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The cutaneous stress response system in three-spined stickleback and European flounder exposed to oxidative stress: Different mode of action

Konrad Pomianowski [1], Magdalena Gozdowska [1], Ewa Sokołowska, Ewa Kulczykowska [*]

Department of Genetics and Marine Biotechnology, Institute of Oceanology, Polish Academy of Sciences, Powstańców Warszawy 55 Str., 81-712 Sopot, Poland

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ABSTRACT

In fish, the skin is directly exposed to multiple environmental stressors and provides the first line of defense against harmful external factors. It turned out that cortisol and melatonin (Mel) are involved in fish cutaneous stress response system (CSRS) similar to mammalian. This study investigates the mode of action of CSRS in two teleost species of different biology and skin characteristics, the three-spined stickleback and the European flounder, after exposure to oxidative stress induced by a potassium dichromate solution. The cutaneous stress response system presents different ways of action in two studied species: Mel concentration increases in the skin of both species, but cortisol concentration increases in the skin only in sticklebacks. Data suggest that stickleback skin cells can produce cortisol. However, cortisol is not involved in the response to oxidative stress in flounders. In stickleback skin, two genes encoding AANAT and ASMT/HIOMT (enzymes involved in Mel synthesis), *aanat1a* and *asmt2*, are expressed, but in flounder skin, only one, *asmtl*. Because gene expression does not change in stickleback skin after exposure to stress, the source of increased Mel is probably outside the skin. A lack of expression of the gene encoding AANAT in flounder skin strongly suggests that Mel is transported to the skin by the bloodstream from other sites of synthesis. Pigment dispersion in the skin after exposure to oxidative stress is found only in sticklebacks.

1. Introduction

The skin of fish serves many functions. It is involved in maintaining osmotic balance, communication, sensory perception, locomotion, respiration, and thermal regulation and is the site of coloration. The skin of fish provides the first line of defense against a wide variety of chemical, physical, and biological stressors and is vulnerable to harmful factors in the surrounding water that have potential or confirmed detrimental effects on the organism. Although much is known about the biology of fish skin, there is still much to be uncovered, including the local stress response system: How it works and responds to a changing environment or stressors.

The well-known adaptive response to stress to preserve homeostasis is the activation of the hypothalamic-pituitary-adrenal axis (HPA). It involves the production of corticotropin-releasing hormone (CRH) and arginine vasopressin (in fish, arginine vasotocin) that stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH), which induces the synthesis and secretion of glucocorticoids (in fish, cortisol) (Baker et al., 1996; Wendelaar Bonga, 1997; Barton, 2002). Slominski and colleagues proposed for the first time that there is a local mechanism of stress response in human skin that is equivalent to HPA (Slominski et al., 1995; Slominski and Mihm, 1996). Detection of gene expression of CRH and CRH receptors by the Slominski group (Slominski et al., 1999, 2007; Slominski and Wortsman, 2000) and local synthesis and metabolism of glucocorticoids shown in many studies (Slominski et al., 1999, 2007, 2015; Hannen et al., 2011; Cirillo and Prime, 2011) allow researchers to accept that there is a stress response system in mammalian skin, where glucocorticoids play a crucial role. However, in teleost fish, it is commonly approved that cortisol is synthesized in the interrenal cells of the head kidney and released into the bloodstream; thus, the hormone is the result of the HPI (hypothalamic-pituitary-interrenal) cascade (Mommsen et al., 1999). In previous studies (Kulczykowska et al., 2018; Pomianowski et al., 2020; Gozdowska et al., 2022), we showed that glucocorticoid, cortisol, and an indole hormone melatonin (*N*-acetyl-5-methoxytryptamine; Mel) with its metabolite AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine) are involved in the cutaneous stress response system (CSRS) in fish similar to mammalian (for a review, see: Slominski et al., 1999, 2008; Slominski and Wortsman, 2000).

* Corresponding author.
E-mail address: ekulczykowska@iopan.gda.pl (E. Kulczykowska).
[1] These authors contributed equally and are jointly considered the first author.

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Regarding Mel and AFMK, there is evidence that both are highly effective in protecting organisms from oxidative stress (Tan et al., 2000; Hardeland, 2008), including their action in mammalian skin (Fischer et al., 2006; Slominski et al., 2008). Mel is synthesized and secreted primarily in the pineal gland and retina in vertebrates, including fish. It is produced from serotonin (5-hydroxytryptamine; 5-HT) by two consecutive enzymes: (i) aralkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), also known as serotonin *N*-acetyltransferase; and (ii) *N*-acetylserotonin *O*-methyltransferase (ASMT; EC 2.1.1.4), also known as hydroxyindole-*O*-methyltransferase (HIOMT) (Fig. 1). Mel formation and release are low during light and increase during darkness (Klein, 2007; Bolliet et al., 1996; Reiter et al., 2010). These days, many sources describe various localizations and functions of Mel, including those in fish (for review: Falcón et al., 2010, 2011; Acuña-Castroviejo et al., 2014). The presence of the hormone has been confirmed in many organs and tissues because this small molecule crosses natural barriers without difficulties (for review: Kulczykowska, 2002). Furthermore, Mel concentrations in the skin of fish are higher than those in plasma (the examples are given by Kulczykowska et al., 2018). This fact suggests an active uptake or local synthesis of this indoleamine and its unique action at this peripheral site. The mammalian skin is an example of an organ where active synthesis and metabolism of Mel takes place (for review: Slominski et al., 2008, 2017). Today, it turns out that also the skin of fish is a site of Mel synthesis and metabolism (Fernández-Durán et al., 2007; Kulczykowska et al., 2017, 2018; Pomianowski et al., 2020; Gozdowska et al., 2022). First, Fernández-Durán et al. (2007) demonstrated a relative expression of the AANAT2 gene in the skin of rainbow trout (*Oncorhynchus mykiss*) and suggested Mel synthesis at this site. Then we found that AANAT genes are expressed in the three-spined stickleback skin (*Gasterosteus aculeatus*) at noon and midnight (Kulczykowska et al., 2017). Next, we show the expression of genes encoding enzymes involved in Mel synthesis, AANAT, and ASMT, and the activity of AANAT in the skin of the same species (Pomianowski et al., 2020). Considering the presence of transcripts of both the AANAT and ASMT encoding genes and the activity of AANAT in the skin at noon and midnight, local Mel synthesis in stickleback skin is strongly supported. Furthermore, the presence of AFMK in the skin of European flounder (*Platichthys flesus*) indicates that Mel is metabolized here (Kulczykowska et al., 2018). However, there is still no indication if Mel synthesis in fish skin is a rule or a species-specific phenomenon. Furthermore, it remains unclear whether cortisol in fish skin originates solely from interrenal cells and the bloodstream or if skin cells participate in its production.

In our previous study on the three-spined stickleback, we already addressed the response of the skin to oxidative stress; our study indicated that exposure of fish to a potassium dichromate solution ($K_2Cr_2O_7$) increased Mel and cortisol levels in the skin of this species (Gozdowska et al., 2022). However, more studies were needed to clarify some aspects of the action of the cutaneous stress response system. Therefore, we have revisited it in this paper with the example of two species of fish: European flounder (*P. flesus*) and three-spined stickleback (*G. aculeatus*).

This study aims to compare how CSRS works under oxidative stress conditions in these fish. By studying these species, we aim to check whether the action of the cutaneous stress response system is similar or varies in fish from different habitats, species of distinctive biology, and skin characteristics. For the record, the three-spine stickleback is a small pelagic fish with a laterally compressed spindle-shaped body. It is one of the most ubiquitous and adaptable species widely distributed in coastal waters of the northern hemisphere. It inhabits fresh, brackish, or salt water. However, European flounder is a flatfish that inhabits European coastal waters, salt and brackish, and freshwater. It is a bottom-dwelling fish with nocturnal activity, burrowing on the substrate during the day. Although in both, stickleback and flounder, bony plates form a kind of armour, the body covering differs markedly: in pelagic stickleback, it is more delicate, and the skin is thinner, while in bottom-dwelling flounder, it is more armoured, and a thick layer of collagen toughens the skin. We use a mark, that is, the change of pigment dispersion in the skin after exposure of fish to stress to emphasize the difference between stickleback and flounder skin characteristics.

