Comparative Biochemistry and Physiology, Part C 270 (2023) 109640

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Acetic acid-induced pain elicits stress-, and camouflage-related responses in zebrafish: Modulatory effects of opioidergic drugs on neurobehavioral phenotypes

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ARTICLE INFO

Edited by Martin Grosell

Keywords:
Pain-like phenotypes
Stress
Body pigmentation
Opioid system
Zebrafish

ABSTRACT

While pain results from the activation of nociceptors following noxious stimuli, mounting evidence links pain- and stress-related responses in mammals. In zebrafish, the activation of hypothalamic-pituitary-interrenal (HPI) axis may also regulate body pigmentation (the camouflage response). Here, we aimed to investigate a putative relationship between pain-, stress-, and camouflage-related parameters in adult zebrafish. To answer this question, we assessed whether intraperitoneal acetic acid injection can activate the HPI axis, measuring whole-body cortisol and the camouflage response as physiological endpoints in the presence or absence of morphine or naloxone, an opioid antagonist. Acetic acid induced a stereotypic circling behavior in the top of the tank, accompanied by abdominal writhing-like response, a specific phenotype that reflects local nociceptive effect. Both whole-body cortisol levels and camouflage response increased in the acetic acid group, while morphine prevented these responses, and naloxone antagonized morphine-induced effects. Moreover, we observed positive correlations between representative behavioral, physiological and skin coloration endpoints, and a "pain index" was proposed to summarize phenotypic profile of zebrafish under different pharmacological manipulations. Collectively, these findings suggest a coordinated activation of pain, camouflage- and stress-related pathways following acetic acid injection in zebrafish. Our data also support that camouflage response represents a novel and relevant biomarker for future probing pain and stress neurobiology, with a robust sensitivity to opioidergic drugs.

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https://doi.org/10.1016/j.cbpc.2023.109640
Received 6 February 2023; Received in revised form 18 April 2023; Accepted 26 April 2023
Available online 29 April 2023

1. Introduction

An unpleasant sensory and emotional experience associated with actual or potential tissue damage, pain is a biological response that serves to alert the body to a possible injury. Pain results from the activation of nociceptors in response to mechanical, thermal or chemical noxious stimuli (Basbaum et al., 2009; Loeser and Treede, 2008), and around 20 % of adult population worldwide experience painful conditions (Goldberg and McGee, 2011). Noxious stimuli trigger the activation of the hypothalamic–pituitary–adrenal (HPA) axis, facilitating corticosteroid (*e.g.*, cortisol) release (Hannibal and Bishop, 2014). Depending on the intensity and duration of stressor, HPA-axis dysfunction, widespread inflammation, and chronic pain may occur, supporting a molecular link between stress and nociception (Hannibal and Bishop, 2014).

In teleost fishes, stress responses are mediated by the activation of the hypothalamic-pituitary-interrenal (HPI, homologous to mammalian HPA) axis and involve a cascade of hormones (*e.g.*, corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and cortisol) (Fuzzen et al., 2010; Ghisleni et al., 2012; Leclercq et al., 2010; Tran et al., 2014). While melanosome dispersal is absent in CRF morphant zebrafish, the activation of the HPI axis modulates the camouflage response - the (skin color change that facilitates numerous behaviors such as foraging, predator avoidance, reproduction, and social communication (Prakash and Toro, 2019; Wagle et al., 2011), also see Nguen et al. (2013). At the molecular level, CRF stimulates the secretion of pituitary hormones, such as ACTH, and β-endorphin (β-END), both encoded by the proopiomelanocortin (*pomc*) gene (Demin et al., 2021; Ghisleni et al., 2012). Two *pomc* genes, *pomca* and *pomcb*, have been described in zebrafish (Sundstrom et al., 2010). While *pomca* encodes ACTH, leading to the stimulation of inter-renal gland (Gonzalez-Nunez et al., 2013; Sundstrom et al., 2010), α-melanocyte-stimulating hormone (α-MSH) is a product of *pomcb* gene, and promotes melanosome dispersal in cultured melanocytes and larval zebrafish (Sheets et al., 2007).

