Comparative Biochemistry and Physiology, Part A 208 (2017) 46–53

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Review

The time enzyme in melatonin biosynthesis in fish: Day/night expressions of three aralkylamine *N*-acetyltransferase genes in three-spined stickleback

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ARTICLE INFO

Article history:
Received 26 April 2016
Received in revised form 9 March 2017
Accepted 11 March 2017
Available online 16 March 2017

Keywords:
aanat expression
AANAT isozymes
Absolute RT-qPCR
Aralkylamine *N*-acetyltransferase
Day/night changes
Fish
Melatonin
Three-spined stickleback

ABSTRACT

In vertebrates, aralkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87) is a time-keeping enzyme in melatonin (Mel) biosynthesis. Uniquely in fish, there are several AANAT isozymes belonging to two AANAT subfamilies, AANAT1 and AANAT2, which are encoded by distinct genes. The different substrate preferences, kinetics and spatial expression patterns of isozymes indicate that they may have different functions. In the three-spined stickleback (*Gasterosteus aculeatus*), there are three genes encoding three AANAT isozymes. In this study, for the first time, the levels of *aanat1a*, *aanat1b* and *aanat2* mRNAs are measured by absolute RT-qPCR in the brain, eye, skin, stomach, gut, heart and kidney collected at noon and midnight. Melatonin levels are analysed by HPLC with fluorescence detection in homogenates of the brain, eye, skin and kidney. The levels of *aanats* mRNAs differ significantly within and among organs. In the brain, eye, stomach and gut, there are day/night variations in *aanats* mRNAs levels. The highest levels of *aanat1a* and *aanat1b* mRNAs are in the eye. The extremely high expressions of these genes which are reflected in the highest Mel concentrations at this site at noon and midnight strongly suggest that the eye is an important source of the hormone in the three-spined sticklebacks. A very low level of *aanat2* mRNA in all organs may suggest that AANAT1a and/or AANAT1b are principal isozymes in the three-spine sticklebacks. A presence of the isozymes of defined substrate preferences provides opportunity for control of acetylation of amines by modulation of individual *aanat* expression and permits the fine-tuning of indolethylamines and phenylethylamines metabolism to meet the particular needs of a given organ.

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http://dx.doi.org/10.1016/j.cbpa.2017.03.005
1095-6433/

1. Overview

The aralkylamine *N*-acetyltransferase also known as serotonin *N*-acetyltransferase (AANAT; EC 2.3.1.87) belongs to the acetyltransferase enzymes superfamily. In vertebrates, it is a well-recognized enzyme controlling the daily rhythm of melatonin synthesis (Falcón et al., 2009). Melatonin (Mel; *N*-acetyl-5methoxytryptamine) is a well-known component of the endogenous circadian clock and calendar in vertebrates, including fish (Reiter et al., 2010). This timekeeping hormone is synthetized from serotonin by successive action of two enzymes: AANAT and acetylserotonin *O*-methyltransferase (ASMT; EC 2.1.1.4.) at two major sites: pinealocytes in the pineal gland and photoreceptor cells in the retina, mostly during the night (Klein et al., 1997). It is established that the daily rhythm of Mel production is regulated by diurnal changes in AANAT capacity to acetylate serotonin and therefore AANAT has been named the "Timezyme" (Klein, 2007). In animals living in temperate and arctic regions, an annual pattern of Mel synthesis serves as an "endocrine calendar": there are short-night periods of increased synthesis in summer and long-night periods of increased synthesis in winter (García-Allegue et al., 2001; Sokołowska et al., 2004). A pattern of Mel synthesis in fish, as ectothermic, is regulated not only by photoperiod but also temperature which fluctuates on a daily and seasonal basis (Zachmann et al., 1991, 1992; Falcón et al., 1994). In several fish species as rainbow trout (*Oncorhynchus mykiss*), white sucker (*Catostomus commersoni*) and goldfish (*Carassius auratus*), Mel synthesis increases with rising water temperature but only within the optimal temperature ranges which are different for each species (Max and Menaker, 1992; Zachmann et al., 1992; Iigo and Aida, 1995). This effect has been explained by influence of temperature on kinetic properties of the enzymes in Mel biosynthesis pathway; among them is AANAT (Cazaméa-Catalan et al., 2012, 2013).