In this study, we used a potassium dichromate solution as an external factor that causes oxidative stress because of its highly toxic and oxidizing properties. It is identified as an anthropogenic water pollutant that evokes oxidative stress in aquatic organisms, including fish (Ko et al., 2019; Velma et al., 2009).

2. Materials and methods

2.1. Animals and experimental design

The experiments were carried out on three-spined sticklebacks and European flounders of both sexes. Adult fish were caught in the Gulf of Gdańsk (South Baltic Sea) in October (sticklebacks) and December (flounders), out of the breeding season. The fish were acclimatized in 40-L (sticklebacks) and 70-L (flounders) aerated aquaria with brackish water (7 ppt) at a temperature of 10 ± 2 °C for one month at the Institute of Oceanology PAS (Sopot, Poland). In the experiment, sticklebacks (total weight 0.95–2.1 g) and flounders (total weight 78–370 g) were kept at the 12 L:12D photoperiod. Sticklebacks were fed frozen chironomids, while flounders *Mytilus sp.* mussels once a day at 13:00. Feeding of the fish was stopped three days before the experiment.

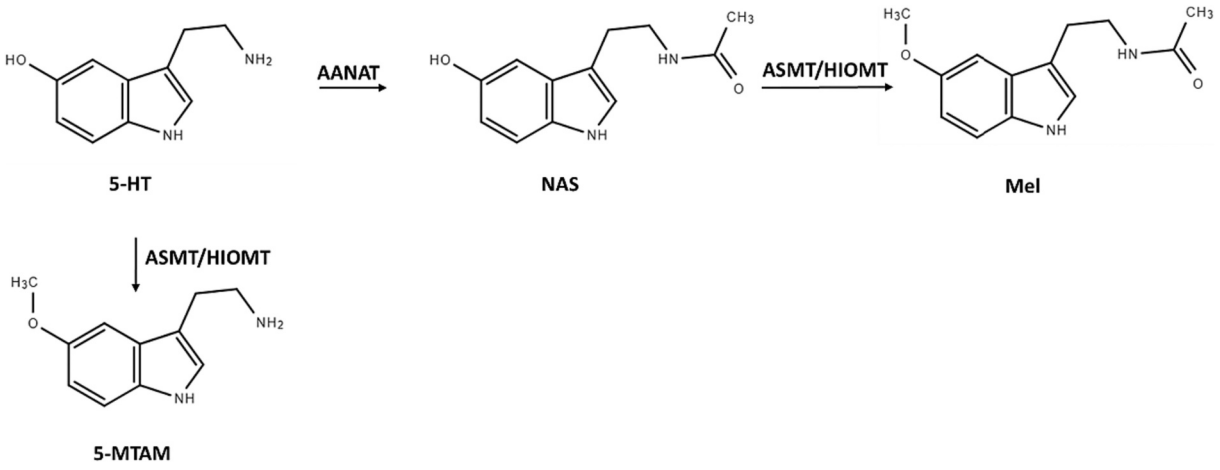

Fig. 1. AANAT and ASMT actions in the serotonin metabolic pathway. 5-HT, serotonin; NAS, *N*-acetylserotonin; Mel, melatonin; 5-MTAM, 5-methoxytryptamine; AANAT, aralkylamine *N*-acetyltransferase; ASMT, *N*-acetylserotonin *O*-methyltransferase; HIOMT, hydroxyindole-*O*-methyltransferase.

2.1.1. Experiment 1 and 2

Before Experiments 1 and 2, the fish were randomly assigned to 2 groups: the control (water without $K_2Cr_2O_7$) and the experimental group (water with $K_2Cr_2O_7$: 50 mg Cr/1 L) for 6 h. The concentration of chromium added to the water was expressed as Cr^{6+} ion in milligrams per liter of water. After Experiment 1, the fish of both groups were sacrificed by decapitation. After Experiment 2, the fish (control and experimental) were rinsed in water without $K_2Cr_2O_7$ for 2 min before sacrificing by decapitation. The sampling started four hours after the light went off. Samples were taken under red light, snap frozen in a cooling bath (dry ice/ethanol) and stored at −70 °C until analysis (Table 1).

2.2. Melatonin analysis

Mel concentrations were measured by HPLC with fluorescence detection preceded by SLE (Solid-Liquid Extraction) according to Gozdowska et al. (2022). Stickleback skin, eyeball, and gill samples (Experiment 2, Table 1) and flounder skin, eyeball, and gill samples (Experiment 2, Table 1) were homogenized (Bead Ruptor Elite, Omni International, USA) in 1 mL of phosphate buffer (0.05 M; pH 6.8), centrifuged (10,000 *g*, 15 min, 4 °C) and supernatants were loaded onto SLE columns (SLE NOVUM, 100 mg/5 mL; Phenomenex). Mel was eluted using 6 mL of dichloromethane: ethyl acetate (1:1, *v*/v). After evaporation of the eluate, a residue was dissolved in 0.2 mL of methanol: PBS buffer (pH 7.4) (1:1, v/v) and analyzed by HPLC. Chromatographic analysis was performed using the Agilent 1200 Series Quaternary HPLC System with fluorescence detector (Agilent Technologies, Germany). Mel separation was achieved on a ZORBAX Eclipse Plus C18 (150 mm × 4.6 mm ID, 3.5 μm; Agilent, USA). A gradient elution was applied. The mobile phase consisted of solvent A (10 mM ammonium acetate; pH 5) and solvent B (methanol). A linear gradient was set at 20% to 50% eluent B in 20 min, with a flow rate of 1 mL/min and column temperature at 20 °C. The injection volume was 50 μL. Fluorescence was recorded at 350 nm after excitation at 230 nm. Mel identification of Mel was performed by comparing the retention time of the sample with that of the standard (Mel, Sigma, Steinheim, Germany).

2.3. AANAT activity assay

The AANAT activity assay was carried out according to Pomianowski et al. (2020) with modifications. Stickleback skin samples (Experiment 2, Table 1) were weighed and homogenized (Bead Ruptor Elite, Omni International, USA) in 500 μL of 0.2 M phosphate buffer (pH 6.8) with 5 μL of 1.2 mM acetyl coenzyme A (AcCoA; Sigma, St. Louis, MO, USA). The homogenates were then centrifuged at 13,000 *g* at 4 °C for 10 min. The enzyme assay was carried out in the presence of a 20 μL aliquot of the supernatant, 5 μL of 74.4 mM serotonin (5-HT; Sigma, St. Louis, MO, USA) and 10 μL of 1.2 mM AcCoA at 10 °C for 60 min. The final concentrations of 5-HT and AcCoA in the reaction mixture were 10.3 mM and 1.03 mM, respectively. The reaction was stopped by adding 5 μL of 6 N perchloric acid (Sigma-Aldrich, St. Louis, MO, USA), the mixture was centrifuged (10,000 *g*, 4 °C, 10 min) and a 20-μL aliquot of supernatant was injected into the HPLC system. The product of the enzymatic reaction, *N*-acetylserotonin (NAS), was measured using the Agilent 1200 Series Quaternary HPLC System with fluorescence detector (Agilent Technologies, Germany) according to Pomianowski et al. (2020) with modifications. Chromatographic separation was achieved on a ZORBAX Eclipse Plus C18 (150 mm × 4.6 mm ID, 3.5 μm; Agilent, USA). A gradient elution was applied. The mobile phase consisted of solvent A (10 mM ammonium acetate; pH 5) and solvent B (methanol). A linear gradient was established at 5–30% eluent B in 20 min and then 30–70% B in 10 min, with flow rate 1 mL/min and column temperature at 30 °C. Fluorescence detection was carried out at 350 nm with excitation at 230 nm. NAS identification was performed by comparing the retention time of the sample with that of the standard (Sigma, Steinheim, Germany).

