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A study of *aanat* and *asmt* expression in the three-spined stickleback eye and skin: Not only "on the way to melatonin"

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ABSTRACT

Melatonin synthesis is controlled by aralkylamine *N*-acetyltransferase (AANAT: EC 2.3.1.87) acetylating serotonin (5-hydroxytryptamine; 5-HT) to *N*-acetylserotonin (NAS), and *N*-acetylserotonin *O*-methyltransferase (ASMT: EC 2.1.1.4) methylating NAS to melatonin (Mel; *N*-acetyl-5-methoxytryptamine). We examined the levels of expression of the *aanat* and *asmt* genes, Mel concentrations as well as AANAT isozyme activity in the eyeball (with retina) and skin of the three-spined stickleback (*Gasterosteus aculeatus*), at noon and midnight. We found mRNA of four genes (*aanat1a*, *snat*, *asmt* and *asmt2*) in the eyeball, and two (*aanat1a* and *asmt2*) in the skin. The presence of two transcripts of genes encoding AANAT and two of ASMT in the eyeball at noon and midnight, suggests activity of AANAT and ASMT isozymes in metabolic pathways besides "the way to melatonin", all the more so because day/night changes in Mel concentration do not follow the changes in either the expression of genes or the activity of AANAT. The high effectiveness of noon NAS synthesis in the eyeball at low substrate concentrations, which is not reflected in high Mel production, suggests the function of eye NAS beyond that of a precursor to the biosynthesis of Mel. The inhibition of AANAT isozyme activity by product observed in the eyeball may be one of the mechanisms of 5-HT husbanding in the eye (retina). The presence of transcripts of genes encoding both AANAT and ASMT and the activity of AANAT, at noon and midnight, supports a local Mel synthesis in the sticklebacks' skin.

1. Introduction

Melatonin (Mel; *N*-acetyl-5-methoxytryptamine) is synthesized from serotonin (5-hydroxytryptamine; 5-HT) by successive action of two 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). It is worth mentioning that Mel was first discovered as a substance lightening frog skin color and inhibiting the melanocyte stimulating hormone by Aaron Lerner et al. (1958). Recent decades have seen a flood of information on the metabolic and physiological effects of Mel in vertebrates. Indeed, Mel, as a potent regulator of circadian and seasonal rhythms, an effective antioxidant, and an important component of the immune system, is implicated in a wide spectrum of physiological and behavioral events (for review, see: Carrillo-Vico et al., 2013; Falcón et al., 2009; Hardeland et al., 2011; Pévet, 2003). In teleost fish, circadian changes in skin coloration drew attention to Mel as a factor responsible for pigment aggregation in melanophores (Reed, 1968), but nowadays various roles of Mel are well documented and discussed (Cowan et al., 2017; Drąg-Kozak et al., 2018; Esteban et al., 2013; Kulczykowska, 2002). Consequently, both enzymes, AANAT and ASMT, are recognized as these which are primarily engaged in Mel production. However, AANAT acetylates not only serotonin (5-hydroxytryptamine; 5-HT), to *N*-acetylserotonin (NAS), the precursor of Mel, but also dopamine to *N*-acetyldopamine (Gaudet et al., 1993; Slominski et al., 2002a, 2003, 2005; Zilberman-Peled et al., 2006; Paulin et al., 2015), while ASMT methylates NAS to Mel as well as 5-HT and its metabolites to 5-methoxytryptamine (5-MTAM), 5-methoxyindole acetic acid (5-MIAA) and 5-methoxytryptophol (5-MTOL) (Pévet et al., 1981; Morton, 1987). So that also other metabolic pathways besides Mel synthesis should be taken into consideration in studies of *aanat* and *asmt* expression.

In teleost fish, there are several AANAT isozymes belonging to two AANAT subfamilies, AANAT1 and AANAT2, which are encoded by distinct genes and differ markedly in their substrate preferences, kinetics, and spatial distribution in various organs/tissues (Cazaméa-

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Catalan et al., 2014; Coon and Klein, 2006; Zilberman-Peled et al., 2004, 2006). It is probably a result of the first round of whole-genome duplication followed by subsequent mutations in duplicated genes occurring during teleostean evolutionary history (Falcón et al., 2009, 2011). Coon et al. distinguished two AANAT encoding genes (*aanat1* and *aanat2*), expressed in pike (*Esox lucius*, L.) retina and pineal gland (Coon et al., 1999). Later research showed expression of three genes *aanat1a*, *aanat1b* (also known as *snat*) and *aanat2* in the pufferfish and medaka (*Oryzias latipes*) (Coon and Klein, 2006), sea bass (*Dicentrarchus labrax*) (Paulin et al., 2015), and three-spined stickleback (*Gasterosteus aculeatus*) (Kulczykowska et al., 2017). Regarding ASMT encoding genes in fish, *asmt* and *asmt2* (also known as *hiomt* and *hiomt2)* transcripts or only, *asmt* transcript has been found in the brain, pineal organ, eye, retina, gastrointestinal tract, liver, muscles, gills, gonads and skin of various species (Khan et al., 2016; Muñoz-Pérez et al., 2016; Velarde et al., 2010; Zhang et al., 2017). According to www.ensembl.org, in the three-spined stickleback, there are also two genes (*asmt* and *asmt2*) localized on two different chromosomes (XVI and XV). A presence of several AANAT and ASMT isozymes in fish may point to their activity in various metabolic pathways when other biologically active compounds besides Mel are formed, i.e. *N*-acetyldopamine, 5-MTAM, 5-MIAA and 5-MTOL. Indeed, there is ample physiological evidence that these compounds are engaged in many processes, for instance, *N*-acetyldopamine as sclerotizing agent of the insect cuticle (Karlson and Sekeris, 1962) and metoxyindoles as potent antioxidants and free radical scavengers (Ng et al., 2000).

