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Moonlight receptor of the "1-h-midge" *Clunio marinus* studied by micro-XRF

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Abstract. Melanin is a pigment widely occurring in animals, plants, fungi and algae. It does not only colour skin, hair and eyes but serves mainly as photoprotectant and prevents overload with minerals induced by inflammations, infections and degenerative diseases. Therefore, the mechanisms underlying melanisation gained increasing interest in the field of biomedical research and clinic. So far, the processes of melanogenesis are only partly analysed, nearly nothing is known on a putative switch between melanins of different types. Here we offer a model organism to study these mechanisms as part of a naturally cycling change of transparency of the retinal shielding pigment. A marine midge, *Clunio marinus*, living in coastal regions, undergoes a complex timing of its development by solar and lunar climatic periodicities, which synchronise biological clocks. The question was how the animals can discriminate changing sunlight from moonlight intensities. For the first time, we could show a "moonlight window" in the larval ocelli of this midge, and propose a hypothesis on the underlying mechanisms. Driven by a lunar clock the image forming ocelli become transparent and convert during moonlit nights to a sensitive photometer, which can record the dynamics of environmental light. High resolution X-ray fluorescence (XRF) measurements of the distribution of trace minerals in single melanosomes combined with their fine structural details in various states of the lunar cycle provide a first insight into the enzymatic pathways for the generation of a dark melanin (like eumelanin) and a light coloured melanin (like phaemelanin). Essential advantage of this approach is the spatial and temporal resolution of the metals associated with melanisation processes, which could never before be demonstrated in these details. The data may stimulate further research projects in biomedicine.

1. Introduction

In organisms melanin is a ubiquitous pigment molecule with a far reaching functional meaning, like pattern of skin or fur, colour of hair and eyes; nevertheless it also serves as photoprotectant, and storage of excess minerals, which might occur along with inflammation, injury and infection. The molecule is highly concentrated intracellularly within membrane vesicles, so called "melanosomes". Understanding genetics and biochemistry of melanogenesis and also hormonal or neuronal controlling pathways has nowadays gained a fast growing relevance in medicine for the early diagnosis and treatment: cancer research, degenerative diseases of the central nervous system (e.g., Alzheimer or Parkinson disease) and other age dependent illnesses; also some genetic defects like albinism and special varieties of blindness can be relayed to mutations in the melanisation processes. Nevertheless, a concise theory about all these complex interrelations is still missing. The tiny midge *Clunio* which lives in Atlantic and Pacific coastal regions [1, 2] offers a surprising model for solving some of the open questions of melanisation. Different from most insects, where ommochromes fulfil this function, *Clunio* has melanin as screening pigment in its larval ocelli [3], and here a clear rhythmic change of the screening pigment transparency can be observed [4, 5]. Endogenously controlled by a lunar clock [5, 6], this mechanism alters the function of the ocelli from image forming eyes - with light access only from a distinct direction like in a pinhole camera (Fig. 1) - to a non-visual photoreceptor for a temporal and spatial integration of environmental light during moonlit nights [6].
Fig. 1 Semithin sections of larval ocelli in various lunar states: LCD 2-3 begin of Full moon; LCD 4-5 Midst of Full moon; LCD 15 New moon, about a week past nocturnal moonlight; LCD 25 New moon about one week before nocturnal moonlight restarts. [Lunar cycle day (LCD), on LCD 1 "moonlight" is switched on for four nights, the entire lunar cycle lasts 30 days; ON = optic nerve, rh = rhabdom, sp = screening pigment]

This moonlight window enables the organism 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 the survival of organisms like *Clunio* which live in an intertidal environment [1, 2]. Here we try to develop a hypothesis how this change of transparency in the larval ocelli could be achieved - possibly by a switch between two different forms of the melanin protein. However, a detailed knowledge of the decisive steps of melanisation is missing mainly caused by the lack of a general concept, which comprises the generation of all the various melanin forms - eumelanins (black-brown colour of e.g., skin, hair and eyes), phaeomelanins (yellow-light red pigment in similar sites of the organism), neuromelanins (in the nervous system) and all "allomelanins", which occur in plants, fungi and algae. In insects melanisation has not been analyzed in detail. We hypothesize that in *Clunio* melanogenesis, at least partially, may take place similarly to the mammalian processes. Enzymes involved in melanogenesis depend on trace metals, which can be associated with certain steps of the pigment formation and are also indicative for the different forms of melanin [7]. Thus, we investigate the elements occurring in the ocellar screening pigment layer of the midge along with the changing transparency in different lunar states.