Teleost fish are unique among vertebrates because they possess several AANAT isozymes belonging to two AANAT subfamilies, AANAT1 and AANAT2, while in most vertebrates only a single AANAT has been identified to date. In terms of sequence, teleostean AANAT1 subfamily is more closely related to AANAT of other vertebrates, while AANAT2 subfamily has no counterpart (Coon and Klein, 2006; Cazaméa-Catalan et al., 2014). Quite recently Cazaméa-Catalan et al. (2014) and Li et al. (2016), in in silico studies, have presented multiple AANAT isoforms in several fish species, but the first data on different characteristic of AANAT isozymes in fish in the retina and pineal have been provided by Falcón et al. (1996), Coon et al. (1999) and Benyassi et al. (2000). For instance, Falcón et al. (1996) have been the first to consider the presence of a different AANAT in the pineal organ and retina in the northern pike (*Esox lucius*), and Coon et al. (1999) have shown a comparable affinity of AANAT1 for indolethylamines and phenylethylamines in the retina and a preferential affinity of AANAT2 for indolethylamines in the pineal in the same species. The later reports on substrate preferences, kinetics and spatial expression patterns of AANAT1 and AANAT2 have demonstrated that AANAT2 is dedicated to Mel synthesis and AANAT1 covers wider range of activities (Zilberman-Peled et al., 2004, 2006). For example, AANAT1 acetylating aralkylamines besides serotonin (Nisembaum et al., 2013; Paulin et al., 2015) can be involved in metabolism of dopamine and preventing toxic reactions (Iuvone et al., 2005; Zilberman-Peled et al., 2006). There are several findings in fish indicating that AANAT1 in the retina has a much wider role beyond "timezyme". Besseau et al. (2006) have shown that the activity of AANAT1 in the rainbow trout retina is low throughout the night and increases during the light period, in contrast to activity of AANAT2 in the pineal organ. They proposed that the function of AANAT1 in the retina is related to aromatic amine detoxification rather than keeping daily rhythm of Mel synthesis (Besseau et al., 2006). It may explain high Mel production in the retina during the photophase that was reported earlier by Gern and colleagues in this species (Gern et al., 1978).

Regarding the genes encoding AANAT in fish, as early as 1998 Bégay and co-workers have detected AANAT transcripts in the pineal organ of the rainbow trout and pike and in the retina and pineal organ of the zebrafish (*Danio rerio*) (Bégay et al., 1998). Coon with co- workers thereupon has distinguished two different AANAT genes (*aanat*) in the pike, *aanat1* expressed in the retina and *aanat2* in the pineal gland (Coon et al., 1999). Three different AANAT genes, two related to AANAT1, *aanat1a* and *aanat1b*, and third related to AANAT2, *aanat2*, have been shown by in silico analysis in the genome of the pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*) and medaka (*Oryzias latipes*) by Coon and Klein (2006). Later on, Isorna et al. (2011) have cloned two *aanat* cDNAs corresponding to the *aanat1a* and *aanat1b*, and analysed the relative expression of these genes by RT-qPCR in the retina of the sole (*Solea senegalensis*) (Isorna et al., 2011). Authors have postulated the different functions of two isoforms, because the profiles of *aanat1a* and *aanat1b* expression changed during sole development: *aanat1a* mRNA predominated before metamorphosis and did not exhibit rhythmicity and the *aanat1b* mRNA predominated after metamorphosis and displayed a daily rhythm. Quite recently Paulin et al. (2015) have demonstrated in the sea bass (*Dicentrarchus labrax*) that *aanat1a* and *aanat1b* are expressed in different brain regions, retina and peripheral organs/tissues, i.e. *aanat1a* and *aanat1b* in gonads, intestine, liver and muscle, and *aanat1b* in gills and heart whereas *aanat2* only in pineal and gonads. The findings of rhythmic expression of *aanat2*, lower at midday and higher at midnight, in the turbot (*Scophthalmus maximus*), zebrafish and sole (*Solea senegalensis*) at different stages of development (Vuilleumier et al., 2007; Isorna et al., 2009) are in agreement with the role of AANAT2 in timekeeping (Falcón et al., 2009).

In our study, for the first time, the levels of *aanat1a*, *aanat1b* and *aanat2* mRNAs are analysed by absolute quantitative real-time polymerase chain reaction preceded by reverse transcription (RT-qPCR) in the whole brain, eye, skin, stomach, gut, heart and kidney of the three-spined stickleback (*Gasterosteus aculeatus*). A quantitative comparison of three *aanat*s expression in various organs of the three-spined stickleback at daytime and night is the first step to recognize physiological status of AANAT isozymes at different locations in fish. There is a lot of evidence that AANAT role extends far beyond that of the time enzyme in Mel biosynthesis, thus AANAT as a multi-faceted enzyme merits consideration and extensive studies.

1.1. Why the absolute quantitative RT-qPCR?

In real-time PCR, there are two strategies: genes mRNA levels can be quantified by relative or absolute RT-qPCR. So far, in most studies of expression of *aanat*s the relative method has been applied. In such a case, one or more housekeeping genes are recommended for data normalization and the expressions of genes of interest in different organs/tissues represent the relative values versus the reference (organ or tissue) (Livak and Schmittgen, 2001; Pfaffl, 2001). However, such scientific approach where arbitrarily chosen organ/tissue is used as a reference has its limitation because results strongly depend on this choice and may mislead. What is more, there is no justification from either physiological or anatomical point of view for the choice of any particular organ/tissue as a reference. The pitfalls associated with using the reference genes in qPCR technique and the relative method in calculation of mRNA levels are extensively discussed by Kozera and Rapacz (2013). Also Paulin et al. (2015) have found the comparison between relative amounts of *aanat* mRNAs in the retina and pineal problematic. The absolute quantitative RT-qPCR method where mRNA levels are calculated on the basis of a standard curve helps us to deal with the problem and enables us to compare the expressions of genes encoding AANATs in different organs.

