Comparative Biochemistry and Physiology, Part A 318 (2026) 112030

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Short Communication

Short communication: Skin melatonin and cortisol responses to water acidification and basification within the optimal pH range in three-spined sticklebacks

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ARTICLE INFO

Editor: Michael Hedrick

Keywords:
Fish
Skin
pH shifts
Water acidification
Water basification
Carbonate system
Melatonin
Cortisol

ABSTRACT

Fish skin functions not only as a passive protective barrier but also as an active site of key physiological processes, including a local stress response system. In fish, this system involves the hormones cortisol and melatonin (Mel), which contribute to counteracting environmental stressors and maintaining homeostasis. In this study, we examined the sensitivity of both components of the cutaneous stress response system (CSRS) in three-spined sticklebacks (*Gasterosteus aculeatus*) exposed to acidic ($pH = 6.54$) and basic ($pH = 8.70$) water conditions, representing the boundary values of the species' optimal pH range, under either rapid or gradual pH change regimes. Water pH in the aquaria was precisely controlled using a custom-designed gas-exchange system regulating dissolved CO_2 levels. Mel concentrations were measured in the skin, brain and eyeball, while cortisol was determined in the skin. Samples were collected during the day. Skin Mel levels were significantly higher under acidification than under basification ($P = 0.036$; rapid change regime), whereas cortisol remained stable across all conditions. Ocular Mel levels were not affected by treatments. Brain Mel concentrations were generally very low but tended to be slightly higher under basification than under acidification in both regimes ($P = 0.05$, borderline significance). The marked differences in skin Mel levels between acidic and basic pH water conditions, accompanied by stable cortisol concentrations, indicates that cutaneous Mel, but not cortisol, is highly sensitive to subtle water pH fluctuations even within the species' optimal range.

Fish inhabit nearly the full spectrum of aquatic pH, from 3–4 in Amazonian blackwaters to 10–11 in soda lakes (Randall et al., 1989; Wilson et al., 1999), though species richness peaks in mildly acidic to neutral waters. The acid-base properties of natural waters are governed by the carbonate system, defined by four interrelated parameters: pH, partial pressure of CO_2 (pCO_2), total alkalinity (TA), and dissolved inorganic carbon (DIC). Any change in one of these parameters leads to a redistribution of carbonate species altering the entire system. In natural environments, variability in dissolved CO_2 is primarily driven by biological processes, such as primary production (CO_2 uptake) and organic matter mineralization (CO_2 release), which induce fluctuations in acid-base conditions. These processes are further amplified by climate change, as rising atmospheric CO_2 levels and altered biogeochemical cycles affect the carbonate system and, consequently, water pH. Life in aquatic environments imposes unique acid-base challenges absent in terrestrial habitats, demanding dynamic physiological adjustments by organisms (Tresguerres et al., 2020). Fish adapt to low and high pH primarily by modifying ion and acid-base regulation, adjusting nitrogen excretion, and developing structural and molecular specializations that reflect local environmental conditions. In adult teleosts, the gills are the main site of ion and acid-base regulation, supported by the kidney and gut, and are the main locus for compensating both metabolic and environmental acid-base disturbances (Gilmour and Perry, 2009). Gill ionocytes use Na^+/K^+ATPase, apical H^+-ATPase, Na^+/H^+ exchangers, and Cl^-/HCO_3^- exchangers, whose expression and activity are modulated by environmental pH to manage H^+ excretion and HCO_3^- handling during acidosis or alkalosis (Claiborne et al., 2002; Gilmour and Perry, 2009; Pritchard, 2003). Carbonic anhydrase within gill cells speeds CO_2 hydration, providing H^+ and HCO_3^- to support coupled Na^+ and Cl^- transport, thereby linking ion uptake with acid-base regulation (Gilmour and Perry, 2009). In naturally acidic and basic systems, factors such as dissolved organic carbon, ionic composition, and other chemical characteristics modulate the gill ion transport, thereby mitigating or exacerbating pH-related ionoregulatory challenges (Morris et al., 2021).