Notably, melanosome distribution in zebrafish is responsive to pharmacological agents, making it a tractable system to explore the neural circuits involved in the camouflage response (Wagle et al., 2011). It is conceivable that both cortisol and opioids (which elicit analgesic properties) modulate neuroendocrine stress responses in mammals (Kreek et al., 2005). However, there are no data showing that acetic acid (AA)-induced pain in zebrafish (Costa et al., 2019a; Costa et al., 2019b) alters cortisol release, and whether the HPI axis is pharmacologically sensitive to opioidergic drugs in zebrafish experiencing pain. Here, we examined a putative association of pain-, stress-, and camouflage-related parameters. Specifically, we investigated whether the intraperitoneal AA injection can activate the HPI axis in adult zebrafish and modulate the camouflage response, measuring whole-body cortisol and body coloration as physiological endpoints. Moreover, the potential influence of the opioidergic system on pain-like behaviors and the HPI axis activation in acetic acid-challenged fish was tested in the presence or absence of morphine (MOR), an opioid receptor agonist, and naloxone (NAL), an opioid receptor antagonist.

2. Methods

2.1. Animals

Subjects were 40 adult zebrafish (*Danio rerio*) of both sexes (4–6 months-old, weighing 0.250–0.300 g) of *short-fin* phenotype obtained from a local commercial distributor (Hobby Aquarios, RS, Brazil). Animals were acclimated in 50-L thermostatic aerated housing tanks (50 × 35 × 30 cm, length x height x width) filled with dechlorinated aerated water for two weeks before the experiments. The water temperature was set at 27 ± 1 °C, pH = 7.0–7.2, conductivity at 1,300–1,500 $\mu S \cdot cm^{-1}$, dissolved oxygen at 6.0 ± 0.1 mg/L, total ammonia at <0.01 mg/L, nitrate (NO_3^-) < 50 mg/L, nitrite (NO_2^-) < 0.1 mg/L, alkalinity and hardness at 75 mg/L $CaCO_3$ as described elsewhere (Avdesh et al., 2012). Illumination was provided by ceiling-mounted fluorescent light tubes on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am and off at 9:00 pm). Fish were fed thrice daily with commercial flake fish food (Alcon BASIC™, Alcon, Brazil) until apparent satiety. All subjects were experimentally naive and maintained in accordance with the US National Institutes of Health Guide for Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee (protocol number 5438310817).

2.2. Pharmacological treatments

Zebrafish (n = 5 per group) were randomly selected from four housing tanks and assigned to each experimental group using a computerized random number generator (*e.g.*, www.random.org). Since the abdominal writhing-like behavior and cortisol levels are biomarkers that show replicable data in zebrafish research using a small number of experimental subjects, the n used was consistent with previous reports in zebrafish pain models (Costa et al., 2019a; Costa et al., 2019b). Importantly, the use of a smaller number of rodents (Gewehr et al., 2013; Trevisan et al., 2013) and zebrafish (Dewberry et al., 2016) in translational pain research fully adheres to the principles of 3Rs in animal experimentation. Because no interaction or sex effect on the endpoints measured were observed (Supplementary Fig. S1), both sexes were mixed in the experiments (3:2 male:female per group). These data corroborate our previous report, in which zebrafish can be randomly assorted for measuring pain-like responses as a ~50:50 male:female ratio (Costa et al., 2019b), also see the Results section further.

Animals were gently handled, anesthetized by cooling (*i.e.*, immersion at 4 °C until the absence of movements and markedly reduced opercular movements) (Kinkel et al., 2010), briefly immobilized using a small wet fishing net, and injected intraperitoneally (i.p.) (*i.e.*, into the midline between the pelvic fins) (Costa et al., 2019a; Costa et al., 2019b) with either phosphate buffer saline (PBS, control group), AA (5.0 %), morphine alone (MOR, 2.5 mg/kg), acetic acid and morphine (AA + MOR), naloxone alone (NAL, 5.0 mg/kg), or acetic acid with morphine and naloxone (AA + MOR + NAL). Injections were performed using a BD Ultra-fine™ 30-U syringe (needle size 6 mm × 0.25 mm) with a volume of 10 μL (which did not impair zebrafish swimming activity) (Kinkel et al., 2010; Richetti et al., 2011). All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Subjects were then placed individually in experimental tanks (15 × 13 × 10 cm, length x height x width; with a 10 cm dechlorinated water column height) to record behavioral activity for 6 min using a digital camera (Nikon Coolpix P900, Tokyo, Japan). Importantly, chemical and physical conditions of water in the experimental tanks were similar to those in maintenance tanks. Behavioral recordings started as soon as the fish regains equilibrium (±45 s post-injection), allowing a more precise observation of acute responses (Kinkel et al., 2010). All doses were selected based on previous reports using adult zebrafish (Costa et al., 2019a; Costa et al., 2019b).

