Modeling acute visceral pain in adult zebrafish

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ABSTRACT

This protocol describes a reliable procedure for assessing acute pain responses in adult zebrafish (*Danio rerio*) based on the abdominal writhing-like phenotype following a single intraperitoneal injection of acetic acid. The method is an inexpensive, fast, and easy-to-use protocol for measuring pain-like responses in adult zebrafish. The protocol involves five steps: analyses of baseline behavior, drug injection, post-injection recordings of behavior, euthanasia, and data analyses/interpretation. Intraperitoneal injection of 2.5–5.0% acetic acid elicits a robust pain-like behavior by changing zebrafish body curvature, which can be easily quantified using freely available imaging software. This response is sensitive to pharmacological manipulations, as morphine prevents altered body curvature, while naloxone blocks these effects. Pretreatment with diclofenac sodium (a non-steroidal anti-inflammatory drug commonly used as an analgesic) also prevents writhing-like behavior. Demonstrating high predictive and face validity, this unbiased protocol can be performed over the course of ~2 days, enabling a reliable assessment of acute pain-like responses in zebrafish, thus fostering in-depth analyses of complex pain-related mechanisms and anti-pain drug discovery.

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KEYWORDS

zebrafish, nociception, acute abdominal pain, abnormal body curvature, acetic acid, pharmacological manipulations, opioid system, analgesic properties

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GUIDELINES

Strikingly paralleling the abdominal constriction writhing-like response observed in rodents, the present protocol **(Fig. 1)** is easy-to-use, inexpensive, and does not require complex trials or sophisticated automated tools. Specifically, a single acetic acid administration (2.5–5.0%, i.p.) elicits overt changes in zebrafish body posture that persist for 30 min after injection. This phenotype can be easily analyzed using freely available software (*e.g.*, Image J) for Windows, Mac OS X, and Linux systems, and can also be complemented with automated video-tracking tools (*e.g.*, Any-Maze^{TM}, Stoelting, CO and/or NOLDUS EthoVision XT, Wageningen, Netherlands) to assess other relevant zebrafish behaviors, as locomotion and exploration. Albeit sensitive and unbiased, the protocol has limitations to be considered for further refining and model development. For example, the present protocol is based on a relatively narrow (2.5–5.0%) range of algogenic (acetic acid) response that affects the body curvature. Our pilot experiments using 7.0% of acetic acid unaltered body curvature (vs. 5.0%) but caused a high (~50%) mortality rate. Furthermore, a highly trained researcher is needed to perform an i.p. injection, because if poorly performed, it may induce unwanted side-effects, including such non-specific phenotypes as hyperactivity, corkscrew swimming or and/or the loss of posture (ataxia). The ability of other chemicals (beyond acetic acid) to evoke the observed writhing-like responses also merits further testing. Finally, potential individual, and/or strain differences in nociception merits further scrutiny in zebrafish.