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https://doi.org/10.1016/j.cbpa.2026.112030
Received 10 April 2026; Received in revised form 22 May 2026; Accepted 31 May 2026

Beyond the gills, the skin also functions as an active transport epithelium in fish (Glover et al., 2013). Although the skin primary acts as a passive biological barrier, protecting the organism from harmful environmental factors, it also maintains concentration gradients of ions, gases, nutrients, and metabolites between the body and the external environment. Thus, fish skin fulfills essential physiological roles, including gas exchange, ion transport, nitrogenous waste excretion, and nutrient acquisition (Glover et al., 2013). This is especially vital in larvae with immature gills (Glover et al., 2013). In adult fish, the skin also acts as an active stress-response organ, constituting a cutaneous stress response system (CSRS), partly similar to that described in mammals. Research by Slominski and colleagues has shown that mammalian skin is an active neuroendocrine organ (for review see Slominski et al., 2025) capable of synthesizing key components of the hypothalamic-pituitary-adrenal (HPA) axis, including corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol, and also produces melatonin (Mel) and its biologically active metabolites (Slominski and Wortsman, 2000; Slominski et al., 2008, 2013, 2018; Acuña-Castroviejo et al., 2014). In mammalian skin, the presence of an autonomous neuroendocrine system integrating HPA-axis elements, immune signaling, and local steroidogenesis is well established. Notably, de novo corticosteroid synthesis (cortisol, corticosterone) occurs in epidermal and dermal compartments and is dynamically regulated by inflammatory mediators, environmental stressors, and cytokines (see review Slominski et al., 2021). Our studies have demonstrated the presence of a CSRS in fish (Gozdowska et al., 2022; Kulczykowska et al., 2018; Pomianowski et al., 2023), in which both cortisol and Mel play functional roles (Gozdowska et al., 2022; Kulczykowska et al., 2018; Pomianowski et al., 2020, 2023). Local Mel synthesis in fish skin has been shown (Gozdowska et al., 2022; Kulczykowska et al., 2017; Pomianowski et al., 2023). However, in contrast to mammals (Slominski and Wortsman, 2000; Vukelic et al., 2011), there is currently no evidence for local cortisol synthesis in fish skin. Recent work from our group demonstrates that the fish CSRS responds to oxidative stress (e.g., exposure of fish to a potassium dichromate, $K_2Cr_2O_7$) (Gozdowska et al., 2022; Pomianowski et al., 2023), however to the best of our knowledge, no studies have yet examined how the fish CSRS responds to fluctuations in ambient water pH.

In the present study, we assessed the sensitivity of two CSRS components, cortisol and Mel, in three-spined sticklebacks (*Gasterosteus aculeatus*) exposed to boundary values of the species' optimal pH range under rapid or gradual change regimes. Fish were exposed to acidic (pH = 6.54) or basic (pH = 8.70) water conditions, starting from the control pH of 7.83, to simulate abrupt versus progressive environmental fluctuations (Fig. 1). This species inhabits cool northern environments and is naturally exposed to a broad range of pH and salinity conditions. The stickleback is also widely recognized as an indicator organism for assessing the biological effects of ambient water chemistry, including oceanic acidification (Glippa et al., 2017; Soor et al., 2024). It has also been a model species in our laboratory for many years.

We mimicked the environmentally relevant dynamics by directly manipulating dissolved CO_2 concentrations through controlled addition or stripping of CO_2 from the water, while keeping total alkalinity (TA) constant and varying pCO_2, thereby isolating the effect of CO_2-driven shifts in the carbonate system. This setup simulates conditions associated with primary production and respiration, as well as broader climate-driven changes in carbon cycling. The ranges of pH and CO_2 variability applied in our experiments correspond to values currently observed or projected for aquatic environments under ongoing climate change, including ocean acidification scenarios and regional conditions in coastal systems such as the Baltic Sea (Jansson et al., 2013; Pajusalu et al., 2024). Physiological impacts of elevated CO_2 and ocean