1.2. Why the sticklebacks?

The abundance, wide distribution in the northern hemisphere, in salt and fresh water, ease of collecting, and interesting reproductive behaviour of male and female (*vide* Niko Tinbergen's pioneering work that resulted in his Nobel Prize in Physiology and Medicine in 1973) have made the three-spined stickleback a popular research organism also in our laboratory (for example: Sokołowska et al., 2004; Gozdowska et al., 2006; Kleszczyńska et al., 2012; Kleszczyńska and Kulczykowska, 2013; Kulczykowska and Kleszczyńska, 2014). As early as 1970 Chen and Reisman demonstrated 21 cytologically visible chromosomes (Chen and Reisman, 1970) and nowadays the NCBI MapView shows three genes encoding AANAT in in this species: two, *aanat1a* and *aanat2* are co-located on chromosome 11 and *aanat1b* is separately located on chromosome 9. In 2012, David Kingsley and his colleagues at Stanford University have sequenced the entire genetic code of sticklebacks from different habitats (Jones et al., 2012). It has enabled us to design the primers for *aanat*s amplicons on the basis of known sequences and use the absolute RT-qPCR method to calculate and compare *aanat1a*, *aanat1b* and *aanat2* mRNA levels in various organs of the three-spined stickleback.

2. Materials and methods

2.1. Animals and procedures

Adult three-spined sticklebacks (Gasterosteus aculeatus) of both sexes (n = 20) were caught beyond the breeding season in Oliva's Stream (Northern Poland). Fish were kept in an aquarium (60 l) with fresh water at 20 °C and photoperiod 12 L:12D for one month. Fish received frozen food (*Chironomus plumosus*) ad libitum at the same time of the day. Three days before sampling feeding was stopped. Fish were anesthetized by immersion in 0.5% (v/v) 2-phenoxyethanol water solution and after transection of the spinal cord the whole brain (pineal included), eye ball (retina included), skin, stomach, gut, heart and kidney were removed and immediately processed for total RNA isolation. Brain, eye ball, skin and kidney were weighted and stored at −70 °C prior to analyses of Mel. The samples were collected at noon and midnight when the Mel values reach minimum and maximum as has been found in our previous studies in this species.

All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for use and experimentation of laboratory animals and with the Guidelines of the Local Ethics Committee on Animal Experimentation.

2.2. Absolute expression of aanats

Total RNA was extracted using GenEluteTM Mammalian Total RNA Miniprep Kit (RTN70, Sigma) according to the manufacturer's instructions. First, concentration of RNA was measured using EpochTM Microplate Spectrophotometer (BioTek). Next, aliquots of 1 μg of not pooled RNA samples were transferred to the new tube for removal of genomic DNA using DNase I, RNase-free (EN0521, Thermo Scientific). After that quantitative reverse- transcription followed by real-time polymerase chain reaction (RT-qPCR) was performed. Briefly, the cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit for RT- qPCR (K1641, Thermo Scientific) containing reaction buffer, dNTPs, oligo(dT)18, random hexamer primers and Maxima Enzyme Mix (RT mixture). Reverse transcription was performed in the presence (RT+) and absence (RT-) of reverse transcriptase (RT) in parallel to assess contamination of the RNA samples by genomic DNA. The RT+ and RT- mixtures were incubated, first at 25 °C for 10 min and 60 °C for 20 min to promote cDNA synthesis, then at 85 °C for 5 min to terminate the reaction. Finally, the RT-PCR was carried out using Illumina Eco Real Time PCR System. Each reaction mixture of 10 μl contained 5 μl of Luminaris Color HiGreen qPCR 2 × Master Mix (K0392, Thermo Scientific), 2 μl of forward and reverse primers' mix (4 μM each), 2 μl of water and 1 μl of cDNA (10 ng). The PCR profile was as follows: 50 °C, 2 min; 95 °C 10 min; [95 °C, 15 s; 59 °C, 30 s; 72 °C, 20 s] × 35 cycles; melting curve [95 °C, 15 s; 60 °C to 95 °C, 3 min]. The melting curve analysis was performed to assess whether the single specific products were amplified.

The primers for *aanat*s amplicons were designed with Primer3 software v. 0.4.0 (available in http://frodo.w.i.mit.edu/) based on the *aanat*s sequences from the three-spined stickleback deposited in Ensembl genome browser (http://www.ensembl.org/Multi/GeneTree/Image?gt=ENSGT00390000015579) and were obtained from InvitrogenTM Life Technologies (Carlsbad, CA, USA) (Table 1).

The method of absolute quantitation of gene expression requires a standard curve in order to calculate amplicon concentration. The synthetic single-stranded sense oligonucleotides for the entire amplicons, i.e. *aanat1a*, *aanat1b* and *aanat2* were used as standards. All three single-stranded sense oligonucleotides, 300 ng of each, were purchased from InvitrogenTM Life Technologies (Gene Art Strings products) (Carlsbad, CA, USA). They were dissolved in 15 μl of water to have a stock solution to prepare the 6-point 10-fold serial dilutions starting at 200 fg/μl. Concentrations of the amplicons in organs were calculated by Eco software. Dilutions that made up the standard curve were defined in fg/μl, therefore the amplicons of studied organs were calculated in fg/μl according to the equation: Quantity = 10(Cq − b)/m where b is the y-intercept and m is the slope of the linear regression of the standard curve and Cq is the cycle number at which fluorescence signal exceeds minimal detection level (the threshold). Finally, absolute transcript abundances were expressed as pg per μg total RNA analysed.