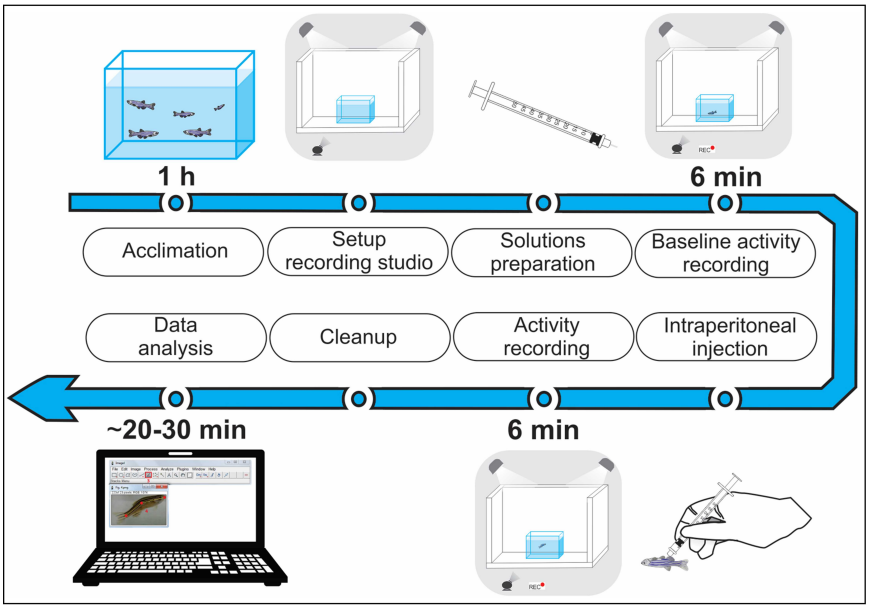

Fig. 1. Schematic diagram showing the experimental procedures to model abdominal constriction writhing-like responses in adult zebrafish and time required for the main steps of the present protocol.

MATERIALS TEXT

Acetic acid glacial **Sigma Aldrich Catalog #ARK2183**

Phosphate Buffered Saline **Thermo Fisher Scientific Catalog #28374**

Sodium chloride **P212121**

Morphine sulfate salt pentahydrate **Sigma Aldrich Catalog #M8777**

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Naloxone methiodide **Sigma Aldrich Catalog #N129**

Diclofenac sodium salt **Sigma Aldrich Catalog #D6899**

Experimental tanks (3.5 L)
Aquarium

Tecniplast **ZB30TK**

3.5 L tank made of blue polycarbonate

Insulin gauge needle (BD Ultra-fineTM II, NJ, USA)
(8 mm x 0.3 mm)

BD **328838**

0.3 mL

Computer
Computer

Dell **v3681w206w**

Desktop or notebook with necessary basic software able to acquire digital video recordings, run appropriate imaging and/or video tracking software, as well as statistical packages (e.g., Excel, GraphPad Prism, SPSS, or similar).

C505e HD BUSINESS WEBCAM
Webcam

Logitech **no number**

High-resolution video cameras (e.g., HD webcams connected to a computer through a USB) or similar.

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SAFETY WARNINGS

Acetic acid is a severe irritant agent. In contact with the skin or eyes, an 80% solution can be corrosive, causing severe burns. Use personal protective equipment (*e.g.*, gloves, lab coat, safety glasses, and respirator) to prevent contamination and limit exposure.

BEFORE STARTING

As subjects, use adult zebrafish (~50% male and female ratio, 4–6 months old). Heterogeneous wild-type (*e.g.*, short-fin), specific (*e.g.*, AB, TU) or genetically modified zebrafish strains can be used. While using the same-batch fish cohorts is desirable, fish from different batches still provide highly consistent data. For testing, animals must be randomly separated from their housing tanks and assigned to specific experimental groups using a computerized random number generator (*e.g.*, www.random.org). Both male and female zebrafish can be used for the experiments since no gender difference in writhing-like responses to acetic acid was observed in the reference paper. Note, however, that if using additional behavioral endpoints and/or treatments, animals from both sexes must be tested separately to avoid false positive or negative findings, and consistent with recent NIH guidelines on the inclusion of both males and females in biomedical experimentation. All animal experimentation should be performed in accordance with the Institutional Animal Care and Use Committee (IACUC) following national guidelines and standards.

ACCLIMATIZATION AND BASELINE BEHAVIORAL RECORDINGS ●**TIMING** ~ 1 h for acclimatization; 6 min per animal 1h 6m

1 ⚠

Transport animals from their holding facility to the experimental room for acclimation 1 h prior to the experiments. Avoid low- or high density of fish to prevent social isolation or crowding stress (tanks must have 1–2 fish per 1 L of water). Fish must acclimate to the facility before testing, and the water used must have similar physicochemical characteristics to those of housing tanks. The use of home tank or holding tank water (with properly adjusted temperature and salinity) is required. **!CAUTION:** Ensure that the experimental tank is filled with non-chlorinated water before use set at optimal temperature (27-28°C).