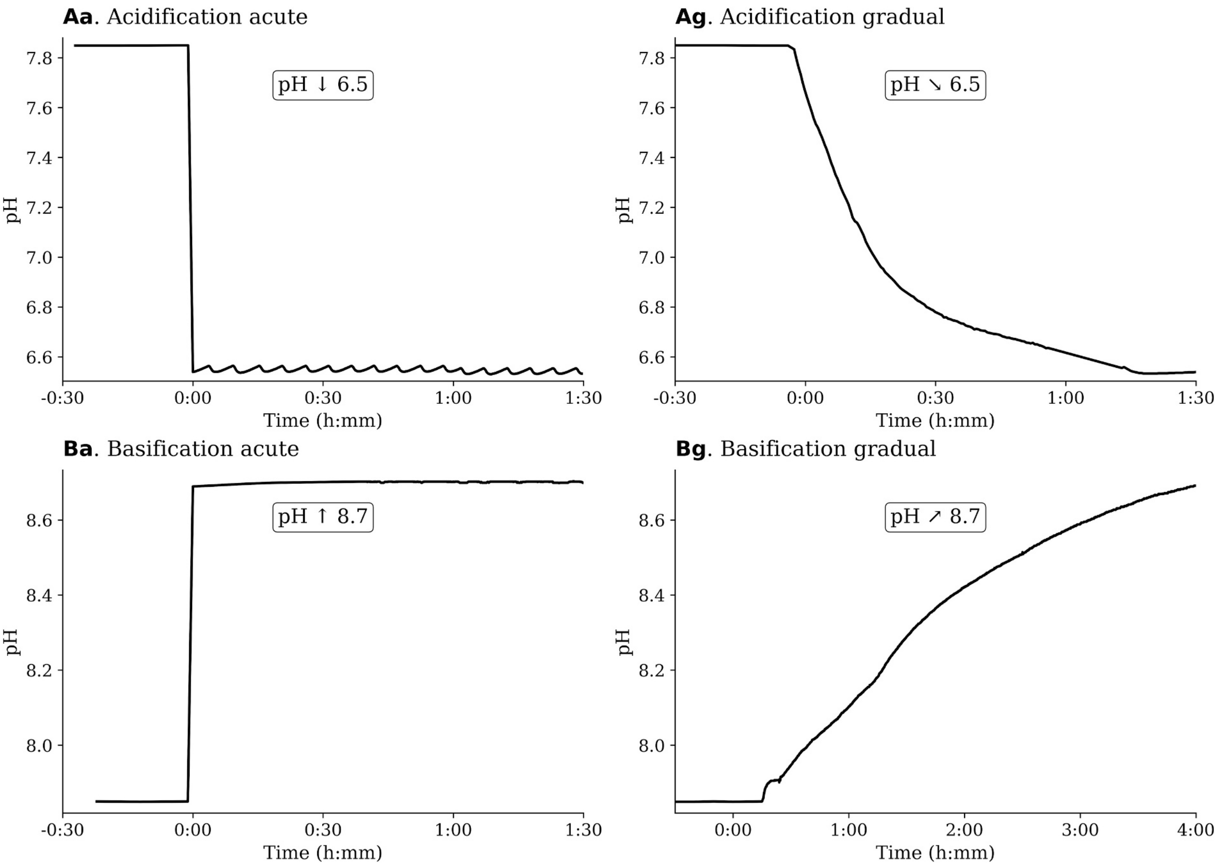

Fig. 1. Profiles of water pH during acute and gradual acidification (*Aa, Ag*) and basification (*Ba, Bg*) treatments. The period before 0:00 represents control conditions, while 0:00 marks the time of fish transfer and the start of experimental exposure.

acidification on fish have attracted considerable research interest (Dennis III et al., 2015; Heuer and Grosell, 2014; Ishimatsu et al., 2005), providing the rationale for the present study. In particular, information on the response of fish skin to changing water pH remains scarce.