2.4. ASMT activity assay

Stickleback skin samples (Experiment 2, Table 1) were weighed and homogenized (Bead Ruptor Elite, Omni International, USA) in 400 μL of 0.1 M phosphate buffer (pH 7.9) with 0.25 mM SAM (S-(5′-adenosyl)-L-methionine chloride; Cayman Chemicals, Ann Arbor, MI, USA). The homogenates were then centrifuged at 10,000 *g*, at 4 °C for 20 min. The enzymatic reaction was carried out in the presence of 30 μL aliquot of the supernatant, 7 μL of 2.5 mM SAM and 8.5 μL of 8 mM NAS at 10 °C for 60 min. The final concentration of SAM and NAS in the reaction mixture was 0.5 mM and 1.5 mM, respectively. The reaction was stopped by adding 14.5 μL of 6 N perchloric acid, the mixture was centrifuged (10,000 *g*, 4 °C, 10 min) and a 30-μL aliquot of supernatant was injected into the HPLC system. The product of the enzymatic reaction, Mel, was measured using an Agilent 1200 Series Quaternary HPLC

Table 1
The type of analysis performed during the experiments.

	Experiment 1			Experiment 2		
	Organ	Analysis	n	Organ	Analysis	n
Three-spined stickleback	Eyeball	Gene expression	12	Skin	Gene expression	12
					Cortisol level	10
					Mel level	10
						10
					AANAT activity	10
	Skin	Gene expression	12		ASMT activity	10
		Cortisol level*	10		Melanophore number	
		Mel level*	10	Gills (with arches)	Cortisol level	10
					Mel level	10
				Eyeball	Gene expression	8
						8
					Mel level	
				Skin	Gene expression	8
					Cortisol level	8
European flounder	Skin	Gene expression	5		Mel level	8
					AANAT activity	8
					ASMT activity	8
					Melanophore number	8
				Gills (only filaments)	Cortisol level	8
					Mel level	8

n – number of analyzed fish.
* Gozdowska et al., 2022

System with fluorescence detector (Agilent Technologies, Germany). Chromatographic separation was achieved on a ZORBAX Eclipse Plus C18 (150 mm × 4.6 mm ID, 3.5 μm; Agilent, USA). A gradient elution was applied. The mobile phase consisted of solvent A (10 mM ammonium acetate; pH 5) and solvent B (methanol). A linear gradient was set at 5–60% eluent B in 20 min, at a flow rate of 1 mL/min and column temperature at 30 °C. Fluorescence detection was carried out at 350 nm with excitation at 230 nm. Mel identification was performed by comparing the retention time of the sample with that of the standard (Mel).

2.5. RT-qPCR analysis

2.5.1. RNA extraction

Stickleback skin and eyeball samples (Experiment 1, Table 1) and skin samples (Experiment 2, Table 1) and flounder skin (Experiment 1, Table 1) and skin and eyeball samples (Experiment 2, Table 1) were taken for RNA extraction. Total RNA was purified according to Pomianowski et al. (2020). The European flounder eyeball was cut in half horizontally immediately after being taken from −70 °C, and half was taken to extraction. RNA extracts from flounder organs were not treated with DNase I.

2.5.2. Primer design and RT-qPCR conditions

Expression analysis conditions (RT-qPCR) for the stickleback *aanat1a, snat, asmt, asmt2* genes were previously described by Pomianowski et al. (2020). Based on the results of the previous research (a very low expression in the eyeball and skin that makes it impossible to optimize the conditions of qPCR), we decided to exclude the *aanat2* gene from the studies. For the European flounder, a new method was developed using molecular probes. At first, the gene transcripts of *aanat*, *aanat-1*, *aanat-2*, *asmt*, *asmt-2* and *asmtl* were identified among the transcriptomic data of European flounder (NCBI BioProject no.: **PRJNA637628**), and the integrity of these transcripts, as well as the intron/exon boundaries in the gene sequences, was verified by comparison with homologous genomic sequences of Japanese flounder (*Paralichthys olivaceus*) (GenBank accession number: **GCA_001904815** and **GCF_001970005**) and three-spined stickleback (Ensemble release 97 genome assembly: **BROAD S1**) as described in detail by Pomianowski et al. (2021). Furthermore, the application of bioinformatic differential expression analysis (Zhou et al., 2013; Soneson et al., 2016; Patro et al., 2017) allowed us to identify four candidates for reference genes among nine different flounder organs. We chose *uba52* (ubiquitin A-52 residue ribosomal protein fusion product 1 n 44) as the reference gene because it showed the most stable expression in the eyeball and skin after potassium dichromate treatment. The primers and TaqMan molecular probes for the RT-qPCR analysis (Table 2) were designed using Primer3 software v. 2.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) with strict input settings that include a location of at least one primer in pairs at the intron/exon junction. The accuracy of qPCR efficiency was confirmed for each gene in the multiplex duplex reaction using standard curves made of serial dilutions of 6-point, 10-fold purified PCR templates (Extractme DNA Clean-up Kit, Blirt, Gdańsk, Poland) consisting of (393-1266 bp) rtPCR amplicons surrounding each qPCR target. Before each run, 50 ng of total RNA was reverse transcribed using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK). Each RT-qPCR multiplex-duplex assay was performed in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) and run in a total volume of 10 μL containing 1 X ABsolute QPCR Mix (Thermo Scientific, Waltham, MA, USA), 0.3 μM of each primer, 0.25 μM of each molecular probe and 2 μL of cDNA solution. The following protocol was used: 95 °C for 15 min, followed by 35 PCR cycles of 15 s at 95 °C (denaturation), 30 s at 65 °C (primer annealing) and 30 s at 72 °C (elongation). Three replicates were used for each combination of organ type and multiplex-duplex assay. The relative expression calculation was based on the difference between the Ct values for reference and target genes, separately for each organ tested, according to the Livak and Schmittgen (2001) equation.

Table 2
Primer and TaqMan molecular probe sequences used in European flounder RT-qPCR analysis. Fluorophores and quenchers are marked for each probe in italics.

Gene	Sequence (5′ → 3′)	Product length (bp)
aanat	F: AGACTCACCACAGAAGCGCT R: ATCTCCGTGAAGGTGAGGC P: *JUN*-CCGCTGCGCCATCACCGTGGGCA-*QSY*	265
aanat-1	F: ACCATGGATGCCCTGACTCT R: CTGCACCTTGAAACCCGACT P: *FAM*-CAGGTGCCTGCCCTACGTCCGCCGT-*MGB*	255
aanat-2	F: GCTTTCCCAAGAGGCCATGA R: TGCATGTTGGACACGGAGAT P: *FAM*-AGCACGTCCCGGACAGCCCCGCT-*MGB*	253
asmt	F: GGAGATAAGTCAGGCGGTGG R: GCTTCCTTCTCTGACAGCGT P: *JUN*-CCGCCTGCAGGGGCCTTCAGCTGC-*QSY*	244
asmt-2	F: GGATGGGACGGCTGTTTACA R: TCAGCATCTCCTCCTCCGAT P: *FAM*-TGGTGGACGCCGTCAGGGAGGGG-*MGB*	230
asmtl	F: GTGAGCCCATGGACAAAGCT R: GTTGTGTGGGAGCTGAGTGA P: *FAM*-TCCGACCAGGAGAGTGCATCCGCTCGT-*MGB*	236
uba52 (reference gene)	F: CCAGGACAAGGAAGGAATTCCC R: CAGACGGGCATAGCATTTGC P: *VIC*-TCAGCAGCGTCTGATCTTCGCCGGCA-*MGB*	220

2.6. Cortisol analysis

The measurement of cortisol in the skin was performed according to Gozdowska et al. (2022). Stickleback skin samples (Experiment 2, Table 1) and flounder skin samples (Experiment 2, Table 1) were homogenized (Bead Ruptor Elite, Omni International, USA) in 1 mL of PBS buffer (0.05 M, pH 7.4) and centrifuged (10,000 *g*, 10 °C, 10 min). The supernatants were diluted (1:1, v:v) with 50 mM sodium phosphate dibasic (pH unadjusted) and subjected to SLE columns (SLE NOVUM, 100 mg/5 mL; Phenomenex). Cortisol was eluted with dichloromethane (6 mL) from the column and after solvent evaporation the residues were reconstituted in 2 mL of Cortisol ELISA buffer. Cortisol levels were analyzed using the competitive enzyme-linked immunosorbent assay kit AChE (acetylcholinesterase) (Cayman Chemical, USA) according to the manufacturer's instructions.

