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ABSTRACT

Much is known about melatonin and its role in the circadian regulation of vertebrate organisms. However, melatonin has not been studied extensively in more primitive bilaterians. The aim of this study was to analyze the relationship between melatonin and the circadian rhythm in the organism *Stenostomum virginianum.* Melatonin assay methods from previous research in the field were optimized for smaller tissue sampling of microscopic metazoans. The optimized assay methodology was then used to identify melatonin in *Stenostomum virginianum* using high performance liquid chromatography (HPLC). Identification of photoreceptors was used to correlate the presence of melatonin to the circadian rhythm. PAX-6 was chosen for study because it is considered the master eye regulatory gene. Immunohistochemistry and confocal microscopy confirmed the presence of PAX-6 in the anterior region of *S. virginianum.* The next step in this project is the examination of melatonin concentration at various time points and the comparison of the fluctuation pattern to the *S. virginianum* sleep and reproduction cycles. Following this comparison, the next logical step is the analysis of melatonin biosynthetic enzymes. This project builds on the work of Dan Stanton and Julian P. Smith, PhD.

INTRODUCTION

The circadian rhythm has been studied extensively in vertebrates, but the evolutionary examination of the biological clock in basal multicellular organisms has yet to be completed. Previous research has revealed that melatonin is highly involved in the biological clock; it synchronizes daily rhythms in vertebrates (Klein et al 1997). Further study has suggested that melatonin is certainly involved in the circadian rhythm of primitive organisms (Roopin & Levy 2012a,b). The model organism of such studies is the planarian. When *Dugesia* were decapitated, their fissioning rates greatly increased, suggesting that an inhibitory compound is released from the head. When decapitated planarians were exogenously exposed to melatonin, asexual reproduction was suppressed. When returned to regular culture water, the normal rate of fissioning resumed. This suggested that melatonin is present in the head of planarians and is also involved in fission inhibition (Morita & Best 1984). In order to further examine the evolution of the circadian clock, a more primitive Catenulid flatworm (Larson & Jondelius 2008) was chosen for study: *Stenostomum virginianum.* Previous study of *S. virginianum* has shown that 0.1mM of melatonin suppressed asexual fissioning when applied exogenously and down-regulates mitosis (Stanton & Smith, unpublished). However, no previous attempts have been made to successfully identify melatonin inside *S. virginianum.* The aim of this study was to develop a melatonin assay technique using high performance liquid chromatography (HPLC) and investigate whether or not melatonin is present in *S. virginianum.*

Melatonin is involved in the synchronization of the circadian clock (Klein et al 1997). In order to correlate melatonin cycling back to the circadian rhythm, photoreceptors must be present. Sensory receptors were discovered in the sister species *Stenostomum leucops* and *S. leucops* exhibited an intense response to illumination when in a resting state (Palmberg and Reuter 1992). The paired box protein 6 (PAX-6) is the highly conserved master eye-regulatory gene and is expressed in both developing and mature eyes across phyla (Callaerts et al 2006). Therefore, a second aim of this study was to identify PAX-6 in order to locate possible photoreceptors in *S. virginianum* using immunohistochemical analysis and confocal microscopy.

MATERIALS & METHODS

Stenostomum virginianum (Platyhelminthes, Catenulida), was obtained from college lake at Winthrop University and kept cultured in artificial pond water (APW) on a 14:10 [L:D] cycle at 21.0 °C. Organisms were fed *Paramecium multimicronucleatum* purchased from Carolina Biological Supply. New culture dishes were formed approximately every 1.5 weeks in order to maintain optimal fissioning conditions.

Optimization of high performance liquid chromatography

A solution of melatonin standard was prepared using anhydrous melatonin and ultrapure water. 150 µL of solution was analyzed for optimal excitation wavelength using a spectrofluorometer. Using this wavelength, the optimal emission wavelength was then obtained. Using the observed optimal excitation and emission wavelengths, the optimal pH of the 50 mM ammonium acetate mobile phase was observed using a spectrofluorometer. The optimized values obtained from the previous experiments above were used on the HPLC to optimize the mobile phase and retention time. Using a standard solution of melatonin and serotonin in ultrapure water, the retention time was optimized beginning at 22/78 (v/v) of acetonitrile and 50mM ammonium acetate (5.0 pH) using an incremental decrease of acetonitrile. The optimization occurred at the shortest retention time with two distinct melatonin and serotonin peaks.