The main sites of Mel production in vertebrates, including fish, are always the pineal gland and retina, but there are many examples of Mel synthesis in different peripheral organs (for review: Acuña-Castroviejo et al., 2014). For instance, there is the gut, a quantitatively relevant site of Mel production (Bubenik and Pang, 1997), and skin where Mel is a key component of the local mechanism of response to environmental and endogenous stressors (Slominski et al., 2017). Functions of Mel in mammalian skin are well recognized by Slominski's research group and have been reviewed quite recently (Slominski et al., 2018). It is worth stressing that the phenomenon of alternative splicing of AANAT and ASMT leading to different isoforms of enzymes was discovered just in the skin of mammals (Slominski et al., 2002b, 2018). Thus a presence of more than one form of each AANAT and ASMT is neither unique nor restricted only to fish.

In most fish species, Mel synthesis peaks at night and reaches its nadir at midday (Falcón et al., 2010) but there are exceptions to this rule, e.g. Mel secretion from salmonids' pineal organ is independent of the light regime (Gern and Greenhouse, 1988; Iigo et al., 2007). Moreover, Mel synthesis in ectothermic organisms (as fish are) is evidently controlled by temperature (for review: Popek and Ćwioro, 2010). For instance, Mel synthesis rises together with water temperature to the optimum level, beyond which it declines (Iigo and Aida, 1995; Max and Menaker, 1992; Zachmann et al., 1992). This happens most probably because of the influence of temperature on AANAT and/or ASMT activity (Falcón et al., 1996; Morton and Forbes, 1989; Paulin et al., 2015).

In this study, we examined mRNA expression of the *aanat* and *asmt* genes by quantitative real-time polymerase chain reaction preceded by reverse transcription (RT-qPCR) and Mel concentration by radioimmunoassay (RIA) in the eyeball (with retina) and skin of the three-spined stickleback, at noon and midnight. We performed our study in the laboratory at low water temperature of 12 ± 2 °C to prevent sticklebacks from spawning. We aimed to answer the following questions: (i) which of the *aanat* and *asmt* genes are expressed in the eyeball and skin, (ii) whether there are day/night differences in gene expression, (iii) whether there are day/night differences in the activity of AANAT isozymes, and (iv) whether the day/night changes of Mel concentration in the eyeball and skin do follow the changes in either the expression of genes encoding AANAT/ASMT isozymes or the activity of AANAT isozymes.

This study is linked with our previous research on the expression of the *aanat* genes in various organs of the three-spined stickleback and a role of Mel as a component of the cutaneous stress-response system in fish (Kulczykowska et al., 2017, 2018). We have performed our study in the three-spined stickleback, a small teleost fish widely distributed across the Northern Hemisphere in both marine and freshwater environments, because it is easy to collect and maintain in the laboratory at low cost and its genome sequence is readily available (www.ensembl.org), and we have many years of experience with this species.

2. Materials and methods

2.1. Animals and sample collection

Adult three-spine sticklebacks (*Gasterosteus aculeatus*) of both sexes (n = 32) were caught in Oliva's Stream (Northern Poland) in October (out of breeding season) and transported to the Institute of Oceanology PAN (IO PAN Sopot, Poland), where they were acclimatized in 40-L aerated aquaria in freshwater at a temperature of 12 ± 2 °C and a 12L:12D photoperiod for 7 days before sampling. Temperature and photoperiod simulated those in nature in October. During that period fish were fed frozen food (*Chironomus plumosus*) once a day at 15:00. All individuals were out of breeding phase what was confirmed by the analysis of gonads. Gonads were examined using standard histological procedures including embedding in paraffin, sectioning and staining with haematoxylin and eosin. Sections were scanned with a Leica DM500 (Leica, Germany) and an Olympus BX60 light microscope (Olympus, Japan) at magnification 20× and 40×. Midnight samples were taken under the red light from randomly selected individuals sacrificed by decapitation. The eyeballs (with retina) and skin samples were collected around noon (n = 17) and midnight (n = 15) and stored at −70 °C until RNA extraction, AANAT activity assay and Mel measurement. The whole eyeball serves as a benchmark because we have not managed to separate the retina.