Stickleback gill samples (Experiment 2, Table 1) and flounder gill samples (Experiment 2, Table 1) were homogenized in 1 mL of PBS buffer (0.05 M, pH 7.4) and centrifuged (13,000 *g*, 10 °C, 10 min). The supernatants were diluted 10 times with Cortisol ELISA buffer and centrifuged (13,000 *g*, 10 °C, 10 min). The final supernatants obtained were analyzed using the competitive AChE ELISA Cortisol kit (Cayman Chemical, USA) according to the manufacturer's instructions.

2.7. Melanophore analysis

Three-spined stickleback skin samples of (n = 10, 6 control fish, 4 $K_2Cr_2O_7$ treated fish) were taken from the left body flank. Samples of 10 mm × 5 mm (n = 30, 3 samples of each fish) were collected from the dorsal (above the midline), lateral, and ventral body regions. (Experiment 2, Table 1). Skin samples from European flounder (n = 8, 4 control fish, 4 $K_2Cr_2O_7$ treated fish) were taken from the ocular surface. Samples of 20 mm × 10 mm (n = 24, 3 samples of each fish) were collected from the head, middle, and tail body regions (Experiment 2, Table 1). Skin samples were fixed in 4% buffered formalin, mounted on Eukitt slides

(Sigma-Aldrich, USA), scanned with an Olympus BX60 light microscope (Olympus, Japan) and photographed by transillumination with an Olympus XC10 digital camera (Olympus, Japan) coupled with the Olympus microscope. Skin photographs were loaded into ImageJ 1.53e image analysis software and the number of melanophores was evaluated.

2.8. Statistical analysis

Statistical analysis of data was performed using Statistica 13.3 software. Values are presented as mean ± standard error of the mean (SEM). Differences in hormone concentrations (Mel, cortisol), enzyme reaction products, number of melanophores and *aanat*s, *asmt*s, *asmtl* mRNA levels between control fish and fish exposed to oxidative stress were examined with variance analysis (one-way ANOVA) followed by Tukey's honestly significant difference (HSD) *post hoc* test for equal or unequal numbers of fish. Statistical significance was considered at $P < 0.05$.

3. Results

In the three-spined stickleback, after exposure to $K_2Cr_2O_7$, the Mel level increased significantly in the skin and eyeball ($P < 0.01$) but not in the gills (Fig. 2A, B, C). In the European flounder, after exposure to $K_2Cr_2O_7$, the Mel level increased significantly in the skin and gills ($P < 0.01$; $P < 0.05$) but not in the eyeball (Fig. 2D, E, F).

In stickleback skin, the activity of AANAT decreased significantly (*P*

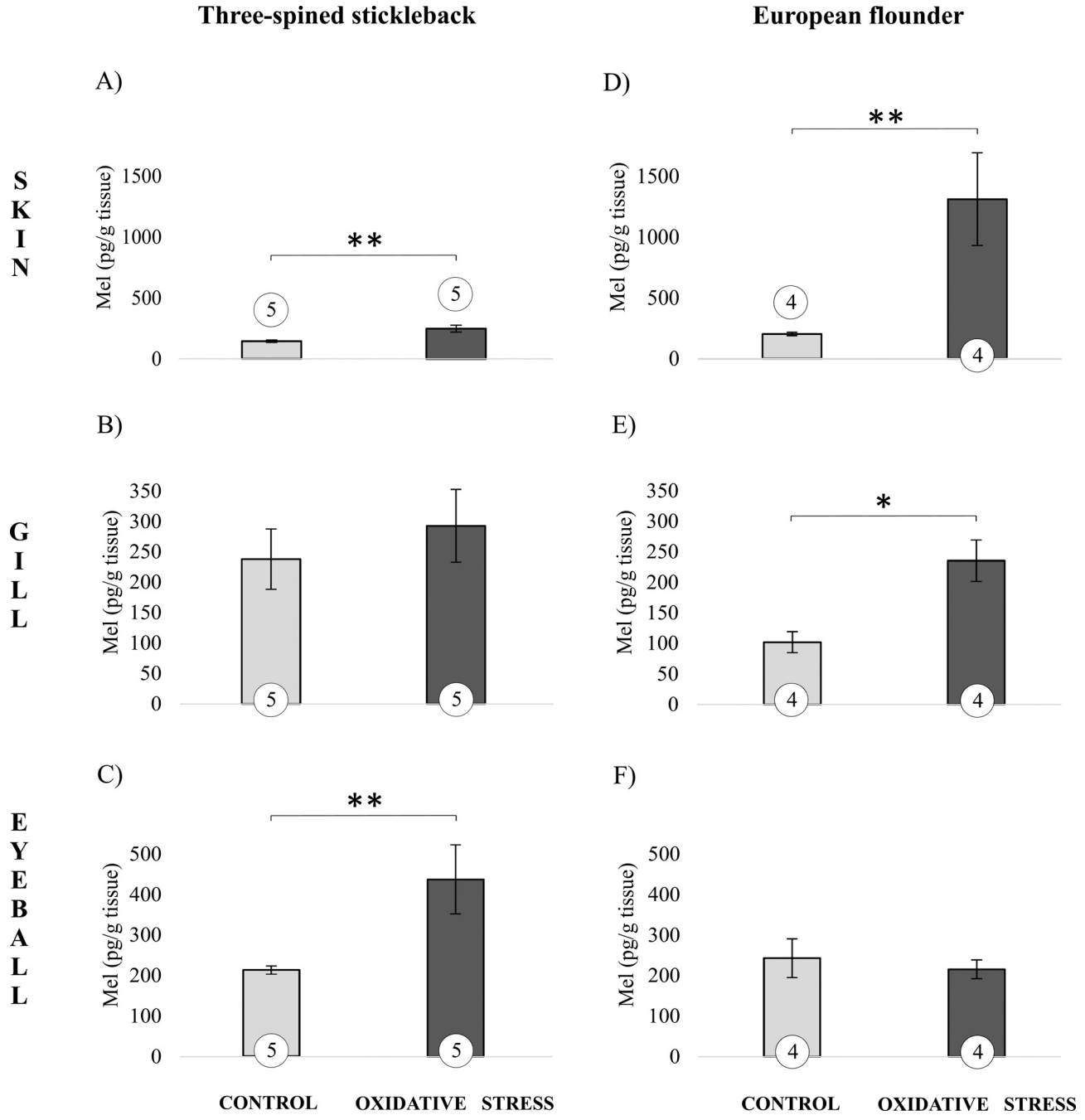

Fig. 2. Melatonin levels in the skin (A), gills (B) and eyeball (C) of three-spined stickleback and skin (D), gills (E) and eyeball (F) of European flounder (Experiment 2): control fish (light bars) and $K_2Cr_2O_7$-treated fish (dark bars). Values are presented as means ± SEM. Significant differences are indicated as $^*P < 0.05$, $^{**}P < 0.01$ (Tukey's HSD *post hoc* test). The number of fish is given in circles.

< 0.01) after exposure to $K_2Cr_2O_7$, in contrast to ASMT, where its activity increased ($P < 0.05$) (Fig. 3).