2 ⚠

Mount a camera on the frontal side of tank test and connect video recorder to the power in a switch-on mode. Provide adequate illumination (fluorescent light bulbs), to ensure that the enlightenment is proper to differentiate the subject from the apparatus, allowing a precise detection of fish. However, avoid excessive brightly lit environments (stressful for zebrafish) and dark areas (poor fish recognition and video-tracking detection of locomotion if desirable).
? TROUBLESHOOTING

? TROUBLESHOOTING

Problems: (1) Inappropriate video lighting.

Possible reasons: (1) Lack or excess of brightness, heterogeneous background.

Solutions: (1) Measure the light intensity above the experimental tank with a lux meter or open Android or IOS application (*e.g.*, Lux meter, Crunchy ByteBox, Germany, or similar) to ensure adequate illumination. Use a styrofoam background to improve contrast.

3 Prepare the acetic acid solutions in 1.5 mL microcentrifuge tubes (2.5% or 5.0% diluted in PBS or 0.9% NaCl).

4 ⚠

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Transfer the fish from the holding tank to the experimental tank and start recording for 6 minutes to measure the baseline behavioral phenotypes. Animals should be transferred individually to the experimental tank. Ensure adequate transport to minimize handling stress. **!CAUTION:** If more than a single injection is applied, the baseline behavior should be recorded after the first injection to avoid false positive/negative results. **?TROUBLESHOOTING**

? TROUBLESHOOTING

Problem: (1) Pretreatment alters the normal zebrafish behavior.

Possible reasons: (1) The drug used affects the swimming activity of fish and/or elicits abnormal phenotypes. Incorrect injection.

Solutions: (1) Run a pilot experiment using different concentrations of the drug. Analyze zebrafish behavior at different time intervals after pretreatment. Use highly-trained researchers to perform the injections.

INTRAPERITONEAL INJECTIONS ●**TIMING** ~ 3 min per animal 3m

5

Before i.p. injections, video recordings must be stopped.

6

Remove the fish from the tank with a fishing net and proceed with the i.p. injection using a BD Ultra-fine™ 30U syringe (needle size 6 x 0.25 mm) with a volume of 10 µL (which does not impair normal zebrafish behaviors). Animals must be gently handled, briefly anesthetized, and later immobilized using a small wet fishing net (< 5 s). The i.p. injections should be quickly performed into the midline between the pelvic fins. To minimize potential interference of drugs in the behavioral endpoints measured and to allow a fast evaluation of the swimming activity, avoid using anesthetics (tricaine or other similar drugs). Injections can be quickly performed through the net in fish previously anesthetized in cold water without affecting complex behaviors (*e.g.*, depth preference, immobility, and locomotion). **?TROUBLESHOOTING**

? TROUBLESHOOTING

Problem: (1) Unconventional responses (*e.g.*, fish do not exhibit curvature, fish die).

Possible reasons: (1) Incorrect injection, incorrect acetic acid concentration.

Solutions: (1) Use highly-trained researchers to perform the injections. Ensure the appropriate acetic acid concentration is used.

FINAL BEHAVIOR RECORDING ●**TIMING** 6 min per animal 6m

7

Relocate the fish into the experimental tank to record the behavioral phenotypes following i.p. injections for 6 minutes.

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CLEANUP ●**TIMING** 1–2 min per animal 2m

8 Stop the behavioral recording and remove the fish from the experimental tank. Animals must be euthanized following national guidelines and standard protocols.

9 Discard the water used previously, wash the tank thoroughly with tap water and refill it with non-chlorinated water before testing another fish.

10 Repeat steps 4–9 for the next subject tested.

VIDEO ANALYSES ●**TIMING** 20–30 min per animal 30m

11

Digital pictures of fish at sagittal plane **(Fig. 2)** must be taken every 30 s, totaling 24 photos per fish. We strongly recommend the use of "Prt Sc" (*print screen*) Windows tool.