High performance liquid chromatography

Analysis of melatonin using HPLC was completed using the optimized procedure by collection of 500 *S. virginianum* in a 1.5 mL microcentrifuge tube. Ice cold Qiagen RLT buffer (100 µL) was added and allowed to sit on ice for 5 minutes; 200 µL of ice cold extraction solution (0.15M perchloric acid with 0.1% ascorbic acid and 0.01% disodium EDTA), was added and homogenized using vigorous pipetting; the tube was centrifuged for 10 minutes at 10,000 rpm. One hundred and fifty microliters (150 µL) of the supernatant was injected into reverse-phase HPLC with a C18 stationary phase column (5µm particle, 4.6 mm x 150mm column). Using an isocratic elution with 70/30 (v/v) of 50mM ammonium acetate pH 5.0 and acetonitrile as the mobile phase, the flow rate was set at 1.0mL/min. Relative fluorescence detection was set to an excitation wavelength of 296 nm and emission wavelength of 348 nm.

Immunohistochemistry

Four *S. virginianum* were separated from culture and flash-frozen in APW on a copper block immersed in liquid nitrogen. The specimens were freeze substituted in ethanol cooled in liquid nitrogen and placed in a freezer for 13 days. The four specimens were then divided into two groups: experimental and control. Organisms were warmed to room temperature and transferred to 4% formaldehyde in 1x PBS with 10% sucrose. Specimens were then rinsed three times in twenty minute increments in 1x PBS with 0.1% BSA, followed by blocking in BSA-T for one hour on a rocker in the refrigerator. Dilute anti-PAX-6 primary antibody (1:100) was applied to the experimental specimens in 1X PBS with BSA-T and was incubated overnight in the cold. Both groups were rinsed three times in sixty minute intervals with 1x PBS with 0.1% BSA on a rocker in the refrigerator. Specimens were all incubated in the refrigerator overnight in a cocktail of secondary antibody (Cy3-labeled Donkey anti-mouse) 1:400 to highlight PAX-6, Hoechst 33342 1:500 to highlight nuclei, and Alexa488/phalloidin to highlight muscles. The following morning all *S.virginianum* were rinsed 3x30' in BSA-T on a rocker in the refrigerator. Twenty-five microliters of VectaShield was added to a slide, following the placement of the specimens on the slide in one drop of glycerin. A cover slip supported by aluminum foil was placed above the specimen and secured using Sally Hansen's clear nail polish. Specimens were imaged using confocal microscopy.

RESULTS

Spectrofluoremeter experiments resulted in an ideal excitation wavelength of 296 nm (Figure 1), an ideal emission wavelength of 348 nm (Figure 2), and an optimal 50mM ammonium acetate pH of 5.0.

Optimization tests revealed a retention time of 4.45 minutes, using 70/30 (v/v) of 5.0 pH 50mM ammonium acetate and acetonitrile.

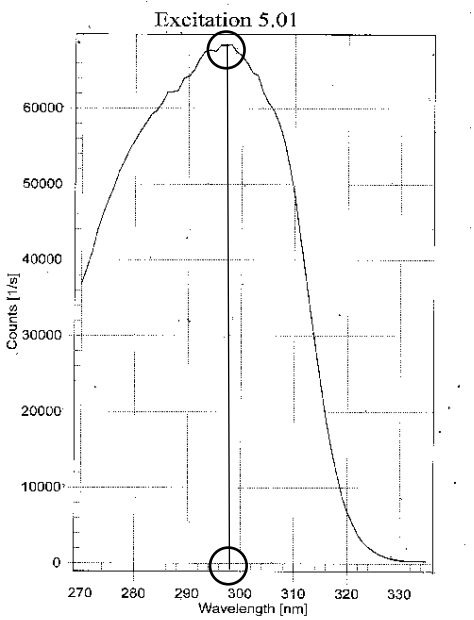


Figure 1: Spectrofluoremeter scan of excitation wavelength: optimal wavelength and peak circled at 296nm.

