CIRCADIAN CLOCK LOCALIZATION IN THE GHOST SHRIMP, *PALAEMONETES PALUDOSUS* AND THE NORTHERN CRAYFISH, *ORCONECTES VIRILIS*

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Introduction

Circadian rhythms are daily anticipatory patterns of behavior and physiology, which are conserved across the animal kingdom. These rhythms are maintained by endogenous biological clocks, which are comprised of a network of specific genes and proteins. These genes and proteins function in various feedback loops, with circadian changes in gene expression and protein abundance (Bell-Peterson et al., 2005). In *Drosophila*, mutation of the *period (per)* gene, which is responsible for PER protein synthesis, causes alterations to the species circadian rhythm (Lin, 2005) and thus PER has been identified as a key clock regulator (Meyer et. al 2006). PER has been conserved across taxa, and localizing the protein has been used as a proxy for specific clock location (Hege et al., 1997). While the majority of research has focused on vertebrate organisms (Bell-Peterson et al., 2005), clock localization has been determined in some invertebrate species in addition to *Drosophila*. The marine gastropods *Aplysia* and *Bulla* (Siwicki *et al.* 1989), the ground crickets *Dianemobius nigrofasciatus* and *Allonemobius allardi* (Shao et al., 2006), and the scorpion, *Andoctonus australis* (Heinrich et a., 1987), have all had putative clock locations demonstrated via PER localization. Only one species of crustacean, however, the southern crayfish, *Procambarus clarkii* has had PER localized in this way. Immunopositive staining for PER was found in the retina, eyestalks and parts of the brain of *P. clarkii* (Escamilla-Chimal et al. 2010).

The purpose of this study was to use immunohistochemistry to localize PER in two closely related crustacean species; the ghost shrimp, *Palaemonetes pauludosus* and the northern crayfish, *Orconectes virilis*, thus potentially locating the species circadian clock.

Methods

Animals and Environmental Conditions

The Ghost shrimp, *Palaemonetes paludosus* and the northern crayfish, *Orconectes virilis*, were acquired from a commercial supplier. Both species were put on a reverse light cycle of 12:12 for an adequate period for circadian entrainment.

Experimental Procedure

Specimens were dissected on ice in a saline solution to prevent fast acting protein degradation. Intact eyestalks, brain, and nerve cord were extracted from the animals and fixed overnight in a 4% paraformaldehyde solution at 4° C, which was buffered with 0.1M Sorenson's buffer. The tissues were washed several times with buffer over the course of one hour, and dehydrated using ETOH dilutions from 30% up to 95%. After rehydrating, tissues were washed in PTA solution, which helps to prevent growth of microorganisms during the ensuing antibody incubations. Tissues were blocked overnight in a 6% Goat serum, which helps to prevent nonspecific background staining. The neurotransmitter, Serotonin was used as a positive control, as it is highly conserved across taxa. Two initial dilutions were used for the serotonin Ab: 1:500 and 1:250 in 6% goat serum. Tissues were incubated at 4° for 48 hours, with constant agitation. Tissues were washed with PTA for twelve hours with several changes of solution, removing 1° Ab. The 2° used was Alexaflour 488, which is a fluorescent tagging agent used to reveal the binding of the 1° Ab. 2° Ab dilution was 1:100 in PTA, with an overnight incubation at 4° C. After the incubation, the tissues were rinsed with Sorenson's buffer for twelve hours. Tissues were mounted onto slides and stored in a light sealed slide holder at 4°C (preventing degradation of the flourescins).

Analysis

Observed immunofluorescence of PER was quantified by comparison to the negative control (2° Ab only). A grid consisting of 50µm² squares was placed over the images, and random squares were chosen for quantification. Stained cell bodies located in each of 16 randomly selected squares were counted, a total cell count was determined and divided by total area to determine stained cells per mm².