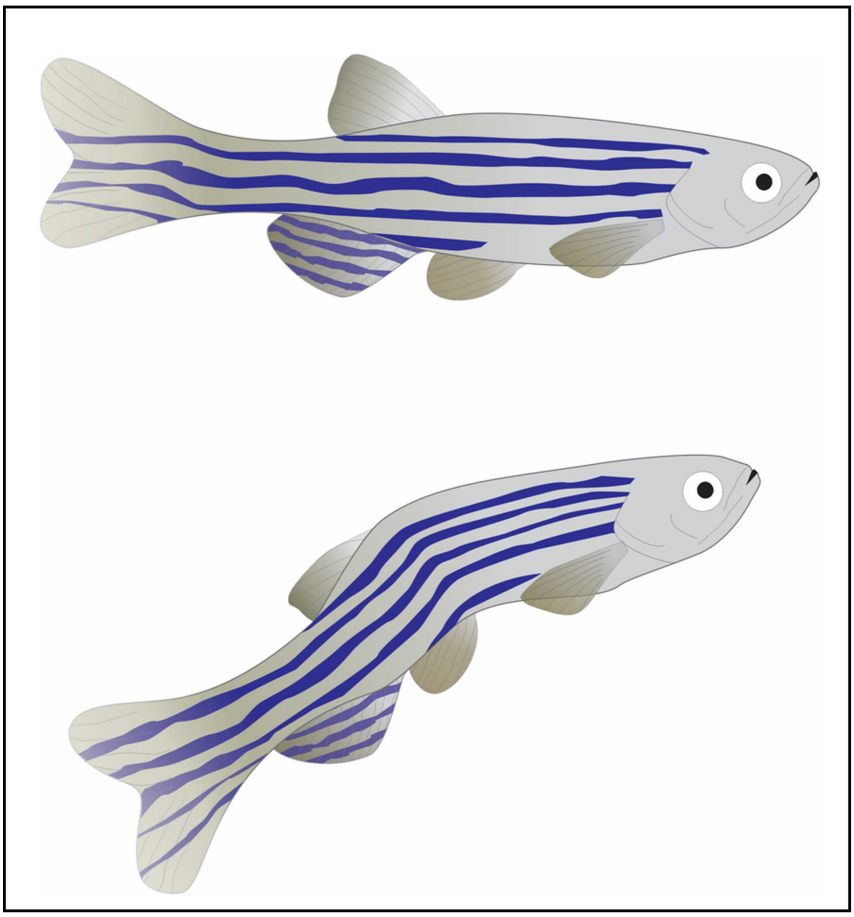

Fig. 2. Representative phenotypes of zebrafish following vehicle (PBS, upper fish) or acetic acid (5.0% AA, lower fish) i.p. administration.

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With ImageJ software (available for download at https://imagej.nih.gov/ij/download.html), open the digital pictures of fish sagittal plane that represent the first 30 s **(1-2)**, and using the angle tool **(3)**, select three positions to estimate the fish body angulation: a frontal (in the front of the head), a central (in the middle of the animal's body – between the anal and dorsal fins), and a posterior one (at the caudal fin) **(4)**. Then, analyze and measure the fish angular value **(5-7)**.
!CAUTION: Results must be subtracted from 180° to calculate a value representing the body curvature index **(Fig. 3)**.
?TROUBLESHOOTING