Water pH in the experimental aquaria was regulated using a dedicated gas-exchange system designed to control dissolved CO_2 concentration. This setup allowed precise and reproducible adjustment of pH without the direct addition of non-gaseous chemical reagents. Acidification was achieved by introducing carbon dioxide into the aeration stream, thereby increasing dissolved CO_2 levels and lowering pH. Conversely, basification was obtained by stripping CO_2 from the water using a CO_2-free gas mixture (synthetic air). High-purity gases (grade 4.0; Linde, Poland) were supplied through diffusers to ensure efficient gas exchange and homogeneous mixing within the aquarium. The addition of CO_2 altered the carbonate system of the water, inducing predictable shifts in pH through changes in dissolved inorganic carbon speciation. Carbonate system parameters and expected pH responses were calculated using CO2SYS (version 2.3), applying the equilibrium constants of Millero (2010), appropriate for low-salinity waters. Gas flow rates were carefully adjusted to achieve the desired pH trajectories while avoiding excessive gas supersaturation that could cause gas bubble disease in fish. Continuous aeration and mixing maintained stable physicochemical conditions throughout the experiments. Water pH was continuously monitored using a calibrated pH probe connected to a multiparameter meter (CX-461, Elmetron, Poland) and maintained within ±0.02 units of the target value. Temporal dynamics of pH variation across the four experimental variants are shown in Fig. 1, illustrating the distinct profiles of acute and gradual acidification and basification protocols.

Adult three-spine sticklebacks of both sexes (total body length: 51–71 mm, total body weight: 0.98–2.32 g) were caught in the Gulf of Gdańsk (southern Baltic Sea) out of the breeding season and transported to the Institute of Oceanology, Polish Academy of Sciences (Sopot, Poland). The fish were acclimatized in 200-L aerated holding aquarium at a temperature (T) of 16 ± 2 °C, salinity (S) of 7.35 (PSU) and pH = 7.83 under a photoperiod of 16L:8D. Water parameters, including temperature, salinity, dissolved oxygen, and pH, were monitored daily using a multiparameter sensor (CX-461, Elmetron, Poland). Sticklebacks were fed frozen chironomids once a day at 15:00.

Four experimental variants (Fig. 1) were conducted: acute decrease in pH to 6.54 (*Experiment Aa-Acidification acute*), gradual decrease in pH to 6.54 (*Experiment Ag-Acidification gradual*), acute increase in pH to 8.70 (*Experiment Ba-Basification acute*), and gradual increase in pH to 8.70 (*Experiment Bg-Basification gradual*).

Prior to *Experiments Aa* and *Ag*, 40 adult three-spined sticklebacks of both sexes were randomly selected from the holding aquarium and placed in a 100 L aquarium for a 3-day acclimation period ($S = 7.35$, $T = 19.0 \pm 0.5$ °C, and $pH = 7.83$) under a 16 L:8D photoperiod, with continuous aeration and mechanical filtration. Similarly, prior to *Experiments Ba* and *Bg*, 40 adult three-spined sticklebacks were randomly selected and acclimated for 3 days in a 100 L aquarium under identical conditions. Feeding was stopped 3 days before the experiments.

In *Experiment Aa*, 20 fish were randomly divided into two groups: a control group ($n = 10$) kept in water of $pH = 7.83$, and an experimental group ($n = 10$) exposed to water of $pH = 6.54$ for 1.5 h. In *Experiment Ag*, the control group ($n = 10$) was kept in water of $pH = 7.83$, and the experimental group ($n = 10$) was exposed to a gradual decrease in pH from 7.83 to 6.54 over 1.5 h. Fish were kept in 40 L aquaria.

In *Experiment Ba*, 20 fish were divided into two groups: a control group ($n = 10$) kept at $pH = 7.83$, and an experimental group ($n = 10$) exposed to water of $pH = 8.70$ for 1.5 h. In *Experiment Bg*, the control group ($n = 10$) was kept in water of $pH = 7.83$, and the experimental group ($n = 10$) was exposed to a gradual increase in pH from 7.91 to 8.70 over 4 h. Fish were kept in 40 L aquaria.