2.2. RT-qPCR analysis

2.2.1. RNA extraction

Total RNA was purified with GenElute™ Mammalian Total RNA Miniprep Kit (RTN70, Sigma-Aldrich, St. Louis, MO, USA) with minor modifications. The whole eyeball and approximately 5 × 5-mm skin segments were homogenized on ice in 500 μL of Lysis Solution supplemented with proteinase K (0.6 mg/mL, E4350, EURx) and 2-mercaptoethanol (0.6% *v*/v, M3148, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min at 55 °C. Total RNA concentrations were measured on Epoch™ Microplate Spectrophotometer (BioTek, Winooski, USA). Each sample was treated with RNase-free DNase I (EN0521, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). For 1 μg of total RNA, 3 U of DNase I and 3 μL of 10× reaction buffer were added, followed by incubation at 37 °C for 30 min. Finally, EDTA (0.3 mM) was added to inactivate DNase I (10 min at 65 °C).

2.2.2. Primer design and RT-qPCR conditions

The primers for RT-qPCR analysis (Table 1) were designed using Primer3 software v. 2.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) based on mRNA sequences from Ensembl genome browser. Primers targeting *asmt* transcript (Ensembl transcript accession no. **ENSGACT00000008780.1**) were located in the first and the second exons while primers targeting *asmt2* transcript (Ensemble transcript accession no. **ENSGACT00000006073.1**) were in exons seven and eight. Primers for *aanat1a* and *snat* were previously described by Kulczykowska et al. (2017). Three genes, encoding 18S rRNA, beta-Actin (*actb*), and ubiquitin (*ubc*) were tested as reference candidates appropriate for the examined tissue types (Hibbeler et al., 2008). More than one splice variant was shown by *actb* in the skin while ubiquitin showed the most stable expression in the examined tissues and was

Table 1
Primers used in RT-qPCR.

Gene	Primer name	Strand	Length [bp]	Tm (°C)	5′ → 3′ sequence
asmt	asmt f	+	20	60.1	GCTGACACTTTCCCCAAAAA
	asmt r	−	20	59.9	CAGCCAGCAGTCTCTCAGTG
asmt2	asmt2 f	+	20	60.0	TCTTCAGCGGTGAACTTCCT
	asmt2 r	−	20	60.0	CCCGTCTGTTCTCAAAGAGC
aanat1a[*]	aanat1a f	+	20	60.1	CACATCAGGATGGAGCCTTT
	aanat1a r	−	20	60.0	GGATGCCCTCACTCTCCATA
snat[*]	snat f	+	20	59.8	GACACAGCGTGAGGAAATGA
	snat r	−	20	60.2	GCCATATGCGTCTTCGAGAT
ubc[**]	ubc f	+	19	61.4	AGACGGGCATAGCACTTGC
	ubc r	−	19	60.6	CAGGACAAGGAAGGCATCC

[*] Kulczykowska et al., 2017.
[**] Hibbeler et al., 2008.

chosen as a reference gene (Table 1).

RT-qPCR was performed in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using SensiFAST™ SYBR No-ROX One-Step kit (BIO-72005, Bioline, London, UK). Reaction efficiencies (E) were evaluated from the slopes of standard curves made of 3-point, 2-fold serial RNA dilutions starting at 40 ng/μL, according to $E = 10^{-1/slope} - 1$ equation (Pfaffl, 2004). The optimal conditions, ensuring efficiency in the 89%–108% range for all qPCRs, were established for each assay, except *aanat2*, which showed very low expression in both organs, so it was impossible to optimize qPCR conditions using existing and newly designed primer sets. Each RT-qPCR was run in a total volume of 10 μL containing 1× SensiFAST mix, 0.4 μM of each primer, 1× Reverse Transcriptase, 2 U of RiboSafe RNase Inhibitor, and 20 ng of total RNA. The following 3-step protocol was used: 45 °C for 10 min (RT step), 95 °C for 2 min (initial denaturation), followed by 35 PCR cycles of 5 s at 95 °C (denaturation), 10 s at 64 °C (primer annealing), and 5 s at 72 °C (elongation). Analysis of the melting curve (60–95 °C) concluded the protocol. Fluorescence data were collected after the elongation step and in 0.1 °C steps on the melting curve. Two technical replicates were used for each combination of organ type and primer set.

The relative expression calculation was based on the difference between Ct values for reference and target genes, separately for each tested tissue in biological replicates, according to the Livak and Schmittgen (2001) equation.

2.3. AANAT activity analysis

In order to test a difference between the compositions of AANAT isozymes in the eyeball and skin, analysis of AANAT activity was performed in both organs at corresponding optimal temperatures. Different shapes of AANAT activity curves would indicate a difference between the compositions of AANAT isozymes in the eyeball and skin. For this purpose, the standard enzymatic assay was carried out.

2.3.1. Sample preparation

The single eyeballs (with retina; $n = 10$) and skin samples (10 × 10 mm; n = 10) were weighed and sonicated (Microson XL, Misonix, USA) in 500 μL of 0.2 M phosphate buffer (pH 6.8). Homogenates were then centrifuged at 9200*g* at 4 °C for 20 min. Supernatants were kept on ice for no more than 15 min until AANAT activity assay.