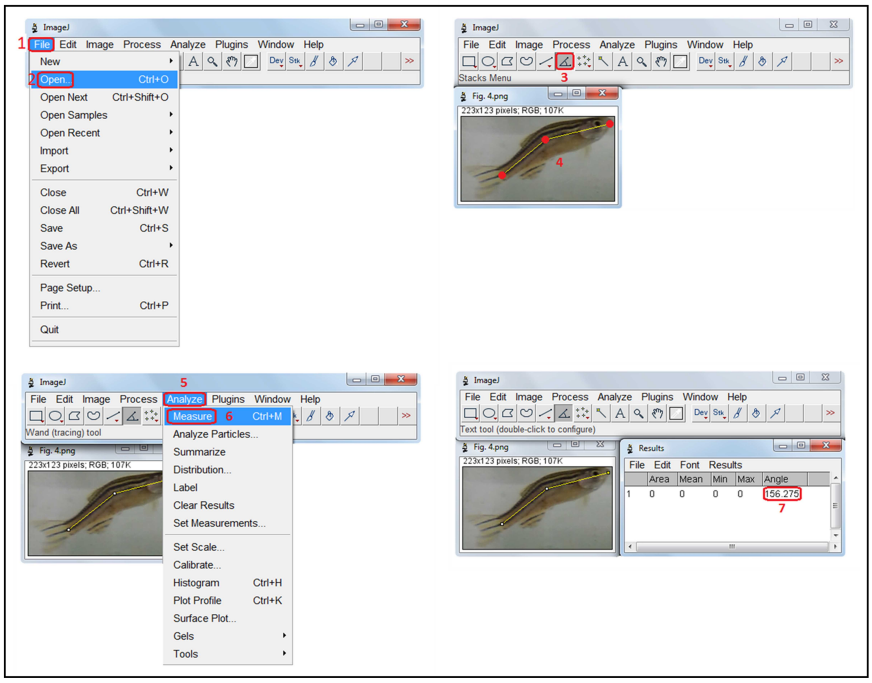

Fig. 3. Main steps to analyze the abdominal writhing-like phenotype using the Image J software. After opening, click on "file" **(1)** and "open" **(2)** to analyze the sagittal pictures taken. Using the angle tool **(3)**, select three points (frontal, central, and posterior) **(4)** to estimate the body angulation. Then, select "analyze" **(5)** and "measure" **(6)** to obtain the angle value **(7)**, which must be subtracted from 180°.

?TROUBLESHOOTING

Problem: (1) Fish do not swim at the sagittal plane. **(2)** The image is not appropriate to measure the body curvature.

Possible reasons: (1) Fish freeze during the entire test period due to increased stress responses; **(2)** The quality of video and/or image acquisition makes the analysis difficult. High-speed swimming in the experimental tank due to increased erratic movements.

Solutions: (1) Ensure adequate manipulation to minimize handling and/or other stressful conditions. Use a narrow experimental tank to restrict the lateral swimming activity. Alternatively, record videos from frontal- and top-view positions to generate 3D traces; **(2)** Ensure a proper manipulation and/or injection to minimize stress. Improve video/image resolution. Use a high-resolution camera to record the behaviors. Select a more appropriate image to quantify the writhing-like response at the respective time interval by using the Prt Sc" (*print screen*) Windows tool (to obtain high definition screenshots).

STATISTICAL ANALYSES ●**TIMING** 20–30 min, depending on amount of data collected 30m

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Several options exist for analyzing the data, based on the specific experimental design. Use the nonparametric Wilcoxon-Mann-Whitney U-test or parametric Student's *t*-test (if data are homoscedastic or normally distributed) for comparing two groups. For more than two groups, use analysis of variance (ANOVA), followed by appropriate post-hoc comparison (*e.g.*, Tukey, Dunn, Student-Newman-Keuls or Dunnet tests). Depending on the study design, an *n*-way ANOVA can generally be applied to assess various factors (*e.g.*, drug/treatment, dose, sex, strain, time, trial, age). Even if a small number of cohorts is used ($n = 5$), robust differences can be detected in acetic acid-treated fish vs. PBS (control group) (*e.g.*, effect size calculated using Cohen's $d = 3.646$). Thus, the use of adult zebrafish to assess pain-like responses following a single i.p. acetic acid injection brings direct "3R's" benefits (refinement, replacement, reduction) of animal experimentation. Use ANOVA with repeated measures to assess time-dependent modulation of the observed phenotypes, if necessary. To facilitate data analysis, export data from every 30 s into separate Excel spreadsheets (one spreadsheet per fish tested in a 6-min trial). The area under curve (AUC) from each individual can be further calculated to express the specific behavioral endpoint using specific statistical packages (*e.g.*, GraphPad Prism).

ANTICIPATED RESULTS

14 The anticipated results described below show robust pain-like responses in zebrafish as reported elsewhere.

Costa FV, Rosa LV, Quadros VA, Santos ARS, Kalueff AV, Rosemberg DB (2019). Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model.. Behavioural brain research. https://doi.org/10.1016/j.bbr.2018.10.009

Intraperitoneal injection of 5.0% acetic acid (AA) induces a typical, well-defined abdominal constriction writhing-like phenotype (measured as body curvature index) in zebrafish, analogous to writhing response observed in rodents. **Fig. 4** illustrates typical results obtained using the present protocol. Notably, these results are robust and replicable even with a small number of subjects per cohort (*e.g.*, $n = 5$), obtained by trained researchers blinded to the experimental condition with inter-rater reliability > 0.85–0.90. Acetic acid-treated fish show a prominent increase in the body curvature index when compared to control (PBS). Interestingly, intraperitoneal injections do not affect the baseline curvature since no differences are observed in PBS-treated group.