The expression of genes encoding AANAT and ASMT is presented in Fig. 4 (three-spined stickleback) and Fig. 5 (European flounder). In the skin of three-spined stickleback, the mRNA level of *aanat1a* and *asmt2* was similar in the control fish and the $K_2Cr_2O_7$ treated fish in both experiments (Fig. 4A, B). The expression of *snat* and *asmt* in $K_2Cr_2O_7$ treated fish was at the detection level in both experiments (Fig. 4A, B). In the eyeball, four genes, *aanat1a, snat*, *asmt,* and *asmt2,* were expressed. Significantly higher expression ($P < 0.05$) was found in fish treated with $K_2Cr_2O_7$ (Fig. 4C). However, in the skin of European flounder, only one gene, *asmtl*, was expressed in the control fish and the $K_2Cr_2O_7$ treated fish in both experiments (Fig. 5A, B). In Experiment 2, the expression level of *asmtl* was significantly higher ($P < 0.05$) in $K_2Cr_2O_7$ exposed fish compared to the control (Fig. 5B). For the record, one difference between the experiments was that in Experiment 2, the fish (control and experimental) were rinsed in water without $K_2Cr_2O_7$ before sacrificed by decapitation. In flounder eyeballs, four genes, *aanat-1*, *asmt*, *asmt-2,* and *asmtl,* were expressed; *aanat-1* expression was significantly higher in the fish treated with $K_2Cr_2O_7$ compared to the control ($P < 0.05$) (Fig. 5C).

In the three-spined stickleback, skin cortisol level was significantly higher in $K_2Cr_2O_7$ treated fish than in the control ($P < 0.05$) (Fig. 6A). On the contrary, gill cortisol level was similar in both groups (Fig. 6B). In European flounder, neither the skin nor the gill cortisol level was affected by $K_2Cr_2O_7$ (Fig. 6C, D).

Mel and cortisol measurement in the gills was a substitute for the analysis of circulating hormones, which requires blood collection, which is not possible in small fish such as the three-spined stickleback. According to Gesto et al. (2015), gill cortisol levels correlate well with plasma cortisol levels in rainbow trout and zebrafish exposed or not to acute stress. We suppose that it is similar in the case of Mel: changes in gill Mel level are related to changes in circulating Mel.

Exposure of three-spined sticklebacks to $K_2Cr_2O_7$ caused a significant decrease in the number of melanophores in the skin ($P < 0.05$), resulting in skin lightening (Fig. 7A). The European flounder kept in $K_2Cr_2O_7$ showed no changes in skin pigmentation (Fig. 7B).

4. Discussion

In our previous studies, we proposed that the skin of fish is a site of action of the system that responds to stress – the cutaneous stress response system (CSRS) (Kulczykowska, 2019; Kulczykowska et al., 2018; Gozdowska et al., 2022). In this study, we have revisited the subject in two species of fish, the three-spined stickleback and European flounder, exposed to oxidative stress. Herein, we have analyzed: (i) the concentration of Mel in the skin, gills, and eyeball (with retina) in both species, (ii) the activity of AANAT and ASMT in stickleback skin, (iii) the expression of genes encoding the AANAT and ASMT enzymes in the skin and eyeball (with retina) in both species, (iv) cortisol in the skin and gills of both species, and (v) pigment dispersion in the skin of both species.

We have shown that skin Mel concentration increases in both species (Fig. 2), and skin cortisol concentration increases only in sticklebacks exposed to stress (Fig. 6). Thus, cortisol appears to be involved in the response to oxidative stress in sticklebacks, but not in flounders. Furthermore, we have found mRNA expression of four genes, *aanat1a*, *snat*, *asmt*, and *asmt2*, in stickleback eyeballs and two, *aanat1a* and *asmt2*, in stickleback skin; *snat* and *asmt* expression in the skin is at a detection limit (Fig. 4). In the eyeball of flounders, we have found mRNA of four genes, *aanat-1*, *asmt*, *asmt-2*, and *asmtl*, but only one gene, *asmtl*, is expressed in the skin (Fig. 5). In sticklebacks, the expression of the genes, *aanat1a*, *snat*, *asmt*, and *asmt2*, vary significantly between control and stressed individuals only in the eyeball (Fig. 4). In flounders, *asmtl* expression is enhanced in the skin and *aanat-1* in the eyeball (Fig. 5). In teleosts, the *asmtl* gene is a product of the fusion between the *maf* and *asmt* genes, and to date, it has been identified in the genomes of several species of fish and transcribed in peripheral organs of fish, including skin (Zhang et al., 2017). Low levels of *asmtl* mRNA were also shown in nine European flounder organs, among them skin (Pomianowski et al., 2021). The function of the gene is unknown and can differ from that of *asmt* taking into account its peripheral distribution (Zhang et al., 2017; Pomianowski et al., 2021). This topic deserves attention and will be addressed in future studies.

The transcripts of *aanat* and *asmt* (Fig. 4) together with the activity of AANAT and ASMT in stickleback skin (Fig. 3) presented in this study indicate that the enzymes of AANAT and ASMT are acting in the skin of this species. It agrees with an earlier finding by Pomianowski et al. (2020). However, gene expressions in the skin do not change in the three-spined stickleback subjected to oxidative stress, although the expression of the four genes increases in the eyeball of stressed fish (Fig. 4A, B, C). Therefore, Mel synthesis outside the skin of sticklebacks,

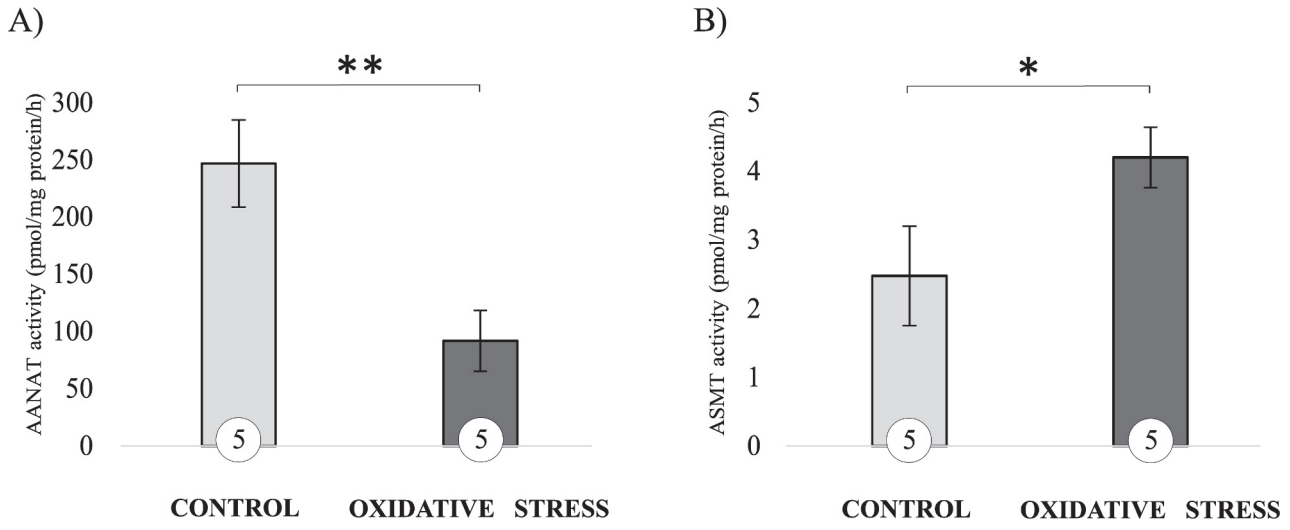

Fig. 3. AANAT activity (A) and ASMT activity (B) in skin homogenates of three-spine stickleback from Experiment 2: control fish (light bars) and $K_2Cr_2O_7$-treated fish (dark bars). Enzymatic reactions were carried out for: 1) AANAT activity at 10 °C with optimal concentrations of serotonin (10.3 mM) and AcCoA (1.03 mM) in phosphate buffer (0.2 M, pH 6.8) and 2) ASMT activity at 10 °C, with the optimal concentration of NAS (1.5 mM) and SAM (0.5 mM) in phosphate buffer (0.1 M, pH 7.9). Values are presented as means ± SEM. Significant differences are indicated as *$P < 0.05$, **$P < 0.01$ (Tukey's HSD *post hoc* test). The number of fish is given in circles.