PER Staining in Subesophageal Ganglion

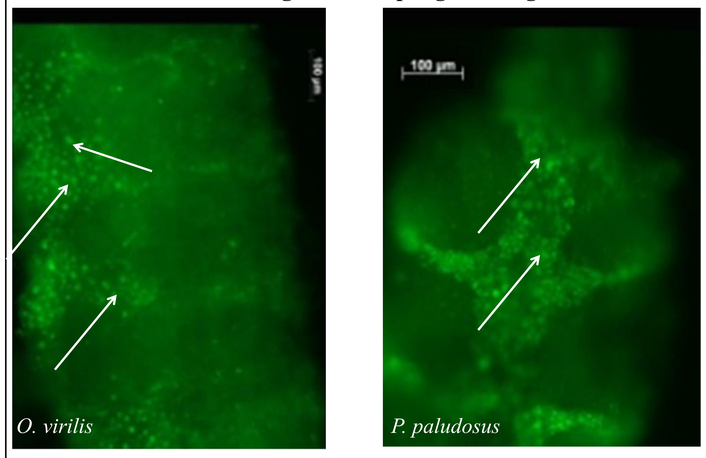

Figure 1: Positive PER staining in the subesophageal ganglion of *P. paludosus* and *O. virilis*, arrows indicate stained cell bodies.

Serotonin (5-ht) and Negative Controls

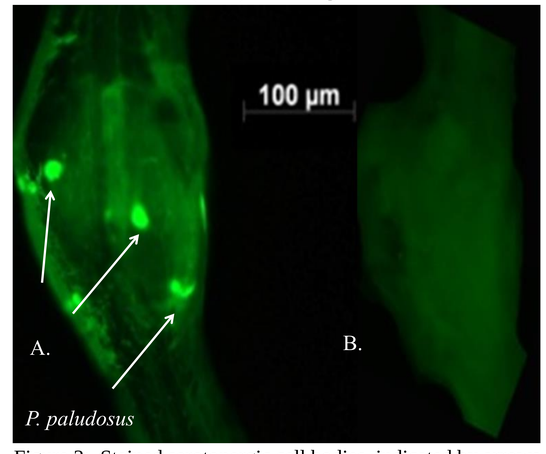

Figure 3: Stained serotonergic cell bodies, indicated by arrows (A) were visualized and 2° Antibody only incubations (B), show lack of nonspecific binding.

PER Staining in Ventral Nerve Cord

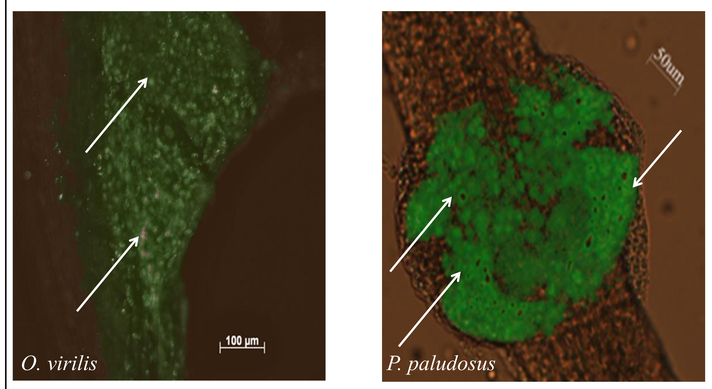

Figure 2: PER staining in ventral nerve cord ganglia of *O. virilis* and *P. paludosus*. Arrows indicate stained cell bodies.

Light Vs. Dark PER Staining

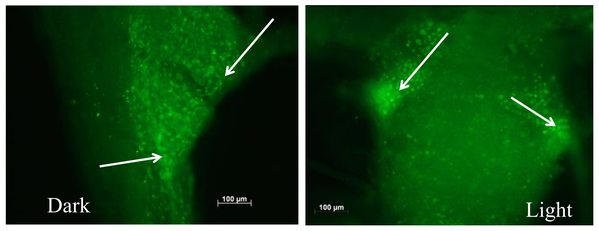

Figure 4: *Orconectes virilis* PER staining in the ventral nerve cord ganglion. Number of stained cell bodies appeared more abundant in the dark phase compared to light phase. Arrows indicate stained cells.

Conclusions

- Potential clock localization to VNC ganglia and subesophageal ganglion
- PER appears to increase at night compared to day
- Limited PER staining in brain and eyestalks (not shown)
- Serotonin (5-HT) localized for first time in *O. virilis* and *P. paludosus*

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