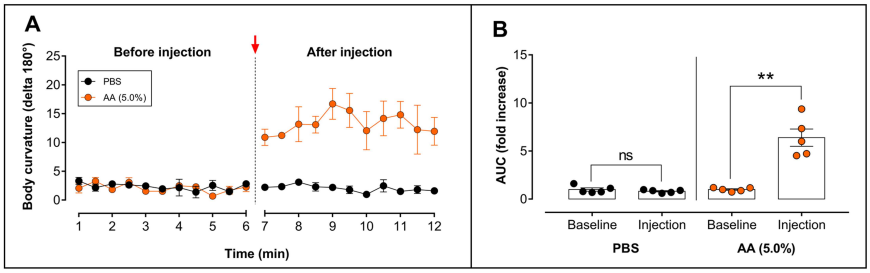

Fig. 4. Intraperitoneal AA injection elicits abdominal constriction writhing-like behavior in adult zebrafish. **(A)** Temporal variations in the body curvature in the vehicle (PBS) and 5.0% AA groups before and after i.p. injection. The red arrow indicates the moment of injection. **(B)** Changes in the area under curve (AUC) following i.p. injections. Results are expressed as means ± S.E.M. and analyzed by paired Student's *t*-test. (**$p < 0.01$; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Our protocol also enables studying the involvement of the opioid system in zebrafish pain-like behaviors, as well as the effects of potential pro- and anti-pain drugs. For example, co-administration of 2.5 mg/kg morphine (MOR), an opioid agonist classically used as an analgesic drug, inhibits the effects of acetic acid on the body curvature index, while a common 'reference' non-specific opioid antagonist naloxone (NAL, 5.0 mg/kg) predictably antagonizes morphine-evoked analgesic responses **(Fig. 5)**.

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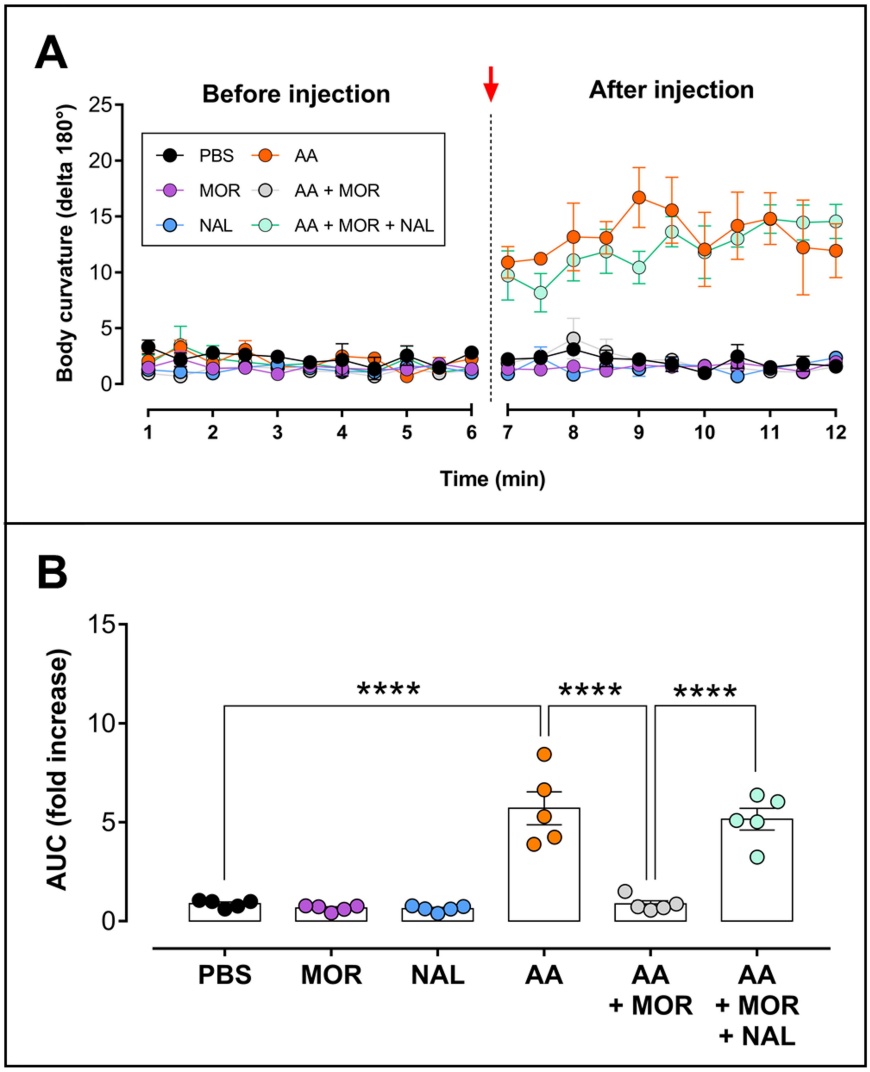