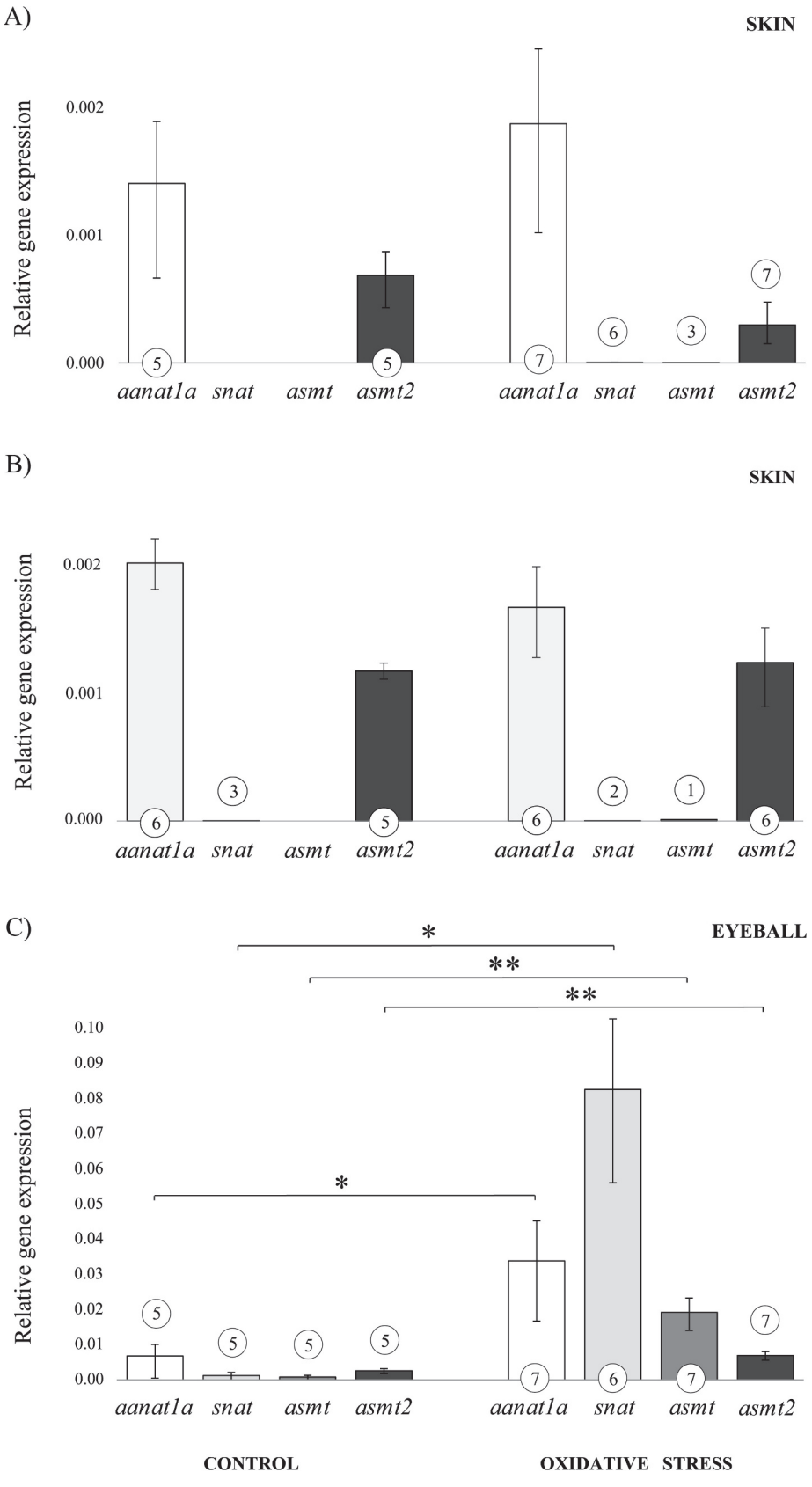

Fig. 4. Relative gene expression in the skin of three-spined stickleback: Experiment 1 (A), Experiment 2 (B), and eyeball: Experiment 1 (C). Fish belong to the control group or the $K_2Cr_2O_7$ treated group. The number of fish where gene expression was found is given in circles. Values are presented as a fold change relative to the reference gene. The measure of variation is derived from the respective SEM of the Cq values. Significant differences are indicated as $*P < 0.05$, $**P < 0.01$ (Tukey's HSD *post hoc* test).

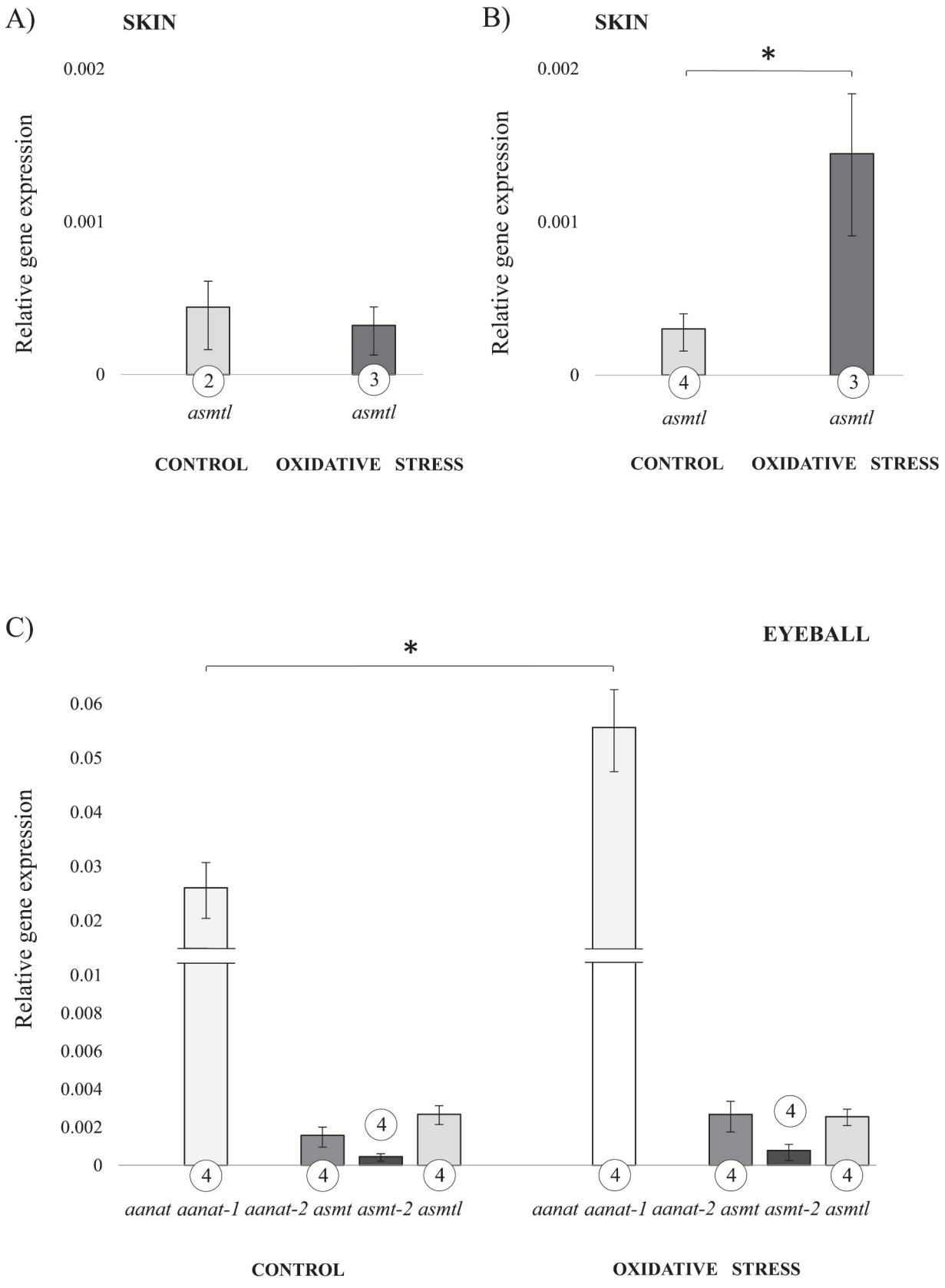

Fig. 5. Relative gene expression in the skin of European flounder: Experiment 1(A), Experiment 2 (B), and eyeball: Experiment 2 (C). Fish belong to the control or $K_2Cr_2O_7$-treated group. The number of fish where gene expression was found is given in circles. Values are presented as a fold change relative to the reference gene. The measure of variation is derived from the respective SEM of the Cq values. Significant differences are indicated as $^*P < 0.05$ (Tukey's HSD *post hoc* test). Neither genes *aanat, aanat-1, aanat-2, asmt,* nor *asmt-2* was expressed in the skin.