2.3.2. AANAT activity assay

The standard enzymatic assay was carried out in the presence of 20-μL aliquot of the supernatant, 5 μL of 174 mM 5-HT (Sigma, St. Louis, MO, USA) and 10 μL of 4.2 mM acetyl coenzyme A (AcCoA; Sigma, St. Louis, MO, USA) at two optimal temperatures (24 °C for eyeball and 30 °C for skin) for 45 min. The final concentration of 5-HT and AcCoA in the reaction mixture were 24.8 mM and 1.2 mM, respectively. The reaction was stopped by the addition of 5 μL of 6 N perchloric acid (Sigma-Aldrich, St. Louis, MO, USA), the mixture was centrifuged (9200*g*, 4 °C, 20 min) and a 20-μL aliquot of supernatant was injected into the HPLC system. The conditions for enzyme studies as the optimal temperature for the eyeball (24 °C) and skin (30 °C), saturated concentration of 5-HT (24.8 mM) and AcCoA (1.2 mM), optimal molarity and pH of phosphate buffer (0.2 M, pH 6.8) were established in the preliminary studies. To analyze the effect of substrate concentration (5-HT) on AANAT activity, 5-HT concentrations ranging from 0.004 to 24.84 mM were tested in the presence of a 20-μL aliquot of the supernatant and 10 μL of AcCoA (final concentration 1.2 mM) for 45 min.

2.3.3. HPLC analysis

The product of enzymatic reaction, NAS, was measured using Agilent 1200 Series Quaternary HPLC System with fluorescence detector (Agilent Technologies, Germany). Chromatographic separation was achieved on a ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 μm; Agilent, USA). An isocratic elution with 10% acetonitrile in 50 mM ammonium formate (pH 4) at 20 °C and a flow rate of 1 mL/min during 20 min was applied. The fluorescence was recorded at 360 nm after excitation at 280 nm. The identification of NAS was performed by comparing the retention time of the sample with that of the standard (NAS; Sigma, Steinheim, Germany).

2.4. Melatonin analysis

The eyeballs and skin samples were weighed and sonicated in 0.05 M phosphate buffer (pH 7.4) containing 0.01% Thimerosal (Sigma-Aldrich, St. Louis, MO, USA) using Microson XL (Misonix, USA). After centrifuging of homogenates at 15,000*g* for 20 min, supernatants were collected and frozen at −70 °C prior to analysis. Mel concentrations were determined in all supernatants using RIA preceded by an extraction procedure in accordance with the method published by Kulczykowska et al. (2006). The solid phase extraction was conducted using a 10 μm Octadecyl C18 Speedisk Column (J.T. Baker, Philipsburg, NJ, USA). The eluates obtained after extraction were dried under vacuum and stored at −70 °C until analysis. Before RIA procedure, dried samples were suspended in Dulbecco's phosphate buffered saline (Sigma-Aldrich, St. Louis, MO, USA) containing 0.01% Thimerosal. Mel concentration was analyzed using total Mel kit (IBL International, Hamburg, Germany). All samples in duplicate were counted in the Wallace Wizard gamma counter (PerkinElmer). The intra-assay coefficient of variation for tissue samples was 8.7%. Mel recoveries were in the range of 88%–110%. The inter-assay coefficient was not determined, because all samples were measured in the same assay. The detection limit was 3.5 pg mL^{-1} of the extract.

2.5. Statistical analysis

Statistical analyses of data were done using Statistica 8.1 software. Values were presented as mean ± standard deviation (SD; AANAT