2.3. Pain-like behaviors

To confirm the induction of pain-related responses, relevant behavioral phenotypes were chosen based on their specificity in the zebrafish model reported here (Costa et al., 2019a; Costa et al., 2019b). The abdominal writhing-like behavior (Costa et al., 2019b) was measured using digital pictures of fish sagittal plane, taken every 30 s, totaling 24 photos per fish. Then, the body posture of fish was measured with ImageJ 1.52 software for Windows. Briefly, three points of fish body were selected as a frontal (the front of the head), central (middle of the body between anal and dorsal fins), and a posterior (the caudal fin) points. Results were subtracted from 180° to estimate a value representing the body curvature (Costa et al., 2019b). We also quantified the time spent in the top and the number of circling behaviors, which

represents a stereotypic swimming pattern, using automated video-tracking software (Any-Maze™, Stoelting, CO, USA) at 30 frames/s.

2.4. Measurement of camouflage response

Following behavioral recordings, the camouflage response was analyzed based on a protocol described previouslye (Fontana et al., 2016). Fish were briefly anesthetized in cold water (~4 °C, until loss of equilibrium) and immediately placed on a white paper that served as a clear background. Using a Digital Microscope (USB; 500× digital zoom) positioned 2 cm above, pictures of fish sagittal planes using a 50× magnification were taken and later analyzed using the Image J 1.52 software. The camouflage response was expressed by measuring the body pigmentation as saturation score (SS) and calculated as follows:

$$SS = \frac{1}{MGV} \times 100.$$

where *MGV* represents the mean gray value of the selected region, ranging from 0 (black) to 255 (white).

Two trained observers blinded to the experimental conditions selected the fish images used as representative examples for illustrations. The inter-observer reliability was estimated at 90 % as follows:

$$IRR = \frac{TA}{TR} \times 100$$

where *IRR* is inter-rater reliability (%); *TA* is the total number of agreements; *TR* is the total number of ratings given by each rater.

2.5. Whole-body cortisol measurements

Immediately after assessing the camouflage response, the anesthetized fish were euthanized by decapitation and sex was confirmed by gonadal dissection. Animals were then frozen in liquid nitrogen for 20–30 s for cortisol extraction. Whole-body cortisol was extracted following the ether-based extraction protocol (Mezzomo et al., 2019). Cortisol quantification was performed in duplicates using a commercially enzyme-linked immunosorbent assay kit (EIAgen™ Cortisol test, BioChem ImmunoSystems) (Sink et al., 2008). A strong positive correlation between the curve was observed (r^2 = 0.9413), and samples yielded low inter- and intra-assay CV values (7–10 % and 5–9 %, respectively). Results were expressed as ng cortisol/g tissue.

2.6. Statistics

Normality and homoscedasticity were analyzed by the Kolmogorov-Smirnov and Bartlett test, respectively. Data were analyzed by one-way analysis of variance (ANOVA) and differences among groups were further assessed by the Student-Newman-Keuls *post hoc* test, whenever appropriate. The association between pain-, stress-, and camouflage-related parameters were assessed using Pearson's correlations and an index of pain response was estimated as follows:

$$f(x) = 0.5 \times log_{10}\left(\sum all\ endpoints\ measured\right).$$

This equation was used aiming to combine relevant endpoints measured normalizing the baseline index close to 1. Results were expressed as means ± standard error of the mean (S.E.M.). In order to verify whether the effects of AA occur in a sex-dependent manner, a two-way ANOVA was performed using treatment and sex as factors. The level of significance was set at $p \leq 0.05$ and all data were analyzed by researchers blinded to the experimental condition of the fish.

3. Results

Fig. 1 shows the effects of AA on whole-body cortisol levels in the presence and absence of opioidergic drugs. As a nociceptive agent, AA increased whole-body cortisol compared to PBS group. MOR co-administration prevented AA-induced response, while NAL antagonized the effects of MOR on cortisol levels ($F_{(5, 24)} = 9.304$, $p < 0.0001$). Regarding the camouflage response (Fig. 2), similar effects were verified when the pigment saturation score was measured after AA injection and opioid drugs coadministration ($F_{(5, 24)} = 10.8$, $p < 0.0001$) (Fig. 2A). In fact, AA and AA + MOR + NAL were visibly darker than the other groups as shown by the representative images of fish body coloration after PBS (Fig. 2B), AA (Fig. 2C), MOR (Fig. 2D), AA + MOR (Fig. 2E), NAL (Fig. 2F), and AA + MOR + NAL (Fig. 2G) injections.