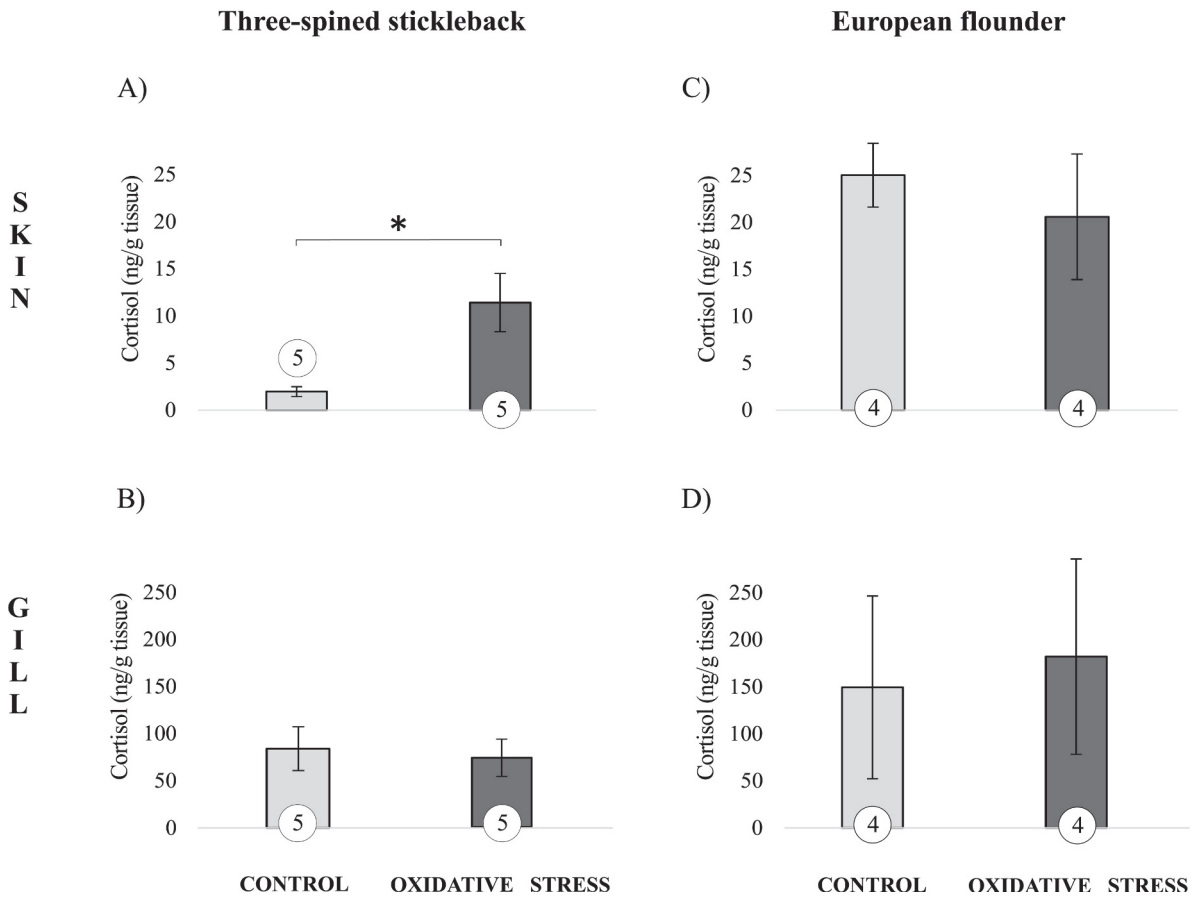

Fig. 6. Cortisol levels in the skin (A) and gills (B) of three-spined stickleback and in the skin (C) and gills (D) of European flounder (Experiment 2): control fish (light bars) and $K_2Cr_2O_7$-treated fish (dark bars). Values are presented as means ± SEM. Significant differences are indicated as $^*P < 0.05$ (Tukey's HSD *post hoc* test). The number of fish is given in circles.

that is, in the eyeball (retina), is affected during the response to oxidative stress. Significantly higher Mel concentration in the eyeball of stressed fish than in the control confirms it (Fig. 2C). Gill Mel concentration (being an approximate measure of circulating hormone level) slightly increases (Fig. 2B). In this way, Mel transported to the skin through the bloodstream may be crucial for the response of the skin to oxidative stress. The pattern of changes in AANAT and ASMT activity in the skin of stressed fish suggests that another metabolic pathway, not directed to Mel, may be privileged in the skin of stressed fish. Increased ASMT and decreased AANAT activities (Fig. 3A, B) may indicate activation of 5-methoxytryptamine (5-MTAM) synthesis after exposure of fish to oxidative stress, but we have no direct evidence that it is true. This topic will be addressed in future studies. It is known that ASMT can methylate *N*-acetylserotonin (NAS) to Mel, as well as 5-HT to 5-MTAM (Fig. 1). However, future studies are needed to confirm the action of ASMT with 5HT as an enzyme substrate in the case of oxidative stress in fish. In mammals, 5-MTAM is a potent agonist of presynaptic 5-HT autoreceptors and modulates serotonin release in the central nervous system (Galzin et al., 1988). The protective effects of 5-MTAM against oxidative stress have also been discussed in mammals (Galano and Reiter, 2018). In fish, Ceinos et al. (2005) provided evidence for the daily cyclic change in 5-MTAM content in the pineal organ of rainbow trout (*O. mykiss*); the 24-h profile of 5-MTAM content reflected that of 5-HT. However, the specific physiological roles of 5-MTAM have not yet been recognized in fish.

In European flounder, we notice a completely different situation: a lack of expression of genes encoding enzymes AANAT and ASMT that could exclude Mel synthesis in the skin of control and stressed fish (Fig. 5A, B). However, it is worth mentioning that Slominski et al. (2003, 2005) observed the acetylation of serotonin to NAS by an alternative *N*-acetyltransferase enzyme (such as NAT-1) in the skin of C57BL/6 mice with AANAT knockout. We did not study the expression of the genes encoding NAT in flounder skin. However, in the absence of any gene responsible for the methylation of *N*-acetylserotonin to Mel in the skin, the source of Mel (Fig. 2D) must be outside the skin and Mel is delivered here by the bloodstream. In the eyeball, the transcripts of *aanat* and *asmt* are evident in control and stressed fish (Fig. 5C), so the necessary conditions are fulfilled for the presence of AANAT and ASMT, and consequently, Mel synthesis here. However, Mel synthesis does not change in the eyeball under oxidative stress (Fig. 2F) despite the increase in the expression of the *aanat-1* mRNA (Fig. 5C). Consequently, the source of the increased Mel in the skin of this species is outside the eyeball. It is probably the pineal organ, a primary source (besides the retina/eyeball) of Mel in fish (Zachmann et al., 1992; Falcón et al., 2011). Furthermore, increased expression of *aanat-1* in the eyeball of flounder, which is not reflected in high Mel production strongly suggests the function of eye NAS beyond that of a precursor to the biosynthesis of Mel. To the authors' knowledge, any distinct role of NAS has not yet been shown in fish; this issue has been discussed in our previous paper (Pomianowski et al., 2020). The eyeball with all four genes expressed in sticklebacks and flounders, made a good reference point and control of the correctness of the analysis of genes expression in the skin.