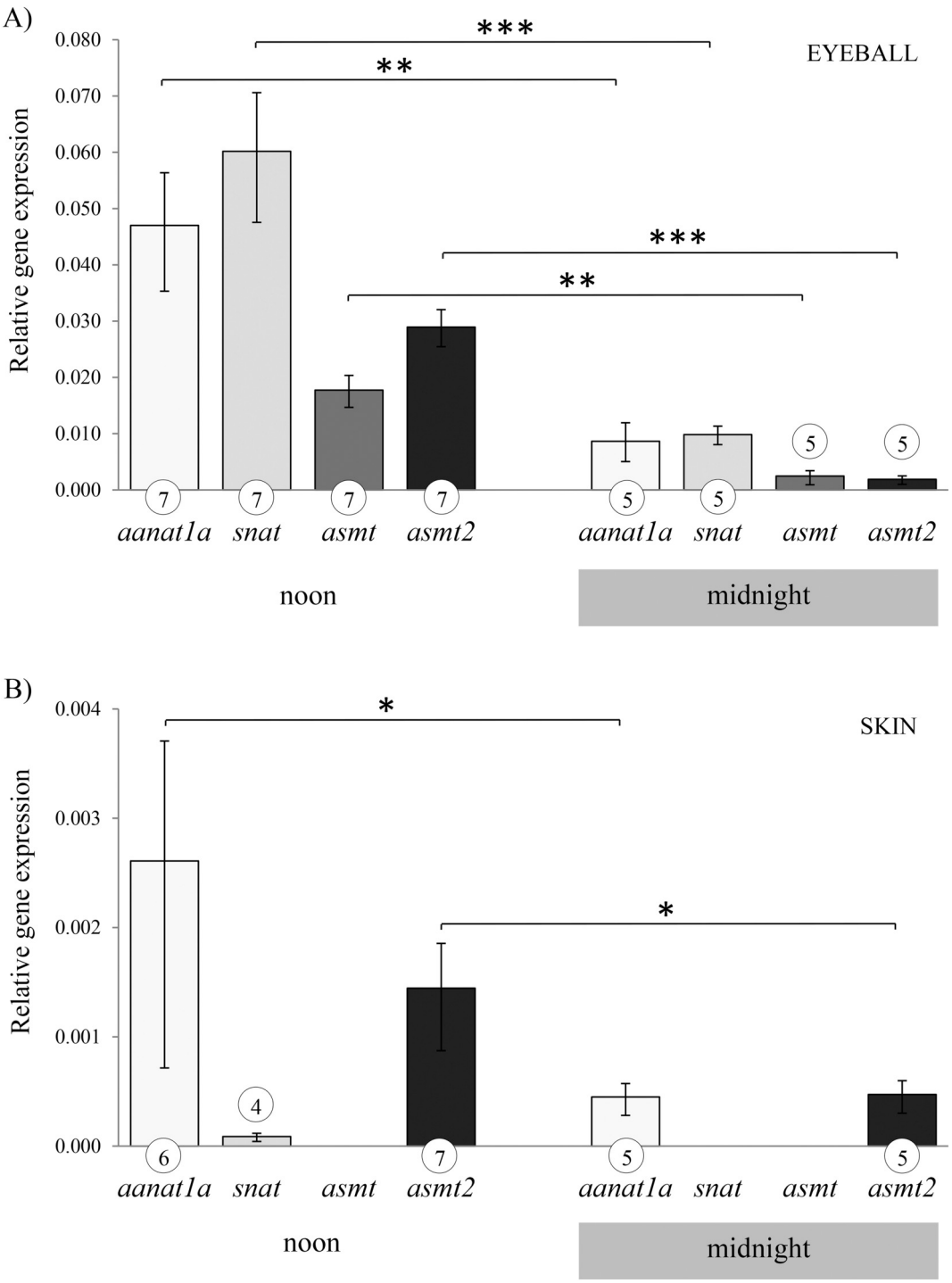

Fig. 1. Relative gene expression in eyeball (A) and skin (B) of the three-spined stickleback (*Gasterosteus aculeatus*) at noon and midnight. Number of sampled fish is given in the circles. Values are presented as fold change relative to reference gene. Measure of variation is derived from respective SEM of Cq values. Significant differences between noon and midnight are indicated in the eyeball as $^{**}P < .01$, $^{***}P < .001$, and in the skin as $^{*}P < .05$. In the skin, *snat* mRNA expression was on the detection limit at noon and below the detection limit at midnight; *asmt* mRNA expression was below the detection limit at noon and midnight.

activity and Mel concentration) or mean ± standard error of the mean (SEM; relative gene expression). For comparisons of day/night differences in *asmt2*, *asmt*, *aanat1a*, and *snat* mRNA levels, Mel concentrations, and AANAT activities in the eyeballs and skin samples, Student's *t*-test was used. Statistical significance was set at $P < .05$.

3. Results

Four genes, *aanat1a*, *snat*, *asmt*, and *asmt2*, were expressed in the eyeball and two, *aanat1a* and *asmt2*, in the skin, and significantly higher levels of their mRNA were shown at noon than midnight (Fig. 1A,B). The skewed shape of the curve depicting the activity of AANAT isozymes in the eyeball that is evident at noon strongly suggests regulation of the isozymes by the reaction product, NAS (Fig. 2). There were no significant day/night differences in AANAT activity either in the eyeball or skin (Table 2). There were significant differences between midnight and noon Mel concentrations with the highest levels at midnight in both eyeball and skin (Fig. 3).

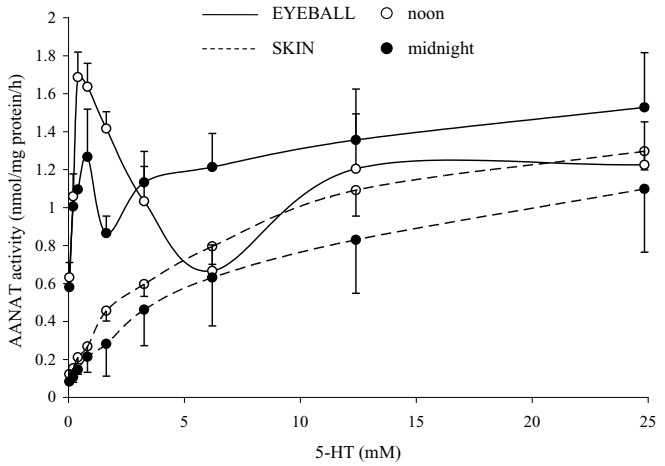

Fig. 2. AANAT activity in skin and eyeball homogenates at noon and midnight. Enzymatic reaction was performed at: 24 °C for eyeball and 30 °C for skin, various concentrations of 5-HT ranging from 0.004 mM to 24.84 mM, AcCoA (1.2 mM), phosphate buffer (0.2 M) and pH = 6.8 for 45 min. Values are expressed as mean ± SD; n = 5.