In the acute treatments (Fig. 1: *Aa* and *Ba*), the target pH was first stabilized in the experimental aquarium, after which fish were transferred directly from control conditions to water at the desired pH level (control; $pH = 6.54$; $pH = 8.70$). In the gradual treatments (Fig. 1: *Ag* and *Bg*), fish were also transferred to a separate aquarium, but the pH adjustment toward the target value began immediately after transfer through controlled modification of the gas composition supplied to the system. In this way, fish in both experimental and control groups experienced identical handling procedures.

After the experiments, the fish were euthanized by decapitation and the following organs were collected: brain, eyeballs, and skin. Brain Mel levels primarily reflect pineal production; in very small fish such as sticklebacks, isolation of the pineal organ is not feasible. Sampling began at 10:00 a.m., about six hours after lights-on when pineal melatonin secretion is low, reducing the risk that nocturnal pineal Mel masks cutaneous Mel. Most of the examined fish had mature gonads but were not yet in spawning condition. Samples were placed on ice and immediately frozen at −80 °C for subsequent analyses of Mel and cortisol.

Melatonin concentrations in skin, eyeball, and brain samples were measured by HPLC with fluorescence detection, as described by Gozdowska et al. (2022). Brain samples were sonicated in 0.4 mL of phosphate buffer (0.05 M, $pH = 6.8$), the homogenates shaken for 15 min and centrifuged at 8000 ×*g* for 20 min at 10 °C. The supernatants were applied to SLE columns (NOVUM PRO SLE, Phenomenex), and analytes were eluted with 2 mL of dichloromethane:ethyl acetate (1:1, *v*/v). After evaporation, residues were dissolved in 0.25 mL of methanol: PBS buffer ($pH = 7.4$, 1:1, v/v) and analyzed by HPLC. Eyeball and skin samples were homogenized (Bead Ruptor Elite, Omni International, USA) in 0.5 mL of perchloric acid (1 M), centrifuged (10,000 ×*g*, 20 min, 4 °C), and the supernatants were analyzed by HPLC. Chromatographic analyses were performed on an Agilent 1200 Series HPLC system with fluorescence detection using ZORBAX Eclipse Plus C18 column (100 × 4.6 mm, 3.5 μm; Agilent, USA). The mobile phases were: A, 10 mM ammonium acetate ($pH = 5$), and B, methanol. A gradient of 10–40% B (0–20 min) followed by 40–60% B (20–40 min) was run at 0.5 mL/min and 30 °C. The injection volume was 20 μL. Fluorescence detection was set at Ex/Em = 230/350 nm, and Mel was identified by comparison of retention times with external standards (Sigma, Germany).

Cortisol levels in skin were measured using a commercial Cortisol ELISA kit (Cayman Chemical, USA). Skin samples were homogenized in 1 mL of PBS buffer (50 mM, $pH = 7.4$), centrifuged (10,000 ×*g*, 20 min, 10 °C), and the supernatants were purified using SLE according to the protocol (TN-0076, Phenomenex), with minor modifications. Specifically, supernatants were diluted (1:1, v/v) with 50 mM sodium phosphate dibasic (pH unadjusted) prior to SLE, and dichloromethane was used as the eluent. The eluates were evaporated to dryness prior to ELISA.

Statistical analysis of data was performed using Statistica 13.3 software. Data are presented as mean ± standard error of the mean (SEM). Because the sample size was limited, data distribution could not be reliably assessed; therefore, non-parametric tests were applied. All differences in Mel and cortisol concentration between control and experimental fish were evaluated using the Mann-Whitney *U* test. Statistical significance was set at $P < 0.05$.

Mel concentrations in the skin were significantly higher in fish exposed to acidification than in those exposed to basification under the rapid change regime ($P = 0.036$; Mann-Whitney U test), with a similar but non-significant trend observed in the gradual change regime ($P = 0.062$; Mann-Whitney U test) (Fig. 2). Skin cortisol concentrations remained stable across all conditions ($P > 0.05$; Mann-Whitney U test) (Fig. 2). Eyeball Mel levels did not differ significantly among treatments ($P > 0.05$; Mann-Whitney U test) (Fig. 2). Mel concentrations in the brain were approximately ten times lower than those in the skin and eyeball and tended to be slightly higher under basification than under acidification in both regimes ($P = 0.05$; Mann-Whitney U test, borderline significance) (Fig. 2). Low Mel levels in the brain, the main site of Mel synthesis in vertebrates, including fish, were observed during the day (sampling time: 10:00 a.m.), reflecting the well-known decrease in