Fig. 5. Influence of opioidergic system in the 5.0% AA-induced abdominal constriction writhing-like responses. **(A)** Changes in the body curvature in vehicle (PBS), AA, 2.5 mg/kg MOR, AA+MOR, 5.0 mg/kg NAL, and AA+MOR+NAL groups across time. The red arrow indicates the moment of injection. **(B)** Area under curve (AUC) values following i.p. injections, expressed as fold increase in relation to PBS group. Results are shown as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Tukey's post-hoc test for significant ANOVA data (**** $p < 0.001$; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Pretreatment with the non-steroidal anti-inflammatory drug, diclofenac (40 mg/kg, for 15 min), another commonly used analgesic agent that does not target the opioidergic system, prevents acetic acid-induced changes in the body curvature index **(Fig. 6)**. These findings suggest that assessing writhing-like responses in the present protocol may be a sensitive and specific approach to evaluate pain-related phenotypes in adult zebrafish.

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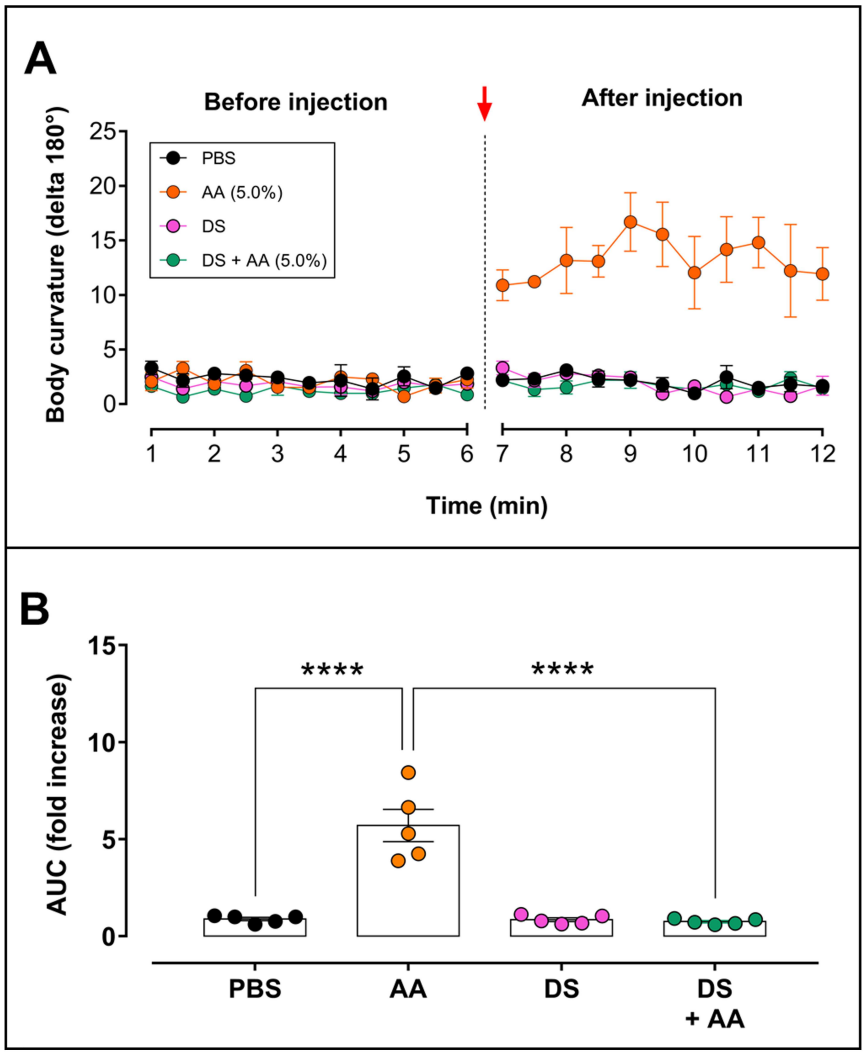