4. Discussion

In this study, we examined the expression of genes encoding the AANAT and ASMT isozymes and measured Mel concentrations in the sticklebacks' eyeball (with retina) and skin at noon and midnight. We also evaluated activity of AANAT in these organs. We found mRNA expression of four genes (*aanat1a*, *snat*, *asmt* and *asmt2*) encoding two AANAT and two ASMT isozymes in the eyeball and two genes (*aanat1a* and *asmt2*) encoding one AANAT and one ASMT isozyme in the skin at noon and midnight (Fig. 1A,B). The expression of the genes was significantly higher at noon than at midnight whereas the opposite was true for Mel concentration (Fig. 3).

Two *aanat* and two *asmt* gene transcripts identified in the eyeball at noon and midnight, strongly suggests activity of two AANAT and two ASMT isozymes just in the eye. We suppose that AANAT and ASMT isozymes in sticklebacks' eye (retina?) can be active in different metabolic pathways in addition to "the way to melatonin", the more so because day/night changes in Mel concentration do not follow the changes in either the expression of *aanat* and *asmt* genes or the activity of AANAT. It is established that AANAT isozymes are involved in acetylation of aralkylamines other than serotonin. Although a first

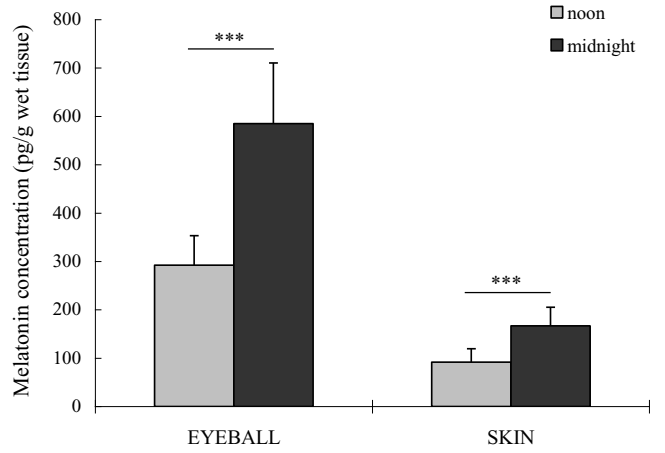

Fig. 3. Melatonin concentration in the eyeball and skin of the three-spined stickleback (*Gasterosteus aculeatus*) at noon and midnight. Values are presented as means ± SD. Significant differences between noon and midnight are indicated as ***P < .001; *n* = 10.

Table 2
AANAT activity in the eyeball and skin of the three-spined stickleback (*Gasterosteus aculeatus*) at noon and midnight. Enzymatic reaction was performed at optimal temperatures (24 °C for eyeball and 30 °C for skin), saturated serotonin concentration (24.84 mM) with AcCoA (1.2 mM) in phosphate buffer (0.2 M; pH = 6.8) for 45 min. Values are expressed as mean ± SD. Statistical significance was taken at $P < .05$; ns – non-significant; $n = 5$.

	AANAT activity (nmol/mg protein/h)			
	Eyeball		Skin	
Noon	1.23 ± 0.22	$P = .12$ (ns)	1.30 ± 0.09	$P = .20$ (ns)
Midnight	1.53 ± 0.27		1.10 ± 0.34	

report on acetylation of dopamine by AANAT in mammals was provided as early as 1993 by Gaudet et al., it was not until 2006 that a possible role of AANAT1 in dopamine metabolism was proposed in fish retina (Zilberman-Peled et al., 2006). Then Paulin et al. (2015) have shown in the sea bass that AANAT1a and AANAT1b can acetylate both serotonin and dopamine, but the affinity and catalytic efficiency for indolethylamines and phenylethylamines of isozymes are different. Also ASMT isozymes are involved in methylation of indolamines other than NAS ("on the way to melatonin"), for instance these involved in 5-HT metabolism: 5-HIAA (5-hydroxyindole acetic acid) and 5-HTOL (5-hydroxytryptophol) (Pévet et al., 1981; Morton, 1987). Furthermore, Tan et al. (2016) has proposed an alternative Mel synthesis pathway where 5-HT is methylated by ASMT to 5-MTAM and then 5-MTAM is acetylated by AANAT to Mel. Indeed, there are many alternatives for AANAT and ASMT action in vertebrates but the specific physiological roles of their products in fish have not been recognized yet. Besides, NAS having antioxidant properties and being produced intensively in the eyeball can have other roles than serving as a precursor of the biosynthesis of Mel. This issue will be discussed in a later section.