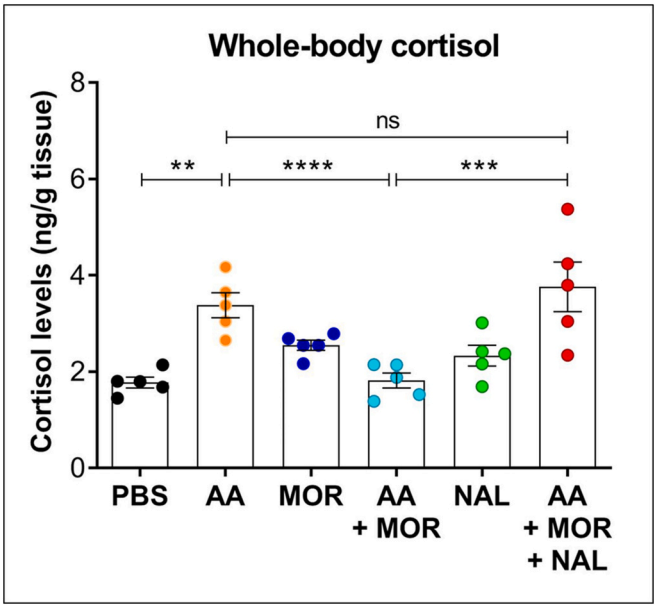

Fig. 1. Effects of 5 % acetic acid (AA) on whole body cortisol levels in the presence or absence of opioidergic drugs. Results were expressed as means ± standard error of the mean (S.E.M.) and analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test (**$p <$ 0.01; ***$p < 0.005$; ****$p < 0.0001$; ns: non-significant). PBS - phosphate buffer saline (control group), MOR - morphine (2.5 mg/kg), NAL - naloxone (5.0 mg/kg).

To confirm the induction of pain-like responses in zebrafish, the effects of AA in the absence or presence of opioidergic drugs were also evaluated in the experimental subjects (Fig. 3). As expected, AA elicited a writhing-like response by increasing the abdominal curvature (~5 fold) compared to PBS. Moreover, fish injected with AA showed a stereotypic circling behavior and spent more time spent in the top. While MOR co-administration prevented these behaviors, NAL antagonized analgesic effects of MOR ($F_{(5, 24)} = 33.83$, $p < 0.001$; $F_{(5, 24)} = 72.42$, $p <$ 0.0001; $F_{(5, 24)} = 5.731$, $p = 0.0013$, for writing-like response, circling behavior, and time spent in the top, respectively).

To link key phenotypes measured, correlations between all endpoints were analyzed here (Fig. 4), revealing strong positive correlations for the abdominal writhing-like behavior with body pigmentation score (r = 0.8792, $p < 0.0001$), whole-body cortisol levels (r = 0.7381, $p <$ 0.0001), and circling behavior (r = 0.9131, $p < 0.0001$). Significant correlations were also confirmed for body pigmentation score with circling behavior (r = 0.8109, $p < 0.0001$) and whole-body cortisol levels (r = 0.7511, $p < 0.0001$). Moderate positive correlations were found for the abdominal writhing-like behavior and time spent in top area ($r = 0.6347$, $p = 0.0002$), as well as for circling behavior and whole-body cortisol levels ($r = 0.6357$, $p = 0.0002$) and time spent in the top (r = 0.6040, $p = 0.0004$). Similarly, time spent in the top correlated with body pigmentation score (r = 0.6450, p = 0.0001) and whole-body cortisol levels ($r = 0.6810$, $p < 0.0001$). The estimative of an index for each experimental condition aiming to express pain (Fig. 5) showed

