Trace metals as indicators of rhythmic melanization of shielding pigment in larval ocelli

G. Falkenberg¹, Ge. Fleissner², P. Alraun¹, U. Boesenberg¹ and Gue. Fleissner²

¹ Deutsches Elektronen-Synchrotron DESY, PETRA III Beamline P06, Notkestr. 85, 22607 Hamburg, Germany

Melanin is a family of pigment proteins occurring in various organisms - animals, plants and fungi – and has a wide spread functional meaning for example as colour of skin, feathers, hairs, and eyes. Melanin molecules, intracellularly accumulated within tiny vesicles, so-called melanosomes, serve as photoprotectant and storage of excess minerals induced e.g., by inflammations, infections or degenerative diseases. Therefore, the mechanisms underlying melanization have gained increasing interest in the field of biomedical research and clinic. However, these processes are only partly analyzed. Nearly nothing is known on a putative switch between melamins of different types and a reversal or prevention of melanin enrichment in distinct cells, e.g. degenerating neurons.

The marine midge Clunio marinus may be a convenient model organism to study melanogenesis as part of a naturally cycling change between two types of melanin proteins in the retinal shielding pigment of larval ocelli. The dark shielding pigment, eumelanin, within the image forming ocelli in the dark moon phase is periodically converted into a transparent protein, phaeomelanin, yielding a retinal photometer device for about a week during full moon. An endogenous lunar clock controls this conversion [1]. This moonlight window enables the organisms to use the dynamics of nocturnal moonshine as a precise Zeitgeber signal for the lunar clock. The complex timing of metamorphosis and reproduction by solar and lunar changes of light intensity is essential for organisms like Clunio, which live in an intertidal environment.

In insects, melanisation has not been analyzed in detail, but we hypothesize that in Clunio marinus melanogenesis at least partially follows the mechanisms analyzed in mammals. Enzymes involved in these processes are known to depend on trace metals, which are indicative for the different types of melanin and can be associated with certain steps of the respective pigment formation [2]. Thus, we have investigated larval ocelli histologically fixated during well-defined lunar states in order to study the rhythmic dynamics of retinal shielding pigment transparency induced by the changing melanogenesis.

Methods: The animals are raised in synchronized cultures under a daily 14:10 h light/dark regime. Every 30 days a nocturnal light was switched on for four nights simulating “full moon” phase. For convenience, we refer to the time span without simulated nocturnal moonlight as “dark moon” phase. Lunar cycle day 1 (LCD 1) is the first of the four days with nocturnal moonlight, the entire lunar cycle lasts 30 days, ending with LCD 30. About every third day of the lunar cycle, midges were decapitated and fixated in 4% Glutaraldehyde. Histological handling was performed with the strict avoidance of metal tools or metal containing chemicals. The heads were resin-embedded in Araldite and cut in semithin (2-3 µm thick) or ultra-thin sections (about 50-80 nm thick; collected on Formvar coated copper grids). We studied the light and electron microscopic views of the ocellar melanosomes in the light microscope (Polyvar Reichert, Vienna, Austria) and transmission electron microscope (Zeiss C10R and C12R, Oberkochen, Germany; for further details see [1]). For XRF measurements, semithin sections parallel to the microscopically investigated sections were mounted on silicone nitride membrane windows (Silson LTD., Northampton, England). The micro-XRF measurements were carried out at the PETRA III beamline P06 at the Hard X-ray Microprobe experiment. A monochromatic beam of 12 keV was focussed by KB-mirrors to a size of 300x300 nm² FWHM (10¹⁰ ph/s). The sample was raster-scanned across the beam at normal incidence and under visual inspection by an in-line video microscope. The micro-XRF signals were collected in the horizontal plane by means of a VORTEX EM silicon drift detector oriented at an angle of 80° with respect to the sample surface normal. Sample dwell times are 1-2 s per scan point (see [3]).

Results: Our pilot studies at PETRA beamline P06 clearly revealed the shielding pigment of Clunio as a melanin, which differs from the metal-free shielding pigment ommochrome generally occurring in insect eyes. Due to the high resolution of the XRF-measurements at PETRA P06, we could identify different trace metals inside the pigment layer and even inside single melanosomes clearly changing according to the fixation time in the lunar phase (Fig. 1). Most important is the result that the transparency of the shielding pigment does not occur as a “demelanization” but rather to a conversion of the dark brown eumelanin into the transparent, resp. light red-yellow phaeomelanin. This process seems to be controlled by trace elements (with Cu probably at the first place) and proceed with the lunar cycle. The advantage of XRF element mapping with a sub-micrometer resolution compared to biochemical analyses of melanisation processes is...
the topological association of metal content with fine structural changes of the melanosomes and the surrounding cytoplasm (e.g. trans-membrane transport enhanced along with high Ca concentration). Another advantage of this project is that we can analyse the dynamics of melanisation during the different lunar phases and are not restricted to a certain status of pigment formation. We will supplement these pilot studies by more data from eyes fixated during the entire lunar cycle. Already now, the shielding pigment of the larval eyes of *Clunio* provides a promising model system for analyses of melanisation mechanisms with great similarity to processes in mammalian tissues.

**Figure 1:** Light microscopic views of semithin section (upper row) and below μ-XRF maps of zinc (Zn), nickel (Ni), copper (Cu), calcium (Ca) within larval ocelli in different lunar phases [from left to right side: LCD 2 = full moon, beginning of simulated nocturnal moonshine, LCD 10 = 6 days after simulated moon off, LCD 19 = midst of dark moon phase, LCD 25 = end of lunar cycle. Column 1-3: *Clunio* strain Vigo; right column: *Clunio* strain Jean; the colour coded intensity scale bars (counts per sec) vary in the different plots]. The element distribution matches a conversion from the dark brown eumelanin in the dark moon phase (maximal at LCD 19) to the transparent phaeomelanin during nocturnal moonshine (LCD 2).

**References**