Relevant up-to-date literature on the genes encoding AANAT isozymes in different organs of several fish species have been recently reviewed (Kulczykowska et al., 2017). In fish as in other vertebrates, mainly *aanat* genes draw researchers' attention in line with the prevailing opinion that just AANAT determines the rate of Mel production. Indeed, for a long time, the changes in AANAT activity have been considered responsible for the daily rhythm of Mel production (Klein et al., 1997). However, Liu and Borjigin (2005), using a combination of various methodological approaches, strongly suggest that AANAT does not determine the rate of Mel formation in rats' pineal gland at night. For instance, they have shown that NAS concentrations greatly exceed those of Mel, and Mel levels do not follow NAS increases observed by long-term pineal microdialysis. In a paper by the same research group, a revised view of the rate-limiting step in Mel formation in mammals in vivo has been discussed (Chattoraj et al., 2009). The vast excess of NAS that is seen in rats is consistent with emerging evidence that NAS might play more roles than that of a precursor of the biosynthesis of Mel. Indeed, NAS itself, having antioxidant properties (Oxenkruk, 2005; Álvarez-Diduk et al., 2015), has been suggested to protect mammalian retinal photoreceptor cells from light-induced degeneration (Shen et al., 2012; Tosini et al., 2012). To the authors' knowledge, any distinct role of NAS has not yet been defined in fish, but the reason to take it into consideration appeared as early as 1984 when Falcon et al. investigated the localization and circadian variation of 5-HT and NAS in pike photoreceptor cells using immunocytochemical procedures (Falcón et al., 1984). We suppose that in sticklebacks' eyeball (retina?) where an activity of AANAT in production of NAS, especially at low substrate concentrations, is very high, NAS itself, independently of serving as a chemical intermediate in biosynthesis of Mel, would have neuroprotective functions, as has been shown in mammals by Tosini et al. (2012) and Shen et al. (2012). Unfortunately, to the authors' knowledge, there are no data on specific actions of NAS in fish eye (retina).

A very high level of *aanat1a* and *snat* mRNAs found in the eyeball is

similar to our earlier results in the three-spined stickleback (Kulczykowska et al., 2017), but in contrast to previous study, day/night changes in Mel concentration do not follow day/night changes in the expression of *aanat* gene in the eyeball. Also in the eyeball, expression of *aanat1a* was shown previously as extremely high at midnight and noon in contrast to current study where *aanat1a* and *snat* expressions are similar. However, previously, fish were kept at a temperature of 20 °C and a 12L:12D photoperiod and could be in early breeding phase when visible signs (e.g. changed coloration) are usually unnoticed in this species. Unfortunately the analysis of gonads had not been done at that time. Indeed, the physiological stage (for instance breeding phase) does matter as there is a clear relationship between brain Mel profiles and reproductive phases in sticklebacks (Sokołowska et al., 2004). In current study, we maintain sticklebacks at a temperature of 12 ± 2 °C and a 12L:12D photoperiod and all individuals are out of breeding phase what is confirmed by the analysis of gonads. Furthermore, a higher temperature (20 °C) could work in favour of *aanat1a* expression and, in this way, could influence the isozymes ratio in the eyeball and in consequence, the substrate preferences and kinetics of AANAT "mixture" in the eyeball. The relationship between temperature and AANAT1 activity was also observed in in vitro experiments with retinas and recombinant AANAT1 in various fish species. For instance, the AANAT1 activity in the pike, sea bass and sea bream increased with the temperature to 36–37 °C and then declined (Coon et al., 1999; Falcón et al., 1996; Paulin et al., 2015; Zilberman-Peled et al., 2004), but in the trout AANAT1 the peak was observed at 25 °C (Benyassi et al., 2000).

Since the role of ASMT in formation of either Mel or other biologically active components should not be neglected, in addition to the expression of *aanat* genes, we have also examined the expression of *asmt* genes. We have confirmed the expression of two genes (*asmt* and *asmt2*) in the three-spined sticklebacks, as predicted after transcriptomic survey (www.ensembl.org). Our data showing different distribution of *asmt* and *asmt2* gene products, i.e. *asmt* transcript only in the eyeball (including retina) and *asmt2* in both eyeball and skin are in agreement with other studies in fish. Indeed, the transcriptomic analysis of *Sinocyclochelius* fish and mudskippers (*Boleophthalmus pectinirosris* and *Periophthalmus magnuspinnatus*) by Zhang et al. (2017) revealed high levels of *asmt* transcript in the eyeball, and *asmt2a* and *asmt2* transcripts in various organs, including skin. Also Velarde et al. (2010) demonstrated *asmt2* (*hiomt2*) transcript in the retina, pineal gland, liver, and gonads of goldfish. It is notable that in our study, the expression of the genes encoding AANAT as well as ASMT was significantly higher at noon than at midnight.

We are aware that the measures of the gene expression (mRNA levels) may not correspond directly to measures of the final bioactive products (AANAT/ASMT isozymes), in part because there are several steps in between the mRNA production and the product formation. Furthermore, a long interval between a peak of mRNA levels of the genes encoding AANAT and ASMT (at noon) and a peak of Mel concentration (at midnight) in the eyeball and skin implies that the rhythmicity of Mel synthesis is controlled rather by translational and posttranslational processes contributing to local clock physiology than by the expression of the genes encoding AANAT and ASMT. Moreover, the activity of the enzymes strictly depends on the chemical environment, which varies in various organs/tissues and is affected by many external (e.g., light, temperature) and internal factors (e.g., physiological condition), and in general is regulated in a very complicated way in the cell. Thus a lack of direct correlation between mRNA levels of the genes encoding AANAT/ASMT isozymes, AANAT activity and final Mel concentration in the organs is not unexpected. However, the expression of two *aanat* genes (*aanat1a* and *snat*) in the eyeball and only one in the skin (*aanat1a*) has led us to the conjecture that the AANAT isozyme composition is different in the eyeball and skin. To address this issue, we analyzed AANAT activity in both organs at corresponding optimal temperatures, 24 °C for the eyeball and 30 °C for the skin. Different shapes of AANAT activity curves (Fig. 2) reflecting different characteristic of isozymes mixture at optimal temperatures tell us about the discrepancy between the compositions of AANAT isozymes in the eyeball and skin. Indeed, a similar expression of both *aanat1a* and *snat* genes in the eyeball may suggest a balance between two AANAT isozymes. In the skin, on the other hand, the expression of only one *aanat1a* gene indicates a presence of only one AANAT isozyme. It agrees with other studies showing AANAT isozymes of different substrate preferences and kinetics which are present in various organs/tissues (Cazaméa-Catalan et al., 2014; Coon and Klein, 2006; Zilberman-Peled et al., 2004, 2006). However, further studies are needed to establish a relationship between temperature and activity of particular isozymes in the eyeball and skin.