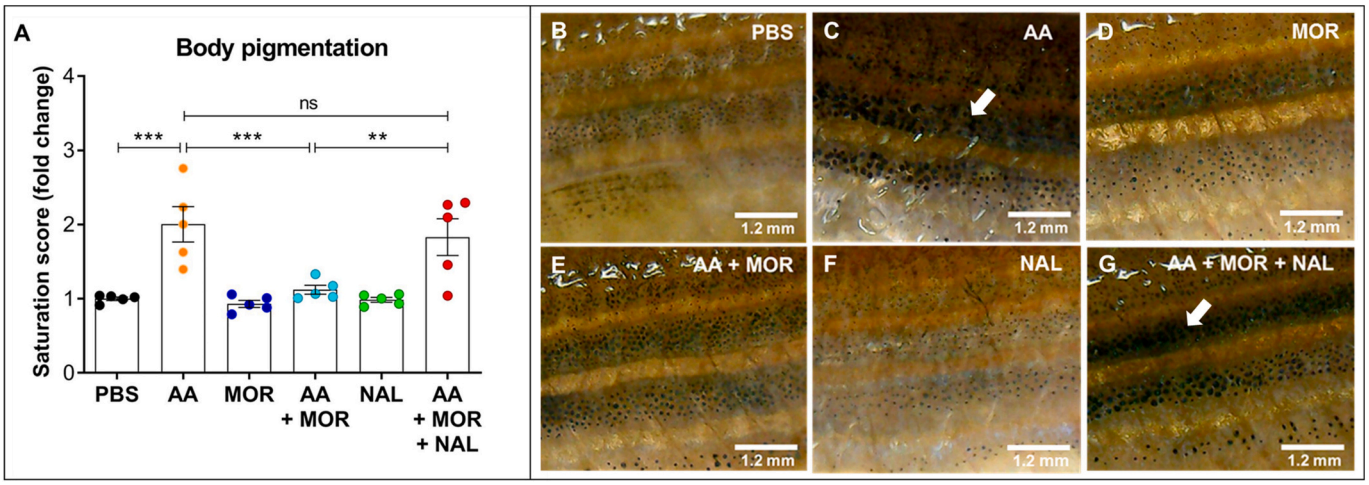

Fig. 2. Modulatory effects of acetic acid (AA), morphine (MOR), and naloxone (NAL) on camouflage response (groups are as in Fig. 1). (A) Quantification of the body coloration as saturation score, with representative images showing the effects of each experimental group on body pigmentation in adult zebrafish using a 50× magnification for (B) PBS, (C) AA, (D) MOR, (E) AA + MOR, (F) NAL, and (G) AA + MOR + NAL. White arrows indicate increased camouflage response represented by the darker body pigmentation pattern. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test (**$p < 0.01$; ***$p < 0.005$; ns: non-significant).

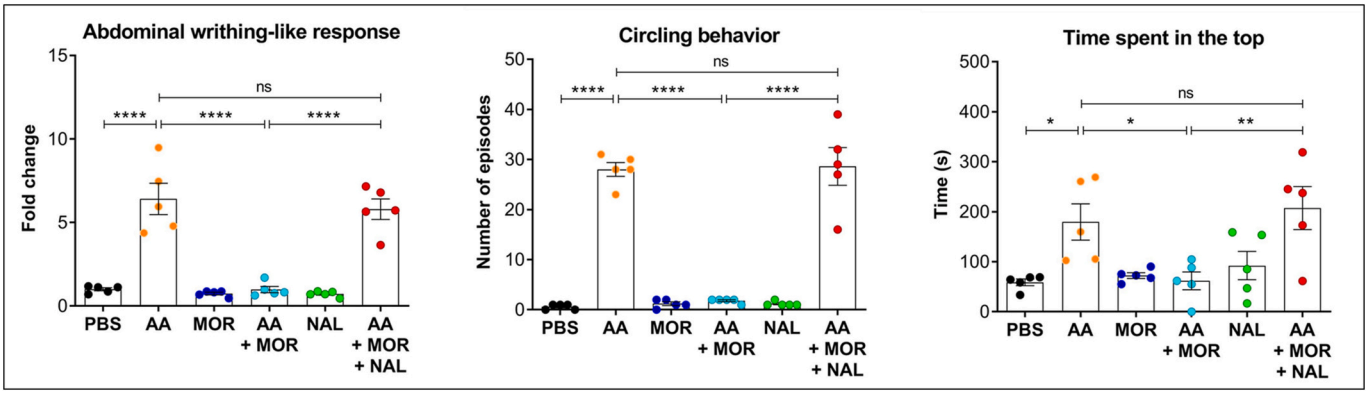

Fig. 3. Main representative pain-like behaviors of adult zebrafish following the intraperitoneal acetic acid (AA) injection in the presence or absence of opioidergic drugs. Results were expressed as means ± standard error of the mean (S.E.M.) and analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; ns: non-significant). Groups included phosphate buffer saline (PBS, control group), AA (5.0 %), morphine alone (MOR, 2.5 mg/kg), AA + MOR, naloxone alone (NAL, 5.0 mg/kg), or AA + MOR + NAL).

consistent data, as AA increased pain index compared to the PBS group, and opioidergic drugs elicit a modulatory effect on such response ($F_{(5, 24)} = 4.956$, $p = 0.0029$). Moreover, the effects of AA on stress-, camouflage, and pain-related behaviors did not differ between male and female zebrafish (Supplementary Fig. S1).

4. Discussion

Our findings show that AA administration modulates both stress- and camouflage-related responses in zebrafish. Consistent with the effects observed in pain-related behaviors, AA increased whole-body cortisol levels and body pigmentation, while MOR prevented these responses. Because NAL antagonized the effects of MOR, an involvement of opioid system in modulating both stress and camouflage can be suggested. Notably, significant correlations between different endpoints measured here support a direct relationship between stress-, pain-, and camouflage-related pathways in zebrafish. For the first time, we also proposed an integration of different behavioral, physiological and skin coloration endpoints as a pain measurement in zebrafish, and the calculated indexes reflected the experimental conditions assessed.