The amplified products were subjected to automated sequencing using 3500 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) to verify their correspondence to the predicted cDNA sequences. Amplicons were prepared after real time PCR reactions without the melting curve step and were identical to the genomic sequence.

Table 1
Nucleotide sequence of specific primers used for the RT-qPCR analyses and the size of amplified products.

Primers	Nucleotide sequence	Amplicon size
aanat1a forward	GACACAGCGTGAGGAAATGA	100 bp
aanat1a reverse	GCCATATGCGTCTTCGAGAT	
aanat1b forward	CACATCAGGATGGAGCCTTT	105 bp
aanat1b reverse	GGATGCCCTCACTCTCCATA	
aanat2 forward	CAGCAAGTGCTCCATCTCCT	102 bp
aanat2 reverse	GACTTCCTGGTGCCCTTCTA	

2.3. Melatonin analysis

Melatonin concentration was analysed in the brain, eye ball, skin and kidney. Organs were homogenized in 1 ml of water and next centrifuged at 9000*g*, 4 °C for 10 min. Mel was extracted using the mixture of 3 × 1 ml of acetonitrile:dichloromethane (1:1, v/v). Organic layer was evaporated in the stream of nitrogen using a Turbo Vap LV Evaporator (Caliper Life Sciences, USA). Next, the residue was re-dissolved in 100 μl of methanol and 40 μl was subjected onto HPLC. Quantitative analyses were performed using Agilent 1200 Series Quaternary HPLC System with fluorescence detector (Agilent Technologies, Germany). Chromatographic separation was achieved on Kinetex C18 column (150 mm × 4.6 mm I.D., 2.6 μm). Gradient elution was applied for separation of Mel. The mobile phase consisted of solvent A (H2O) and solvent B (0.1% TFA in acetonitrile). A linear gradient was 12–20% of eluent B during 20 min. Flow rate was 1 ml/min and the column temperature 20 °C. Fluorescence detection was performed at 350 nm with excitation at 230 nm. Calibration curve was constructed using standard solutions with concentrations ranged from 6 to 743 pg/ml. The detection limit (LOD) for melatonin was 5.8 pg/ml (3:1 signal to noise). Identification