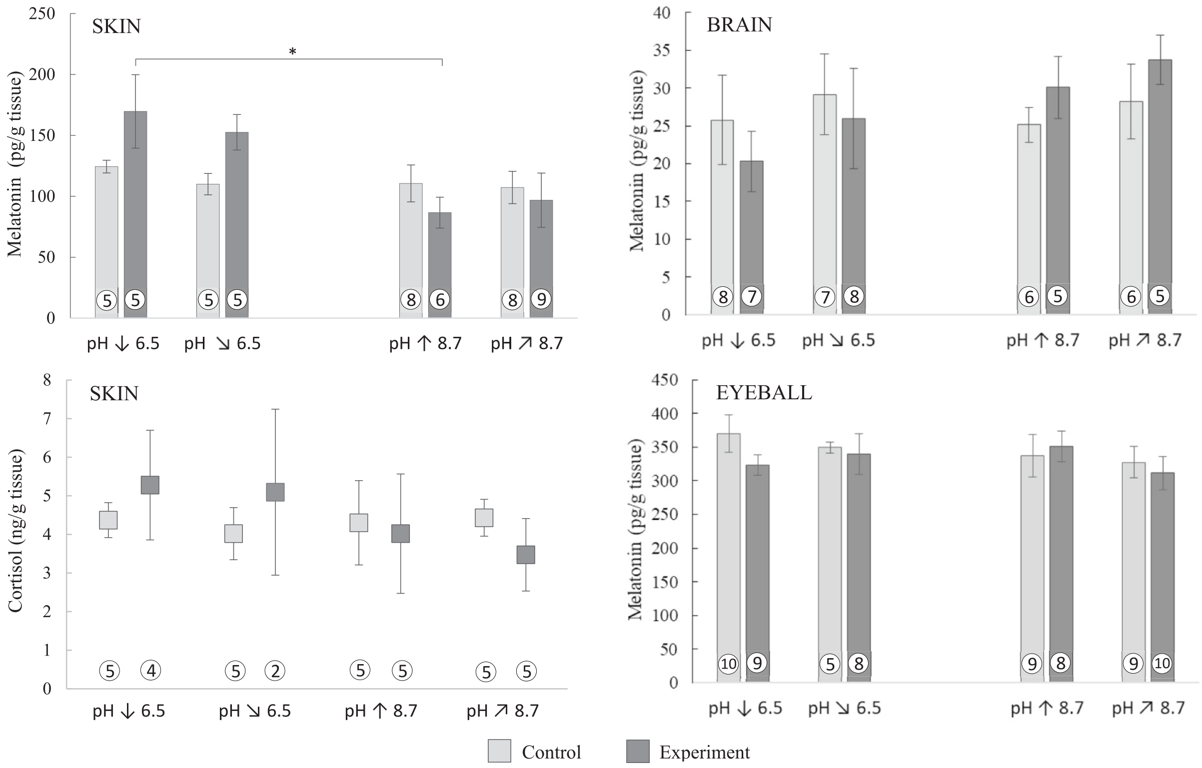

Fig. 2. Melatonin levels in the skin, brain and eyeball, and cortisol levels in the skin of three-spined stickleback exposed to different water pH conditions. Values are presented as means ± SEM. Melatonin levels in all experimental groups were compared both with their respective control groups and with groups exposed to different pH conditions. Significant differences are indicated as $^{*}P < 0.05$ (Mann-Whitney *U* test). The number of fish per group is shown in circles.

pineal organ activity that typically occurs during the light period (Falcón et al., 2010).

