01-0.25%) or citric acid (0.1-5%) was added to
4 tank water in the presence or absence of pharmaceutical compounds, including
5 aspirin (1-2.5 mg/L), morphine sulfate (1-48 mg/L), lidocaine (1-5 mg/L) and flunixin
6 (8-20 mg/L). Fish exposed to 0.1 or 0.25% acetic acid (pH 3.3 or 3.1) were less active
7 than control fish while those exposed to citric acid and 0.01% acetic acid (pH 3.6-2.6)
8 were more active than controls. Administration of the highest doses tested of aspirin,
9 morphine and lidocaine for 30 minutes before exposure prevented the reduction in
10 activity induced by 0.1% or 0.25% acetic acid, but did not change the activity of fish
11 exposed to citric acid or 0.01% acetic acid.

12

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

22

1 A critical omission was failure to report water conductivity, hardness and alkalinity
2 data. These play a pivotal role in determining the magnitude of the acute
3 osmoregulatory effects (particularly ionic and acid-base disturbances) that occur
4 within seconds of exposure of fish to highly acidic water (Wood 1989; Kwong et al.
5 2014). Trials by other researchers using water with different conductivity, hardness or
6 alkalinity profiles could, therefore, generate significantly different results. The
7 immersion assay design also introduces several other unavoidable and uncontrolled
8 interactions inherent with exposure of fish to low pH water (see below). These
9 interactions prevent unequivocal interpretation of the behavioral changes observed.

10

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

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

3

4 In contrast to Currie (2014) and Steenbergen and Bardine (2014), the zebrafish
5 larvae immersion model used by Lopez-Luna et al. (2017) considered reduced (not
6 increased) activity as evidence of “alleged pain behavior” in fish exposed to 0.1 and
7 0.25% acetic acid. Steenbergen and Bardine (2014), interpreted increases in activity
8 and cyclooxygenase-2 (cox-2) gene expression as evidence of nociception in larval
9 zebrafish immersed in 0.0025-0.025% acetic acid. However, cox-2 gene expression is
10 a non-specific marker that is induced by several physiological processes (Wang et al.
11 2016), meaning its expression is also insufficient evidence that nociception is
12 occurring. The fact that larval zebrafish in the present experiment continued to exhibit
13 increased activity when exposed to pH as low as 2.6 in the 5% citric acid experiment
14 is a critical observation. Due to the strong likelihood of acute pathological damage to
15 gills, eyes and other tissues at such low pH (Daye and Garside 1976; Mallat 1985),
16 the absence of “alleged pain behavior” in the citric acid treatment calls into question
17 whether nociception was occurring at all. Furthermore, the fact that both increased
18 and decreased activity are being interpreted by different researchers as evidence that
19 nociception is occurring in larval zebrafish exposed to acetic acid calls into question
20 the construct validity of the assay.

21

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

1 0.25% acetic acid had the opposite effect on behavior (less activity) compared with
2 0.1% citric acid (more activity), even though the pH values were the same (pH = 3.3).
3 They stated their results could indicate there is "*another mechanism affecting the*
4 *response of the nociceptors other than the pH*", but the authors did not elucidate what
5 those mechanisms may be.

6

7 Due to the immersion design, we contend that those other mechanisms may not have
8 anything to do with nociception. Rather, an alternate and more parsimonious
9 explanation is that the behavioral changes were due to detection by, or interference
10 with, chemosensory cells. These are highly sensitive receptors located within the
11 olfactory epithelium (olfactory receptors) as well as inside and outside the mouth (in
12 the case of gustatory and chemosensory receptors) that are triggered by a wide
13 range of chemical substances (Kasumyan 2001; Hara 2011a, 2011b). The
14 chemosensory systems are active, and chemosensory cells are fully developed and
15 functional in zebrafish before 5 dph (Kotrschal et al. 1997; Hansen et al. 2002;
16 Lindsay and Vogt 2004). Dose-dependent behavioral responses to different
17 chemicals are common in studies of chemoreception, and this would help explain the
18 behavioral differences found between the chemicals used as putative noxious stimuli
19 (citric acid vs acetic acid) and importantly, also the pharmaceuticals used as
20 treatments. Indeed, citric acid was identified as a potent gustatory feeding stimulant
21 in zebrafish that can increase pellet feed consumption by 250% over unscented
22 pellets (Kasumyan and Doving 2003; A.O. Kasumyan unpublished). Furthermore,
23 acute exposure to pH < 4.0 can cause pathological alteration of the olfactory

1 epithelium (Daye and Garside 1976) and low pH interferes with chemoreceptors
2 responsible for both olfaction (Tierney et al. 2010; Hara 2011a), and gustation
3 (Kasumyan 2001; Hara 2011b). Acute exposure to low pH can extinguish (Moore
4 1994) or change behavioral responses to odors, including attraction to chemicals that
5 previously elicited avoidance responses (Royce-Malmgren and Watson 1987,
6 Munday et al. 2009), which in some cases is due to neurotransmitter interference
7 (Chivers et al. 2014). Because the chemicals being studied in the larval zebrafish
8 activate chemoreceptors and are also responsible for the drop in pH, this interaction
9 makes it very difficult to determine what mechanism(s) were driving variation in fish
10 behavior amongst treatments.

11

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

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

10

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

18

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

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

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