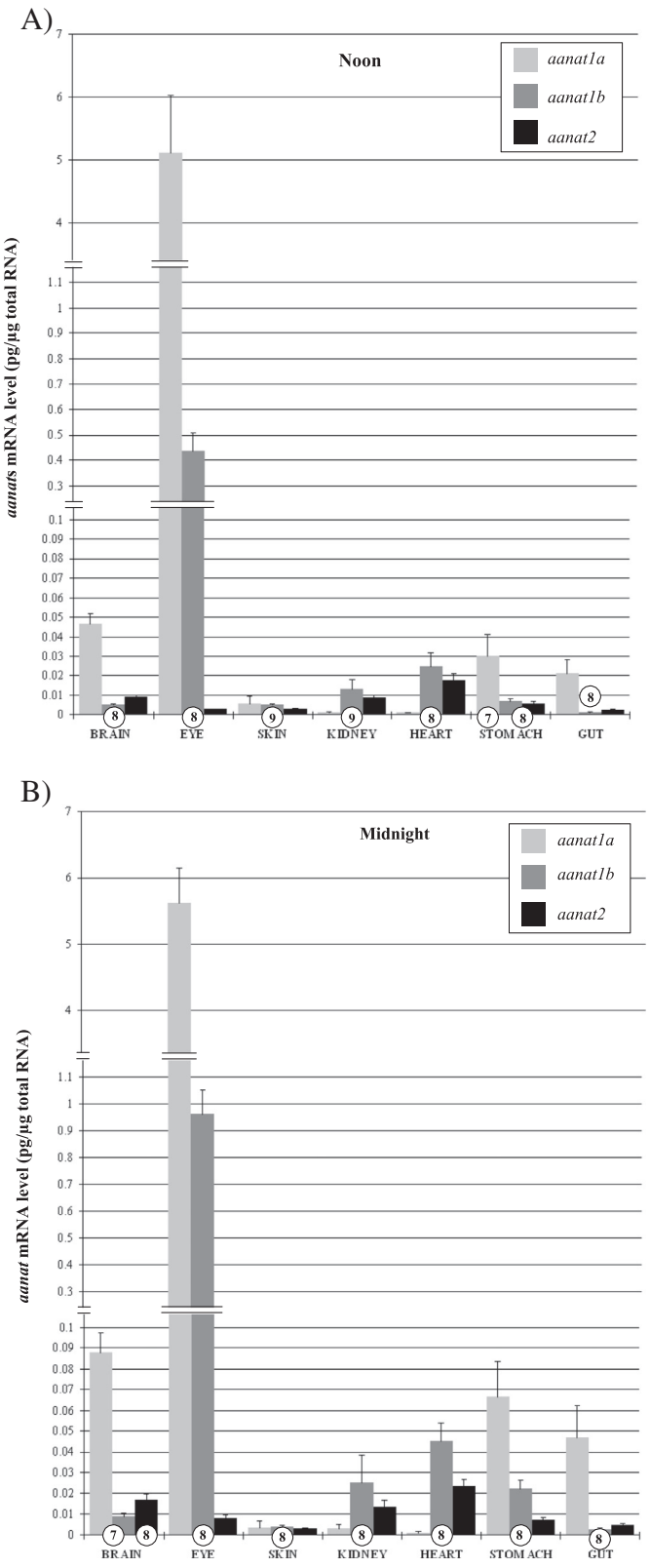

Fig. 1. Levels of *aanats* mRNA in various organs of the three-spined stickleback (*Gasterosteus aculeatus*) at noon (A) and midnight (B). Number of sampled fish is given in the circles. Values are presented as means ± standard error of the mean (S.E.M.).

of Mel was confirmed by comparing the retention time of the sample with that of standard Mel (Sigma, Germany). Melatonin concentrations were expressed as pg per gram wet organ weight.

Table 2
Statistical analysis of differences in *aanat1a* (black), *aanat1b* (blue) and *aanat2* (green) mRNA levels between various organs in the three-spined stickleback (*Gasterosteus aculeatus*) at noon (white area) and midnight (grey area).

	brain	eye ball	skin	kidney	heart	stomach	gut
brain		P<0.001 P<0.001 P<0.001	P<0.001 ns P<0.001	P<0.001 ns ns	P<0.001 ns ns	ns ns ns	ns ns P<0.001
eye ball	P<0.001 P<0.001 ns		P<0.001 P<0.001 ns	P<0.001 P<0.001 P<0.01	P<0.001 P<0.001 P<0.01	P<0.001 P<0.001 ns	P<0.001 P<0.001 ns
skin	P<0.001 P<0.01 P<0.001	P<0.001 P<0.001 P<0.01		ns ns P<0.001	ns P<0.01 P<0.001	ns ns ns	ns P<0.01 ns
kidney	P<0.001 ns ns	P<0.001 P<0.001 ns	ns ns P<0.001		ns ns ns	ns ns ns	P<0.05 ns P<0.01
heart	P<0.001 P<0.01 ns	P<0.001 P<0.001 P<0.01	ns P<0.001 P<0.001	ns ns ns		P<0.05 ns P<0.05	P<0.05 P<0.01 P<0.01
stomach	ns ns P<0.01	P<0.001 P<0.001 ns	P<0.001 P<0.001 P<0.001	P<0.01 ns ns	P<0.01 ns P<0.001		ns P<0.001 P<0.05
gut	ns P<0.01 P<0.001	P<0.001 P<0.001 ns	P<0.01 ns ns	ns ns ns	P<0.05 P<0.001 P<0.001	ns P<0.001 ns	

Significance was taken at P<0.05, ns – non significant.

2.4. Statistical analysis

Statistical analyses of data were done using Statistica 5.1 software. Values were presented as mean ± standard error of the mean (S.E.M.). Analysis of variance (one–way ANOVA) followed by Student's unpaired *t*-test were carried out for comparisons of *aanat*s expressions among analysed organs and within each organ. Significant differences were considered at $P < 0.05$ after Bonferroni correction.

3. Results

The genes *aanat1a*, *aanat1b* and *aanat2* were expressed in all organs at noon and midnight, but there were serious differences in their mRNA levels (Fig. 1A, B). The unquestionable highest levels of *aanat1a* and *aanat1b* mRNAs were in the eye ball at noon and midnight. There were significant day/night differences in *aanat1a* mRNA in the brain, *aanat1b* mRNA in the eye ball and stomach, and *aanat2* mRNA in the brain, eye ball and gut. In most cases, the levels of *aanat2* mRNA were low in comparison to those of *aanat1a* or *aanat1b* at both noon and midnight. Statistical significances were given in Tables 2–4.

There were significant day/night differences in Mel concentrations in the brain, eye ball, skin and kidney (Fig. 2). The highest levels were measured in the eye ball and the second highest in the brain at noon as well as at midnight.

4. Discussion

The first scientific evidence on the non-pineal Mel synthesis was provided as early as 1978 by Gern and colleagues who pointed out that the retina is a second source of Mel (Gern et al., 1978; Gern and Ralph, 1979). Nowadays many works describing various localizations and functions of Mel are available (for review: Falcón et al., 2010; Acuña-Castroviejo et al., 2014) and more and more studies on AANAT are undertaken (see Overview). It is well established that functions of AANAT go beyond producing Mel, e.g. recombinant AANAT1s acetylating dopamine in the retina in the seabream and zebrafish (Zilberman-Peled et al., 2006) and AANAT1 acetylating dopamine in the digestive tract in the goldfish (Nisembaum et al., 2013). Teleost fish with several isoforms of the enzyme encoded by several genes offer an opportunity to broaden our knowledge on specificity of the isozymes and their distinct roles in different organs/tissues. This leads to the need to elaborate

Table 3
Statistically significant differences between noon and midnight levels of *aanat*s mRNA in various organs in the three-spined stickleback (*Gasterosteus aculeatus*).