Studies involving nociception in teleost fishes are important to characterize the functionality of distinct classes of nociceptors across taxa and species (Sneddon, 2004). Mounting evidence suggests that zebrafish express necessary cellular components to recognize nociceptive agents, and display abnormal behaviors in responses to algogens (Malafoglia et al., 2013; Reilly et al., 2008; Maximino, 2011). Since pain perception in animal models is subjective, the recognition of specific phenotypes indicative of "unpleasant sensation" or "discomfort" in fish species constitutes a key aspect to clarify how noxious stimuli influence animal physiology and behavior (Treede, 2006; Vierck et al., 2005). Here, we present behavioral, physiological and skin coloration indices that can be used to assess abdominal pain in zebrafish.

In humans, painful stimuli facilitate the release of CRF from hypothalamic neurons to the anterior pituitary, thereby facilitating the secretion of ACTH and β-END (Ehlert et al., 2001; Jankord and Herman, 2008). Increased levels of plasma ACTH led to the activation of adrenal Gs-coupled melanocortin 2 receptors (MC2R), resulting in cortisol synthesis (Hannibal and Bishop, 2014; Karaca et al., 2021). Although noxious agents facilitate corticosteroid secretion in rodents (Hannibal and Bishop, 2014), there are no data showing whether cortisol levels can be altered in zebrafish tested in an acute visceral pain model. Homologous to the mammalian HPA, the zebrafish HPI axis acts in a similar