Fig. 6. Diclofenac (DS, 40 mg/kg, i.p.) pretreatment prevents 5.0% acetic acid (AA)-induced abdominal constriction writhing-like behavior. **(A)** Temporal variations in the body curvature index in vehicle (PBS), AA, DS, and DS+AA groups across time. The red arrow indicates the moment of AA or PBS injection. **(B)** Area under curve (AUC) values, expressed as fold increase in relation to PBS group. Baseline recordings were performed 15 min after PBS or DS i.p. injections. Results are expressed as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Tukey's post-hoc test for significant ANOVA data (**** $p < 0.001$; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Overall, these robust behavioral and pharmacological responses show high predictive and face validity of the present zebrafish-based pain model. The protocol can be run using animals selected randomly from their home tanks since no differences in the body curvature index was observed when different genders are tested **(Fig. 7)**. Collectively, these data show that the protocol described here enables measuring specific acute pain responses in adult zebrafish.

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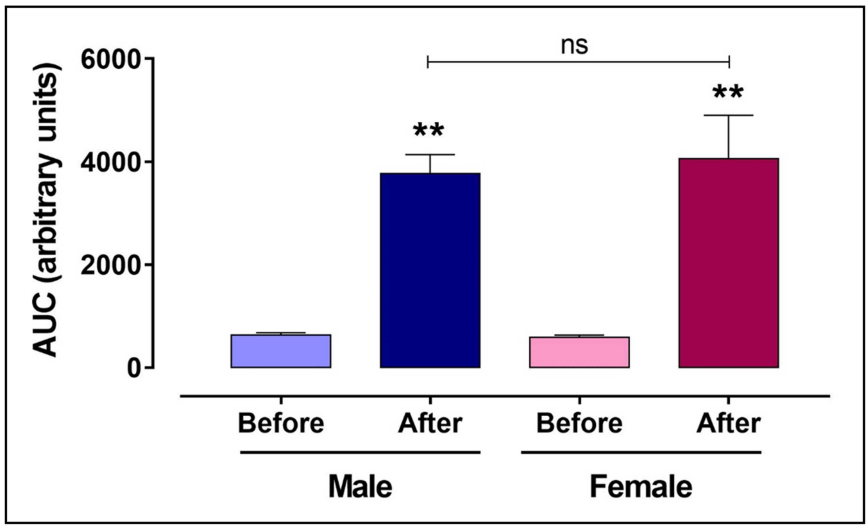

Fig. 9. Male and female show similar effects of 5.0% acetic acid (AA) in the abdominal constriction writhing-like phenotype. Body curvature indexes (before, and after i.p. AA injections) are shown as the area under curve (AUC), expressed as arbitrary units. Data are expressed as means ± S.E.M. and analyzed by repeated measures ANOVA (factors: gender and time), followed by Bonferroni's post-hoc test (** $p < 0.01$ when compared to their respective baseline values; ns = non-significant; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

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