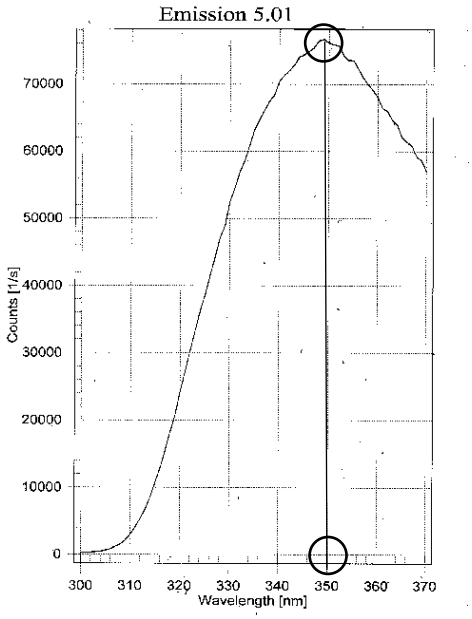
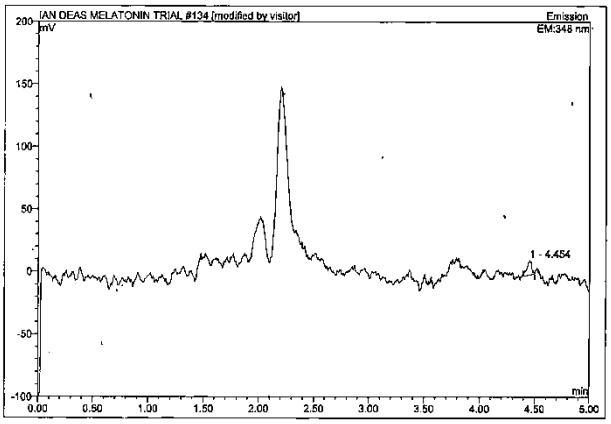


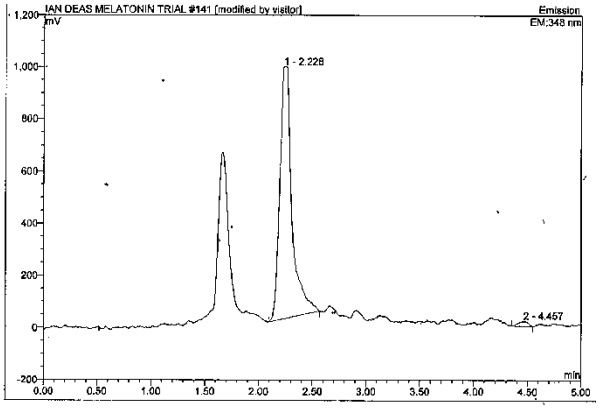
Figure 2: Spectrofluoremeter scan of emission wavelength: optimal wavelength and peak circled at 348 nm.

Melatonin was present in *S. virginianum* using HPLC. The peak area at 4.45 minutes in the ammonium acetate clearing run was 0.574 mV*min (Figure 3). The peak area at 4.45 minutes in the experimental group injection was 2.003 mV*min (Figure 4). The second peak was higher, showing that melatonin is present.



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	4.45	n.a.	12.011	0.574	100.00	n.a.	BMB*
Total:			12.011	0.574	100.00	0.000	

Figure 3: Shows the control run without a significant melatonin peak in the 4.45min range; peak area circled



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	2.23	n.a.	966.984	126.871	98.45	n.a.	BMB
2	4.46	n.a.	18.188	2.003	1.55	n.a.	BMB*
Total:			984.172	128.874	100.00	0.000	

Figure 4: Shows the experimental run with a significant melatonin peak in the 4.45min range; peak area circled

PAX-6 was discovered in the anterior region of *S. virginianum,* near the lobes of the brain (Figs, 5, 7). The control specimen was negative for Cy3/PAX-6 in the same region (Figs. 6, 8) using the confocal observations.