In this study, cortisol is regarded as a component of CSRS in fish exposed to oxidative stress. As mentioned above, cortisol release is controlled by the paraventricular nucleus of the hypothalamus secreting CRH and arginine vasotocin, which stimulate the release of ACTH from the pituitary, which in turn stimulates the release of cortisol from interrenal cells (Baker et al., 1996; Wendelaar Bonga, 1997; Barton,

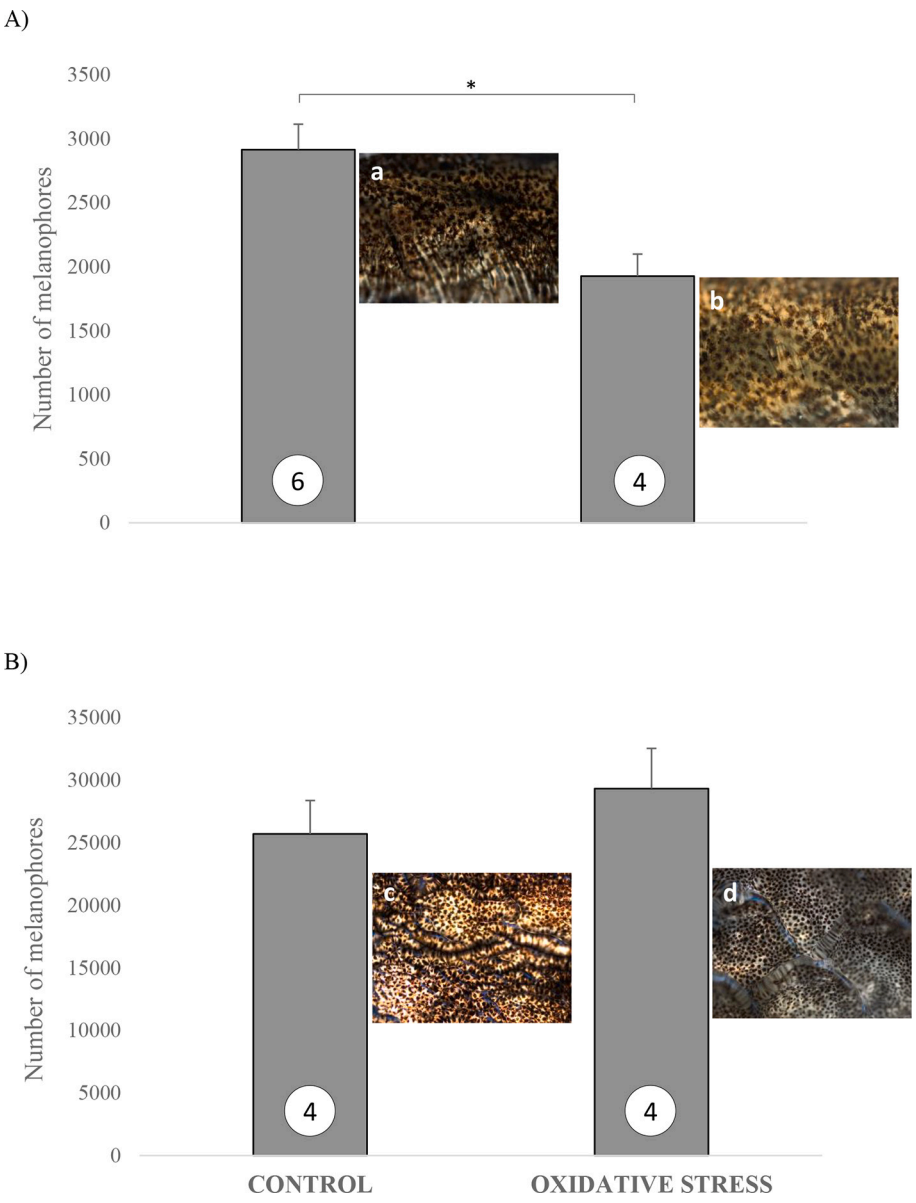

Fig. 7. Number of melanophores in skin samples of three-spined stickleback (A) and European flounder (B) in control and $K_2Cr_2O_7$ treated group (Experiment 2). Photos a − d show melanophores in the control group and after exposure to $K_2Cr_2O_7$, in both species, respectively. A 40-fold magnification was applied. The values are presented as means ± SEM. Significant differences are indicated at $^*P < 0.05$ (Tukey HSD *post hoc* test). The number of fish sampled is given in circles.

2002). As presented in the Introduction, there is evidence that glucocorticoids are synthesized and metabolized in mammalian skin. Recently, it has appeared that cortisol can also be produced in isolated scales of European sea bass (*Dicentrarchus labrax*) while cultured *in vitro* and stimulated with different concentrations of ACTH (Samaras and Pavlidis, 2022). The fish scales are of dermal origin; for this reason, the finding is a starting point for considering skin cells as a source of cortisol in fish. In our study, the cortisol concentration in the skin of stress-exposed sticklebacks is significantly higher than in the skin of control individuals (Fig. 6A). However, there is no difference between cortisol levels in the gills (representing the level of hormone in the bloodstream) in control and stressed fish (Fig. 6B). Therefore, our premise is that the response to oxidative stress in this species involves cortisol synthesized locally in skin cells. However, in the skin of European flounder, there is no difference in cortisol levels between stressed and control individuals (Fig. 6C); cortisol concentration in the gills does not alter after exposure to stress (Fig. 6D). Therefore, the reaction of the European flounder to oxidative stress is not linked to the production and release of cortisol. It is adequate because fish exhibit a wide diversity of physiological responses to stress, reflected in cortisol changes (or lack of changes) under stress conditions (Barton, 2002). Furthermore, the interpretation of experimental data and the comparison between various species can be tricky because the magnitude, duration, and recovery of cortisol changes caused by stress in fish are species-specific (Barton, 2002). It should be taken into account that in many situations, cortisol concentration changes but not in some environmental and physiological conditions (MacDougall-Shackleton et al., 2019); therefore, there are controversies about the value of applying cortisol as a measure of coping with stress (Breuner et al., 2013; Ellis et al., 2012; Romero and Gormally, 2019). It is evident in the case of the three-spined stickleback and European flounder in this study.

Fish with diverse ecological preferences that use different habitats may differ in many features, including body shape and skin characteristics, as in the case of three-spined stickleback and European flounder. The analysis of melanophores in the skin performed in this study shows that exposure of fish to a potassium dichromate solution causes a

significant decrease in their number in sticklebacks but not in flounders (Fig. 7A, B). The different response of the skin to stress in various species is likely due to various characteristics and resistance of their skin to environmental factors and deserves a thorough investigation.

5. Conclusions

The cutaneous stress response system of the three-spined stickleback and European flounder exposed to oxidative stress presents different modes of action: Mel concentration increases in the skin of both species, but cortisol concentration increases in the skin only in sticklebacks. The source of increased skin Mel is outside the skin in both species. On the other hand, cortisol is probably produced in stickleback skin but is not involved in the response to oxidative stress in flounder. Considering the origin of cortisol (flounder) and Mel (both species) in the skin of stress-exposed fish, we suppose that a local stress response system is linked with systems acting on the level of the whole organism, but to what extent? Further research is required to solve this problem. There are also several other issues to be addressed in future studies, among them the expression of the genes encoding NAT, the physiological role of 5-MTAM and the function of *asmtl* in the skin of fish. Moreover, further investigation of species with various ecological preferences and those having skin of different characteristics and resistance to environmental factors can improve our understanding of the general principles underlying the stress response in fish skin and its significance.

Ethics statement

All experiments complied with the Directive 2010/63/EU of the European Commission for animal experiments and with the guidelines and approval of the Ethics Committee for Animal Experimentation of Bydgoszcz University of Science and Technology, Poland; Protocol No 27/2018 and 01/2022.

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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.

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