Gene	Organ	Significance level
aanat1a	Brain	$P < 0.01$
aanat1b	Eye ball	$P < 0.001$
	Stomach	$P < 0.01$
aanat2	Brain	$P < 0.05$
	Eye ball	$P < 0.01$
	Gut	$P < 0.05$

Significance was taken at $P < 0.05$.

such methodological approach that would make it possible to compare quantitatively the levels of the genes mRNAs in different organs and conjecture about the proportion between AANAT isozymes there. Such method as this is the absolute RT-qPCR. In this paper, we report for the first time the levels of mRNAs of three genes, *aanat1a*, *aanat1b* and *aanat2*, in the whole brain, eye ball, skin, stomach, gut, heart and kidney of the three-spined stickleback. Here we demonstrate that all three genes, *aanat1a*, *aanat1b* and *aanat2* are expressed in all studied organs so that three AANAT isozymes are expected to be present at these sites. Furthermore, the levels of *aanats* mRNAs differ significantly within and among organs. The extremely high levels of *aanat1a* and *aanat1b* mRNAs in the eye ball, i.e. at least two orders of magnitude higher than that of *aanat2*, indicate that AANAT1a and AANAT1b can prevail at this site. Moreover, exceptionally high Mel concentrations at noon and midnight suggest that just the eye is an important source of the hormone in the three-spined sticklebacks. It is also evident that within each analysed organ the levels of *aanat1* (*a* and/or *b*) mRNAs are higher than that of *aanat2*, except for the skin, where expressions of three genes are similar and very low. A very low level of *aanat2* mRNA in all organs may suggest that AANAT1a and/or AANAT1b are principal isozymes in the three-spine sticklebacks.

To our knowledge, to date there is only one study, where *aanat*s mRNAs have been analysed by absolute RT-qPCR, namely *aanat1* and *aanat2* mRNAs in embryonic and post- embryonic eye and brain of chum salmon (*Oncorhynchus keta*) (Shi et al., 2004). In numerous studies, the Northern blot or the relative RT-qPCR was used and generated data differ significantly. The list of achievements is worth quoting because it confirms unflagging interests in the subject and justifies the need for development of a new methodological approach to be able to

Table 4
Statistical analysis of differences between *aanat1a*, *aanat1b* and *aanat2* mRNA levels in various organs in the three-spined stickleback (*Gasterosteus aculeatus*) at noon (white area) and midnight (grey area).

		aanat1a	*aanat1b*	*aanat2*
brain			P<0.001	P<0.001
eye ball			P<0.001	P<0.001
skin			ns	ns
kidney	*aanat1a*		P<0.05	P<0.001
heart			P<0.01	P<0.01
stomach			ns	ns
gut			P<0.05	P<0.05
brain		P<0.001		P<0.05
eye ball		P<0.001		P<0.001
skin		ns		ns
kidney	*aanat1b*	ns		ns
heart		P<0.001		ns
stomach		P<0.05		ns
gut		P<0.05		ns
brain		P<0.001	P<0.05	
eye ball		P<0.001	P<0.001	
skin		ns	ns	
kidney	*aanat2*	P<0.05	ns	
heart		P<0.001	P<0.05	
stomach		P<0.01	P<0.01	
gut		P<0.05	ns	

Significance was taken at P<0.05, ns – non significant.

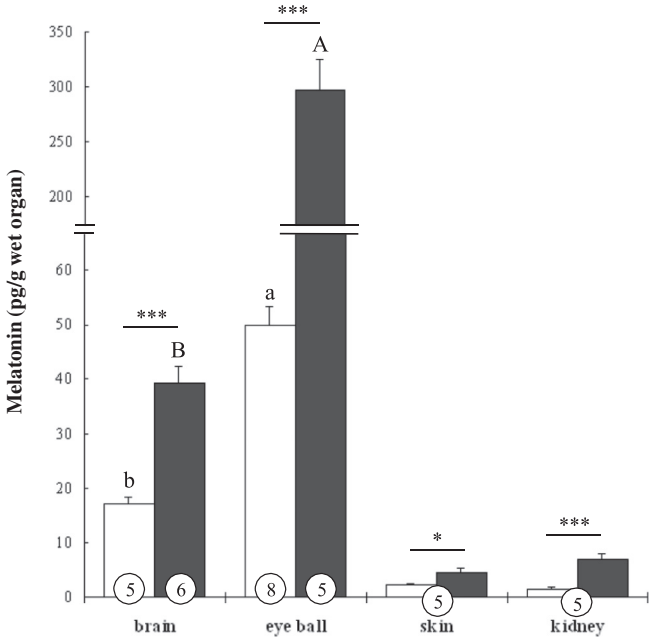

Fig. 2. Melatonin concentrations in various organs of the three-spined stickleback (*Gasterosteus aculeatus*) at noon (white bars) and midnight (black bars). Number of sampled fish is given in the circles. The values are presented as means ± standard error of the mean (S.E.M.). Significant differences are indicated as: a: $P < 0.001$ vs. brain, skin and kidney, b: $P < 0.001$ vs. skin and kidney, A: $P < 0.001$ vs. brain, skin and kidney, B: $P < 0.001$ vs. skin and kidney, $^{***}P < 0.001$ and $^{*}P < 0.05$.