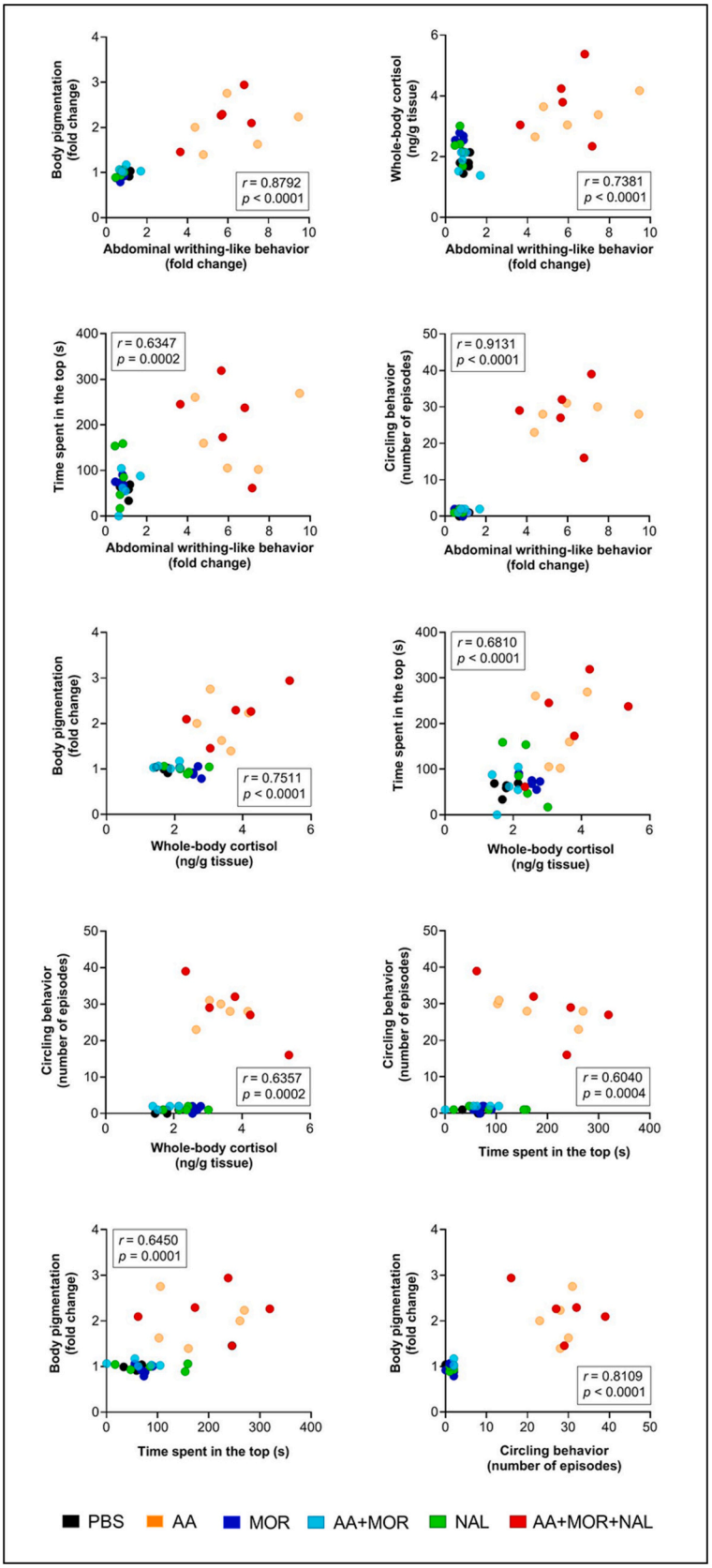

Fig. 4. Correlations between behavioral, physiological and skin coloration endpoints measured under different treatments, including phosphate buffer saline (PBS, control group), acetic acid (AA, 5.0 %), morphine alone (MOR, 2.5 mg/kg), AA + MOR, naloxone alone (NAL, 5.0 mg/kg), or AA + MOR + NAL. Colors denote subjects tested in specific experimental groups: Black – PBS; Orange – AA; Dark blue – MOR; Light blue – AA + MOR; Green – NAL; Red – AA + MOR + NAL. Note that groups with similar responses were clustered in the graphs. Data were analyzed using Pearson's correlation. Pearson's correlation coefficient (r) and p values are shown.

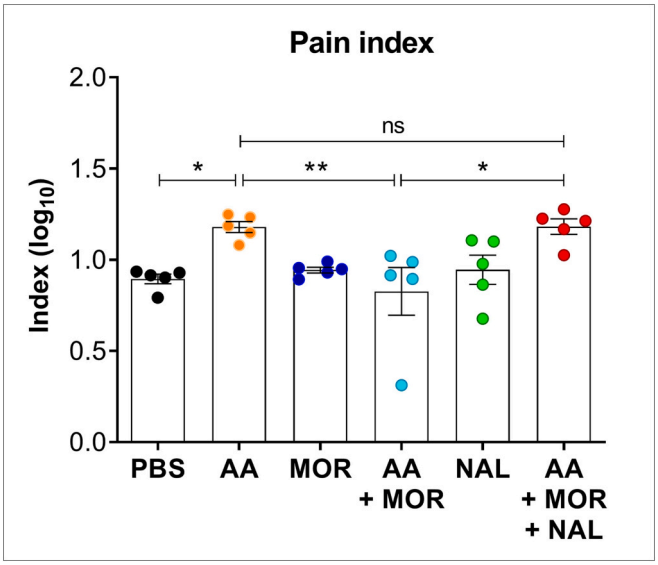

Fig. 5. Estimation of a pain index calculated as $f(x) = 0.5 \times log_{10}(\sum all\ endpoints\ measured)$. Results were expressed as means ± standard error of the mean (S.E.M.) and analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test (*$p < 0.05$; **$p < 0.01$; ns: non-significant). Groups included phosphate buffer saline (PBS, control group), acetic acid (AA, 5.0 %), morphine alone (MOR, 2.5 mg/kg), AA + MOR, naloxone alone (NAL, 5.0 mg/kg), and AA + MOR + NAL.

manner, facilitating stress-related responses. However, in contrast to mammals, zebrafish express two *pomc* genes (*pomca* and *pomcb*). While *pomca* codes for ACTH, γ-lipotropin (γ-LPH), β-melanotropin (β-MSH), and β-END, *pomcb* only codes for α-MSH and β-END (Gonzalez-Nunez et al., 2003).

Similar to other vertebrates, ACTH binds to Gs-coupled MC2R which is expressed on interrenal cells, stimulating cortisol biosynthesis and secretion in fish (Agulleiro et al., 2013; Wendelaar Bonga, 1997). In line with this, a single i.p. injection of AA increases whole-body cortisol level in all zebrafish tested (Fig. 1). Furthermore, the concomitant manifestation of behavioral phenotypes indicative of local nociception and the pharmacological sensitivity to opioidergic modulation (Fig. 3), supporting the link between stress- and pain-related mechanisms in zebrafish. Indeed, our data are in line with previous findings, given an inhibitory effect of endogenous opioids on the HPA axis *via* both μ and κ opioid receptors in humans, corroborating the influence of the opioidergic system on stress-related pathways (Kreek et al., 2005). Thus, future studies of *pomca* and *pomcb* expression and/or their manipulation during pain are needed to further elucidate the precise mechanisms of stress and camouflage responses during painful stimulation.