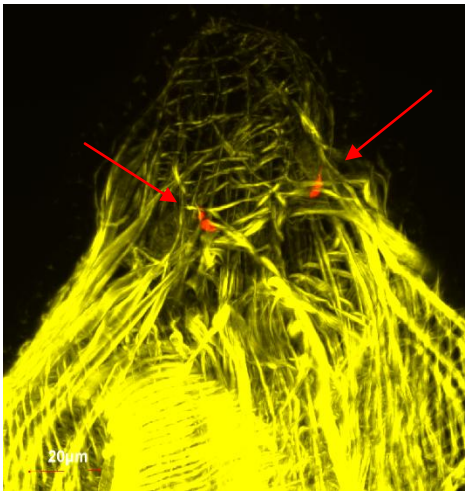


Figure 5: Experimental specimen; maximum intensity z-projection; muscle stained yellow; PAX-6 stained red (arrows)

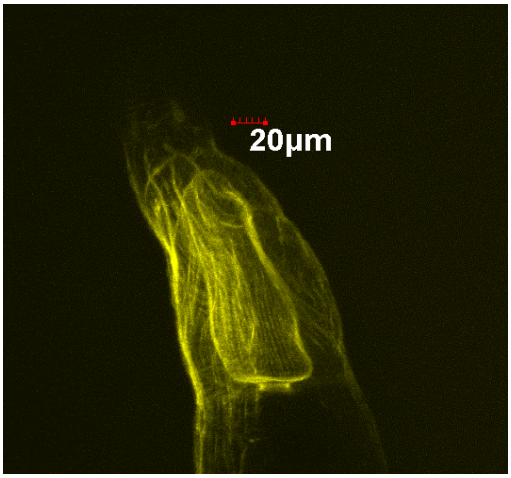


Figure 6: Control specimen; maximum intensity z-projection; muscle stained yellow; note no fluorescence in PAX-6 channel

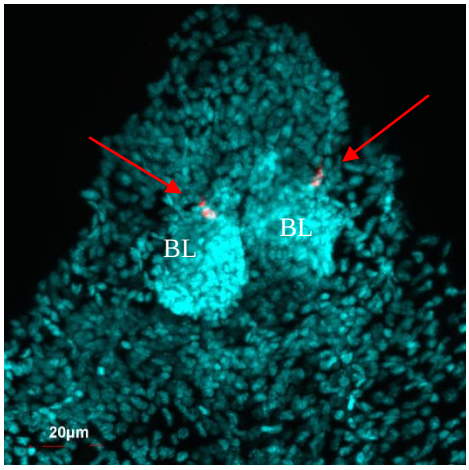


Figure 7: Experimental specimen; maximum intensity z-projection; nuclei stained blue; PAX-6 stained red (arrows); brain lobes (BL).

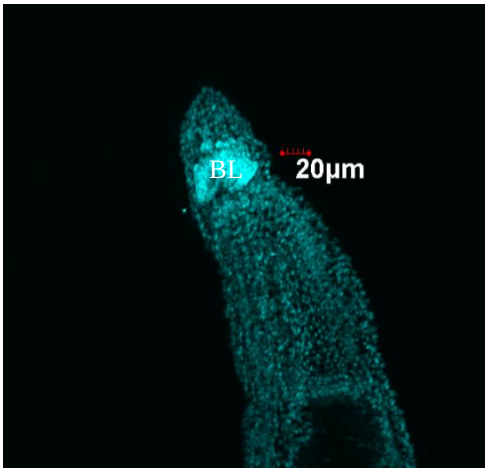


Figure 8: Control specimen; maximum intensity z-projection; nuclei stained blue; note no fluorescence in PAX-6 channel; brain lobes (BL).

DISCUSSION

The optimization of melatonin assay methodology builds upon the previous works of other investigators in the field. This newly developed technique allows for tissue sampling at much lower quantities, building directly on research that used larger samples of tissue (Almeida et al 2011). The methodology also refined the assay approach outlined by Morita and Best used on *Dugesia* (1987). The improvement of sampling methods and the requirement for less tissue sampling allowed for the analysis of much smaller organisms such as *Stenostomum.*

The identification of melatonin using HPLC provides support for Stanton and Smith's (unpublished) hypothesis that melatonin is responsible for suppression of asexual reproduction and the down-regulation of mitosis by showing that melatonin is present inside of the organism. The next step will be to sample *S. virginianum* at different time periods in the circadian cycle to test whether or not melatonin fluctuates in a circadian fashion.

The positive detection of PAX-6 means that there is photoreception in *S. virginianum*. These results are similar to those of Palmberg and Reuter, who examined similar eye-like structures in *Stenostomum leucops* (1992). The next step in this project will be to examine the structure of the eye-like region in *S. virginianum* using transmission electron microscopy (TEM). The analysis of the PAX-6 positive regions will provide further insight into the photoreceptive function of the positive results.

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