compare results between studies. Here are examples. In the trout and pike, AANAT mRNA transcripts were detected in the pineal but not in the retina, brain, kidney, heart, gut, testis, ovaries or liver, however, in the zebrafish, they were detected in the pineal and retina, and also in the ovaries but the signal was weaker (Bégay et al., 1998). Then the presence of two AANAT genes, *aanat1* in the retina and *aanat2* in the pineal gland was proved in the pike (Coon et al., 1999). Similarly, the expression of *aanat1* only in the retina and *aanat2* mostly in the pineal gland but also in the retina but at lower level was shown in the zebrafish (Falcón et al., 2003). Later on Appelbaum et al. (2006) confirmed expressions of both *aanat1* and *aanat2* in the retina in this species. In the goldlined spinefoot (*Siganus guttatus*), *aanat1* was exclusively expressed in the retina among tested pineal, brain, liver, heart, intestine, kidney, spleen, muscle and skin (Kashiwagi et al., 2013). In the rainbow trout, on the other hand, the relative expression of *aanat2* (β-*actin* as a reference gene) was reported in the pineal and at several peripheral sites including gills, kidney, muscle, skin, liver, Brockmann bodies, gall bladder, spleen and gastrointestinal tract (Fernández-Durán et al., 2007). More recently, a strong relative expression of *aanat2* (*rRNA-18S* as a reference gene) was demonstrated in the pineal and considerably weaker in the kidney, liver, foregut, hindgut, retina, and gallbladder in the goldfish (Velarde et al., 2010). In the sea bass, *aanat1a* and *aanat1b* were expressed in the brain, retina and gonads, intestine, liver and muscle, *aanat1b* also in the gills and heart, and *aanat2* only in the pineal and gonads (Paulin et al., 2015). In these investigations, regardless of the different methodological approaches (Northern blot or relative RT-qPCR) and studied species, *aanat2* was mostly expressed in the pineal gland and *aanat1* in the retina, despite the strong differences in the expressions of the genes. However, it should be stressed here again that a quantitative comparison between relative levels of mRNA can be dubious (see the section: *Why the absolute quantitative RT-qPCR*).

In our study of the three-spined sticklebacks, all three genes, *aanat1a*, *aanat1b* and *aanat2* are expressed in all analysed organs but *aanat2* at definitely lower levels. The extremely high levels of *aanat1a* and *aanat1b* mRNAs in the eye ball (retina included) are in line with previously reported expression of *aanat1* in the retina of different fish

species (Coon et al., 1999; Falcón et al., 2003; Kashiwagi et al., 2013). On the other hand, the level of *aanat2* mRNA in the whole brain (pineal organ included) of the sticklebacks is as low as in other organs whereas the expression of *aanat2* has been shown to be very strong in the pineal of the pike, rainbow trout and goldfish (Coon et al., 1999; Fernández-Durán et al., 2007; Velarde et al., 2010). However, it should be stressed here that in our study, *aanat2* mRNA level has been calculated for the whole brain but the gene expression is most likely restricted to the pineal organ, a small area in the brain. Thus the *aanat2* mRNA originating from the pineal organ is diluted in the whole brain extracts and *aanat2* mRNA level is apparently underestimated. However, because it is difficult to compare the results obtained by relative and absolute methods, the discussion, out of necessity, is limited. Therefore we have found it very important to elaborate the absolute RT-qPCR method to be able to compare results between studies in the future.

It is known in fish, that AANAT1 acetylating both indolethylamines and phenylethylamines can be implicated in metabolism of both serotonin and dopamine but its affinity for each substrate may differ (see Overview). In some fish species, including the sticklebacks, AANAT1a and AANAT1b, not AANAT2, seem to be essential for Mel biosynthesis. For instance, in the sea bass, the recombinant AANAT1a and AANAT1b have displayed higher affinity for indolethylamines than phenylethylamines (Paulin et al., 2015). In the pike and gilthead seabream (*Sparus aurata*), AANAT1 having a very high affinity for serotonin is decisive for Mel production (Coon et al., 1999; Zilberman-Peled et al., 2004). The extremely high levels of *aanat1a* and *aanat1b* expressions together with extremely high concentration of Mel in the eye ball at midday and midnight, point just to the eye as an important site of Mel production driven by AANAT1a and AANAT1b in the three-spined stickleback. The second highest Mel concentration is in the brain where the expression of *aanat1a* prevails. Moreover, there are significant day/night variations in Mel levels in the sticklebacks' eye ball and brain. However, the evident day/night differences concern both *aanat1b* and *aanat2* mRNAs in the eye ball and both *aanat1a* and *aanat2* mRNAs in the brain even though the *aanat2* mRNAs levels are very low. We bear in mind that the high expression of the genes may not result in high levels of AANATs and high concentrations of one enzyme in the Mel synthesis pathway may not result in increased concentration of final product of serotonin metabolism – Mel. So that a role of *aanat2* in the rhythmical synthesis of Mel is also considered in both the eye and brain of the sticklebacks, the more so because in many other studies AANAT2 is accepted as a main isozyme involved in Mel synthesis in fish (see Overview).