The lack of significant differences in eyeball Mel concentrations across pH treatments indicates that this organ is relatively resistant to pH fluctuations. The direction of change in skin melatonin between experimental groups exposed to different pH conditions was opposite to tendency observed in the brain, suggesting that regulation of cutaneous melatonin may occur independently of pineal secretion. Moreover, the consistently high Mel levels in the eyeball, similar to those in the skin, together with the very low brain Mel levels (five- to tenfold lower), suggest that both the eyeball and the skin constitute important sources of Mel in three-spined sticklebacks during the daytime. These data are consistent with previous findings for this species under control light conditions(Pomianowski et al., 2020). Moreover, the present results indicate that peripheral, skin-specific Mel is highly responsive to pH changes, independently of central Mel. It is notable that Mel level in the skin is highly responsive to water pH fluctuations even within the optimal pH range for sticklebacks, whereas cortisol level is resistant to these changes. It should be noted that the water pH conditions used in the experiments fall within essentially non-stressful pH levels for sticklebacks; this may explain why skin cortisol levels, typically reflective of stress conditions, remained stable.

Generally, to the authors' knowledge, the influence of surrounding water conditions on fish skin performance is poorly documented, in contrast to the well-studied functioning of the gills. It is well established that water pH affects ion transport in fish gills, primarily by altering epithelial permeability and active transport mechanisms, with low pH often increasing passive ion loss, as previously mentioned in this paper. Recent work has focused on ion transport across the branchial epithelium in acidified waters (Gonzalez et al., 2024). However, we found no comparable studies specifically addressing the impact of water pH on fish skin performance, although cutaneous ion transport in fish from both freshwater and marine environments has been discussed in detail by Glover et al. (2013), and the effects of salinity changes on transepithelial permeability and active transport in rainbow trout skin have been recently investigated (Doyle et al., 2022). Thus, given the current state of knowledge, it remains difficult to explain why skin Mel is sensitive to pH fluctuations. We can only speculate that, in the three-spined stickleback, the skin's pH-sensitive Mel responses may be linked to ion movements across the skin regulated by this hormone under changing pH, similarly to its osmoregulatory action under different salinities (Kleszczyńska et al., 2006; López-Olmeda et al., 2009). Many physiological effects of Mel are mediated via high-affinity receptors present in various peripheral organs, including the skin, gills, and kidney (Confente et al., 2010; Kulczykowska, 2002; Kulczykowska et al., 2006; Sauzet et al., 2008; Falcón et al., 2010). The involvement of Mel in osmoregulation and ion transport through these receptors was first proposed by Kulczykowska (2002) and has been thoroughly discussed by Nisembaum et al. (2021). Therefore, ion movement across skin epithelium under varying pH conditions, where low pH increases H^+ influx and epithelial permeability, while high pH affects bicarbonate buffering, may be associated with the presence and activity of Mel in the skin. Acidic water enhances passive Na^+ and Cl^- loss via gill/skin diffusion, prompting upregulation of Na^+/H^+ exchangers and H^+-ATPase to maintain ion balance. At high pH, reduced H^+ availability shifts reliance toward Na^+/HCO_3^- cotransporters. On the other hand, cortisol levels in the skin remained stable across treatments, although this hormone is known to act not only on the gills but also on the skin, for example by modifying specialized ion-transporting ionocytes (Ridgway et al., 2021). However, it appears that this mechanism is not activated within the well-tolerated pH ranges applied in our experiments.

Taken together, these findings demonstrate that skin Mel, but not cortisol, is highly responsive to subtle pH fluctuations, even within the optimal range for sticklebacks. The action of Mel in fish skin under varying water pH conditions may be associated with its role in ion transport, similar to its involvement in osmoregulatory processes.

CRediT authorship contribution statement

Magdalena Gozdowska: Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Marcin Stokowski:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ewa Sokołowska:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Ewa Kulczykowska:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Ethics statement

All experiments complied with EC Directive 2010/63/EU for animal experiments and the guidelines and approval of the Ethics Committee for Animal Experimentation (Protocol No. 9/2025; University of Science and Technology, Bydgoszcz, Poland).

Funding

This research was supported by a statutory task IV.2 of the Institute of Oceanology, Polish Academy of Sciences, Sopot, Poland.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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