In our study, we do not observe day/night differences in the activity of AANAT isozymes in the eyeball and skin (Table 2). But the skewed shape of the curve depicting the relationship between substrate (5-HT) concentration and AANAT isozyme activity in the eyeball, especially at noon (observed in all five of the five experiments we performed, Fig. 2) strongly suggests an inhibition of the isozymes by the reaction product, NAS. A similar effect has been observed in studies of recombinant AANAT1 isozyme in the sea bream (Zilberman-Peled et al., 2004). The product inhibition may be one of the regulatory mechanisms of 5-HT husbanding in the retina. In retinal cells, 5-HT is an important neurotransmitter and/or neuromodulator per se, not only a substrate in the reaction of NAS synthesis (for review see: Masson, 2019). There are several papers supporting the role of 5-HT as neurotransmitter or neuromodulator in fish retina, which suggests that 5-HT may be involved in the activity of the ganglion cells as well as in regenerating processes, for instance in goldfish (*Carassius auratus*) (Hensley and Cohen, 1992; Lima et al., 1996). Indeed, just at noon (at full brightness), the protection against the light might be physiologically relevant. On the other hand, the inhibition of AANAT activity by substrate (5-HT) could also be considered, as it was presented as early as 1978 by Binkley et al. in the chicken pineal gland, but the shape of our curve at higher 5-HT concentrations does not support it. Moreover, a distinct peak of AANAT isozyme activity at very low, physiological concentrations of 5-HT (Sebert et al., 1985) indicates a very high efficiency of 5-HT acetylation just in the eyeball (with retina) at noon. The peaks are observed in all five experiments curried out in this study, so it is not coincidental.

And last but not least, the expression of genes encoding main enzymes in Mel synthesis pathway, AANAT and ASMT, and the activity of AANAT isozymes in the skin testify to Mel production in the skin of the three-spined stickleback. Although we do not find any noon/midnight differences in the AANAT isozymes activity in the skin, NAS formation in the sticklebacks' skin is evident at noon and midnight. Altogether, these data support our previous strong suggestion that Mel is synthesized locally in fish skin (Kulczykowska et al., 2017, 2018). However, in contrast to the eyeball, the skin does not seem to be an important source of Mel, because its cutaneous concentration is rather low. Also our study on the expression of genes encoding AANAT and ASMT shows that the levels of *aanats* and *asmts* mRNA in the skin are low so that Mel can be derived, to some extent, by circulation.

5. Conclusions

Our data show that: (i) two *aanat* and two *asmt* gene transcripts are present in the eyeball, (ii) AANAT isozyme activity in the eyeball is most probably regulated by the reaction product, NAS, (iii) day/night changes of Mel concentration in the eyeball do not follow the changes in either the expression of genes encoding AANAT isozymes or the activity of AANAT isozymes. The presence of two transcripts of AANAT and two of ASMT encoding genes in the eyeball suggests the activity of the isozymes here in other metabolic pathways besides Mel synthesis. The high effectiveness of noon NAS synthesis in the eyeball at low substrate concentrations, which is not reflected in high Mel production,

suggests the function of eye NAS beyond that of a precursor to the biosynthesis of Mel. The inhibition of AANAT isozyme activity by product may be one of the mechanisms of 5-HT husbanding in the eye (retina). However, further studies in fish are necessary in order to recognize a physiological role of biologically active compounds such as *N*-acetyldopamine, 5-MTAM, 5-MIAA and 5-MTOL which are formed in addition to Mel in reactions mediated by AANAT and ASMT. Also a distinct function of NAS in fish eyeball (retina?) deserves to be addressed. The presence of transcripts of both AANAT and ASMT encoding genes together with the activity of AANAT in the skin, at noon and midnight, supports a local Mel synthesis in the sticklebacks' skin. This study shows that noon/midnight rhythm in Mel concentration is not regulated by changing activity of AANAT isozymes at the site of their action in the eyeball and skin, so that the activity of ASMT isozymes will be taken into consideration as a potential factor affecting the daily rhythm of Mel production in our future studies.

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Ethical approval

All experiments complied with EC Directive 2010/63/EU for animal experiments and with the guidelines and approval of the Local Ethics Committee on Animal Experimentation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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