In the three-spined sticklebacks, *aanat1a*, *aanat1b* and *aanat2* are expressed also in the skin, stomach, gut, heart and kidney at midday and midnight. In the heart, stomach and gut, we have shown notable expressions of *aanat*s mRNA, especially *aanat1a* in the stomach and gut. It is not unexpected because the gastrointestinal tract (GIT) is an important source of Mel and a site of its action not only in adult fish (Bubenik and Pang, 1997; Kulczykowska et al., 2006), but also in larvae where the indole is probably involved in the control of development and protection against free radicals (Kalamarz et al., 2009). In our study, we have also presented the significant day/night differences in *aanat1b* mRNA in the stomach and *aanat2* mRNA in the gut. It is hard to say for sure that the day/night changes in *aanat*s mRNAs levels are translated into changes in Mel concentrations because we do not measure Mel levels at these sites in the sticklebacks. However, the production of Mel by the enterochromaffin cells of the intestine depends rather on the presence of food in the GIT than on photoperiod (for review: Kulczykowska and Sánchez-Vázquez, 2010) and in goldfish, for instance, Mel level in the GIT is high after feeding, regardless of the schedule or random feeding (Vera et al., 2007). On the other hand, Velarde et al. (2010) have shown circadian rhythms in *aanat2* transcripts in hindgut in this species and quite recently Muñoz-Pérez et al. (2016) have demonstrated daily rhythms of *aanat1* and *aanat2* mRNAs in GIT (with peaks at night) in the rainbow trout. In our experiments, sticklebacks were not fed before sampling but they could still feed continuously on plants in aquarium so that our data need clarification in further studies. The same goes for *aanat*s mRNAs levels in the heart. In the skin and kidney of the sticklebacks, where Mel is measured, its concentration shows day/night variation despite a lack of changes in the levels of *aanat*s mRNAs. Here *aanat*s mRNA levels are low and similar at noon and midnight, so higher Mel values at night result most likely from the supply of the hormone by circulation. In mammals, the skin is an important site of Mel synthesis and just here Mel plays an important role in protection against oxidative stress (for review: Slominski et al., 2007). However, in sticklebacks, there is no evidence for Mel production in the skin, because *aanat*s mRNA levels and Mel concentration are very low. Although our previous findings of the presence of Mel at higher concentrations than would be expected as a result of normal distribution by circulation, together with the specific binding of 2-[125I]iodoMel in the gill, small intestine and kidney (kidney tubules) of rainbow trout, flounder (*Platichthys flesus*) and sea bream, have strongly suggested not only synthesis but also function of Mel at these sites (Kulczykowska, 2002; Kulczykowska et al., 2006; Kleszczyńska et al., 2006), further studies are required to better understand a physiological relevance of the data.

5. Final remarks and perspectives

Our study of *aanat*s expression in various organs of the three-spined stickleback is the first step to recognize physiological status of AANAT isozymes at different locations in fish. There is a lot of evidence in vertebrates that AANAT role extends far beyond that of time enzyme in Mel biosynthesis, thus AANAT merits consideration as a multi-faceted enzyme. What is more, a role of Mel is far beyond timekeeping. For instance, Mel is a potent antioxidant and free radical scavenger protecting organism from oxidative damage (Reiter et al., 2000; Hardeland, 2005) and it is involved in the regulation of gastrointestinal motility (Bubenik, 2002), and modulation of immune system (Maestroni, 1993). In teleost fish, several variants of *aanat* are expressed in the pineal organ, retina and peripheral organs and Mel is also present at these sites so that AANAT isozymes are probably involved in the local Mel biosynthesis. However, it has to be taken into consideration that Mel production depends not only on AANAT but also on ASMT thus further experimental study including ASMT gene/genes expression will be essential to clarify this issue.

In conclusions, AANAT isozymes can play distinct roles at the level of major organs dependent on specific substrate preferences. A unique presence of the isozymes in fish provides opportunity for control of acetylation of amines in the cell by modulation of individual genes expression and permits the fine-tuning of indolethylamines and phenylethylamines metabolism to meet the particular needs of a given organ. Hence further studies are essential to show the specificity of AANAT isozymes roles in various organs in fish. Although any direct relationship between *aanat*s mRNA levels and abundance of AANAT isozymes is not expected, a question remains, if the isozymes can play important physiological roles at the locations where their genes are weakly expressed. In our study, it concerns *aanat2* in particular. The situation is even more complicated because effectiveness of the enzyme can be affected by many factors. Therefore physiological and environmental conditions have to be taken into consideration in any further work on action of AANAT isoforms in various organs/tissues in fishes.

Acknowledgments

The authors would like to thank dr Tadeusz Malewski from Museum and Institute of Zoology of Polish Academy of Sciences in Warsaw for sequencing the *aanat*s amplicons. This research was supported by National Science Centre grant UMO-2012/07/B/NZ9/02144. The authors report no conflict of interest.

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