2. Material and Methods

The midges *Clunio marinus* were raised as synchronized laboratory cultures in the animal houses of the Zoological institutes of the Universities Cologne and the Max F. Perutz Laboratories, Vienna, under a daily 14:10 hrs light/dark regime. Every 30 days an artificial "moon" was switched on for four nights, simulating a nocturnal moonlight around "Full moon" [for more details see 5]. For convenience we refer to the time span without nocturnal moonlight as "New moon". Lunar Cycle Day (LCD) 1 is the day with the first of four moonlit nights, the entire lunar cycle lasts 30 days, ending with LCD 30. Histological processing was performed with the strict avoidance of metal tools for dissection, cutting and further handling, and of metal containing chemicals during fixation and embedding [see 5]. For micro-XRF measurements, semithin sections (2-3 µm) were mounted on silicon nitride membranes windows (Silson Ltd., Northampton, England). In order to control the fine structure of single melanosomes, parallel ultrathin sections were collected on Formvar coated copper grids for the inspection in the transmission electron microscope (ZEISS C10R and C12R, Oberkochen). 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 focused by KB-mirrors to a size of 300 × 300 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.

3. Results and Discussion

Our pilot studies at the P06 microprobe on the cyclic change of transparency of the screening pigment in the larval ocelli of *Clunio marinus* provided surprising results, thanks to 50 times higher spatial resolution of this new experiment compared to the previously applied XRF-setup at DORIS III beam line L [4, 5]. Already the beamline L data have made us assume that the screening pigment in *Clunio* larval ocelli is a melanin, not an ommochrome, which generally occurs in insect eyes, but does not contain metal ions. Now, at P06, we could clearly identify different elements in the retinal shielding pigment layer, and even in individual pigment granules, which are changed according to the time of fixation at different lunar phases. The great advantage of XRF element mapping with sub-micrometer
resolution is the topological association of metal content with fine structural details as shown in the transmission electron microscope - in contrast to biochemical analyses of tissue extracts (Fig. 2).

Fig. 2 Melanosomes inside one of the retinula cells of an ocellus at the beginning of New moon (LCD 10). A, B electron microscopic view shows partly filled pigment granules (B higher magnification of A); C µ-XRF element distribution in a section parallel to A, B. The elements are indicative for ongoing eumelanisation.

We can easily discriminate the individual receptor cells (Fig. 3) as well as single pigment granules inside the ocelli (Fig. 2, 3). This is relevant, as the lunar clock signal is most likely transferred to the eye via the optic nerve. The receptor cells next to the entrance of the eye nerve into the retina are the fastest to change their transparency (see Fig. 1). So far, we could analyze ocelli fixated during "Full moon" (LCD 5), the time with nocturnal moonlight, and ocelli fixated during the first week of "New moon" (LCD 10 and LCD 14) and in its middle phase (LCD 19) (Fig. 2, 3).

As the details of different preconditions (animal age, precision of lunar phase, also section plane, section thickness) and their correlation with the XRF data are not yet systematically investigated, we can here only offer a first hypothesis. Corresponding to the different states of the pigment granules we show that the amount of various trace elements like Zn, Ni, Cu, and Ca, as well as their concentration within the pigment granules is changing. Thus, we do not find melanisation versus demelanisation, but rather a conversion between two molecular configurations of melanin like the dark eumelanin and the transparent phaeomelanin [cf. 7]. This conversion seems to be controlled by trace elements (with Cu in the first place) and is likely to occur as the lunar cycle proceeds.

The transport of trace metals from the melanosomes into the surrounding cytoplasm seems to be enhanced as indicated by the increased Ca content, which is essential for membrane transporter enzymes. These pilot analyses must be supplemented and critically verified by further data from eyes fixated during different phases of the experimental lunar light programme in order to resolve the dynamics of the change of transparency in greater details. But already now, they have lead us to the hypothesis that melanogenesis in Clunio ocelli has great similarity to the same process in mammalian tissues [7].

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Fig. 3 µ-XRF maps of ocelli in different lunar states. Left row: ocellus during Full moon putatively filled with phaeomelanin; Middle row: Dramatic change of element distribution at the beginning of melanisation during New moon; Right row: Midst of new moon phase, element distribution matches eumelanin. [The x, y axes of the XRF-maps are calibrated in µm; notice that the colour coded intensity scale bars (counts per sec) differ in the different plots.]

References