The assessment of innate behaviors represents an excellent tool to unravel the neural circuit assembly and function, as they are often robust and "hardwired" (Wagle et al., 2011). Camouflage response is observed in many vertebrate species, including zebrafish (Wagle et al., 2011) and involves neural crest-derived pigment cells (Fujii, 2000; Nascimento et al., 2003), which undergo aggregation or dispersal in the presence of lit or dark conditions, respectively (Singh and Nusslein-Volhard, 2015). Notably, camouflage response also shows a pharmacological sensitivity to ethanol (Singh and Nusslein-Volhard, 2015; Wagle et al., 2011) and various other drugs (Nguen et al., 2013), implying that drugs able to elicit significant effects on brain function and affecting various neurobehavioral responses would likely modulate melanosome aggregation or dispersal.

Although several neurotransmitter and neuropeptide systems may regulate camouflage *in vitro* (Dulcis and Spitzer, 2008; Fujii, 2000; Kolk et al., 2002), a key role of HPI axis activation in zebrafish body pigmentation has been also proposed (Wagle et al., 2011). In fact, proteolytic cleavage of *pomcb* product increases α-MSH levels (Wagle et al., 2011), which regulate the production of pigment in melanocytes through melanocortin 1 receptor (MC1R) (Logan et al., 2003). In both dorsal and ventral skin, MC1R plays a critical role in the control of melanin synthesis and the melanosome dispersal in zebrafish (Cortes et al., 2014), supporting a cross-talk between stress and camouflage behavior. Furthermore, since opioids can negatively modulate human HPA axis (Kreek et al., 2005), a role of opioid receptors in regulating zebrafish HPI axis (hence affecting camouflage-related endpoints) seems indeed likely. However, the reason why animals become darker when experiencing pain is unclear. Increased camouflage may constitute an adaptive strategy in zebrafish under aversive situations, reflecting a generalized stress response to increase protection during the vulnerable condition. Although pain is inherently stressful and increased cortisol levels are to be expected, our data revealed simultaneous darkening in all tested fish, which may constitute an easy-to-use and valuable tool in further studies of pain and stress in fish.

Here, we show that AA-induced pain increases camouflage response. Although MOR and NAL alone did not change body pigmentation, MOR prevented the increased saturation score of AA, whereas NAL antagonized this effect (Fig. 2). Paralleling the abdominal writhing-like phenotype, we report for the first time aberrant behavior after AA injection, described here as a "circling activity in the top area" (Fig. 3). Although the biological significance of this stereotyped behavior is still unclear, it may explain the markedly increased time spent in top area and fewer vertical transitions following AA injection in adult zebrafish (Costa et al., 2019b). Because MOR co-administration abolished such effects, these endpoints may also reflect a pain-like behavior with high predictive validity. Moreover, positive correlations were found for circling behavior with abdominal writhing-like behavior, whole-body cortisol, and body pigmentation (Fig. 4), corroborating the main effects observed for each experimental condition described here. Although the precise molecular mechanisms underlying skin coloration response merit further scrutiny, our findings support a clear relationship between HPI axis activation, body pigmentation, and pain in zebrafish.

In conclusion, we report that stress and camouflage response show a clear association in zebrafish experiencing pain. By integrating behavioral, physiological and skin coloration endpoints reflecting pain, we estimated index values that corroborate the main effects verified in the absence or presence of opioidergic drugs (Fig. 5). These findings help further explore how different stressors, both in terms of duration and intensity, influence pain responses in this aquatic species. Moreover, analyzing other behavioral endpoints (*e.g.*, freezing, erratic movements), individual variations of pain response in a same or different zebrafish population may be useful in pain research, especially for screening novel pain medicines using both adult and larval zebrafish. Camouflage response, highly sensitive to opioidergic agents, may serve as useful objective and easily tractable phenotypic tool to probe molecular machinery of nociception in translational pain- and stress-related research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2023.109640.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors recognize the financial support and fellowships from

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Finance code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). D.B.R. is recipient of the CNPq Research Productivity Grant (Process no. 307690/2021-0) and his work is also supported by FAPERGS Programa Pesquisador Gaúcho – PQG Fellowship Grant (Process no. 19/2551-0001764-2) and PROEX/CAPES Fellowship Grant (Process no. 23038.002125/2021-85; Grant no. 0036/2021). This study is part of the National Institute of Science and Technology in 3D printing and Advanced Materials Applied to Human and Veterinary Health - INCT 3D-Saúde, funded by CNPq, Brazil (Grant no. 406436/2022-3). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the paper. A.V. K. is supported by St. Petersburg State University budgetary funds (Pure ID: 93020614). The laboratory partially used the facilities and equipment of the Resource Fund of Applied Genetics MIPT (support grant 075-15-2021-684).

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