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Influence of the adult eyes on circadian and
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Verfasser:	Stefan Keplinger
Matrikelnummer	0307989
Studienrichtung:	Molekulare Biologie
Betreuerin / Betreuer:	Dr. Kristin Tessmar-Raible

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THE TIMES THEY ARE A-CHANGIN'

- Bob Dylan

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1 Abstract

The marine annelid *Platynereis dumerilii* exhibits a biological phenomenon concerning its spawning. During the night, 9-13 days after the last full moon the worms will leave their tubes, swim up and spawn. Male and female release their gametes into the water. They do this not randomly but synchronously in huge numbers according to the lunar phase. This circadian and lunar controlled photoperiodic spawning is a strategy to ensure successful mating.

Without temporal concordance external fertilisation would be impossible and the gametes would be diluted in the sea without encountering each other. In the controlled laboratory environment spawning of *Platynereis dumerilii* proved to be synchronized by light. To spawn accordingly on time *Platynereis dumerilii* has to initiate metamorphosis to its mature, epitoke form and the maturation of the gametes early enough. Therefore it needs an internal clock to measure time. This “clock” enables the animal to anticipate certain events, like full moon, night and day. To synchronize the lunar clock entrained to the moon. To synchronize the circadian clock it has to be entrained to the sun. *Platynereis dumerilii* needs both of these clocks to anticipate the right time to spawn. The sensors of the light are photoreceptor cells. In *Platynereis* two opsin based photoreceptor systems exist – rhabdomeric and ciliary.

Rhabdomeric photoreceptor cells constitute the larval eyes and the adult eyes. They are the primary visual organs. In this study the involvement of the rhabdomeric adult eyes in lunar and circadian clock is assessed by UV laser mediated selective destruction. Because animals could not be blinded permanently but transiently I assessed the entrainability of the circadian clock and excluded the lunar clock. The regenerative abilities of *Platynereis dumerilii* antagonized permanent tissue destruction. Yet I could significantly reduce the photopigment of the adult eyes in *Platynereis dumerilii*. These blinded animals could adapt their endogenous circadian clock within 72 hours to an inverted circadian rhythm suggesting either incomplete blindness, independence from adult eyes or functional compensation in the photoreceptive gateway for circadian clock perturbations. My finding of so far uncharacterized rhabdomeric ocelli in older stages argues for independency or compensation. I also cloned putative clock output genes and analysed their expression with WMISH. I cloned *pdf*, a neuropeptide, and found expression within certain clusters of *Platynereis* brain. I cloned *aanat*, *hiomt*, enzymes of the melatonin pathway, and an N-terminal gene fragment of presumably *hiomt like* (whose function is not clear) and tracked their expression to the same circum-oral structure. For phylogenetic footprinting I cloned two orthologue *c-opsins* from *Nereis virens*.

Summarized I tried with success to establish laser mediated destruction of adult eyes in various stages, but obtained an ambiguous result for circadian entrainment. My finding of up to date unknown ocelli in *Platynereis dumerilii* might suggest a role for them in circadian entrainment. I successfully cloned and spotted the expression patterns for four presumptive clock output genes.

2 Zusammenfassung

Der marine lebende Annelid, *Platynereis dumerilii*, ist ein Musterbeispiel für das biologische Phänomen des lunar-periodischen Schwärmens. Nachts, 9-13 Tage nach dem letzten Vollmond, verlassen die epitoken Würmer ihre Gespinströhren, schwimmen an die Meeresoberfläche und schwärmen. Männchen und Weibchen geben ihre Gameten dabei ins Wasser ab. Dieses Paarungsverhalten ist nicht zufällig sondern synchron zur Mondphase in großer Zahl. Diese circadiane und lunare kontrollierte Photoperiodizität der Schwärmfrequenz ist eine Überlebensstrategie, um den Erfolg einer Befruchtung zu maximieren. Ohne diese zeitliche Abstimmung wäre eine externe Fertilisation, wie sein *Platynereis* pflegt, nicht möglich und die Gameten würden sich rasch im Meerwasser verdünnen, ohne sich jemals zu treffen.

Unter kontrollierten Laborbedingungen konnte die Synchronisation des Schwärmens mittels Licht nachgewiesen werden. Um termingerechtes Schwärmen gewährleisten zu können, muß *Platynereis dumerilii* die Metamorphose zu seiner reifen atoken Form und die Reifung der Gameten früh genug einleiten. Zu diesem Zweck ist ein endogene „clock“ vonnöten, welche den Organismus befähigt, äußere wiederkehrende geophysikalische Ereignisse wie Mondphase, Tide, Tag und Nacht zu antizipieren. Zur Synchronisation muss die lunare „clock“ an das Mondlicht gewöhnt werden. Zur Synchronisation muss die circadiane „clock“ an das tägliche Sonnenlicht gewöhnt werden. *Platynereis dumerilii* benötigt beide dieser „clocks“, um den richtigen Zeitpunkt zum Schwärmen zu antizipieren. Die Lichtsensoren sind Photorezeptorzellen. In *Platynereis dumerilii* existieren zwei Opsin basierte Photorezeptorsysteme: rhabdomeres und ciliäres. Rhabdomere Photorezeptorzellen sind die sensorischen Einheiten der larvalen und der adulten Augen, welche die primären visuellen Organe des Wurmes sind. In dieser Studie wird der Einfluss der rhabdomeren Adultaugen auf die lunare wie auch auf die circadiane „clock“ mittels gezielter UV-Laser Ablation untersucht. Jedoch war es nicht möglich die Würmer permanent zu blenden, stattdessen ist eine kurzzeitige Blindheit gewährleistet. Das Regenerationspotential von *Platynereis dumerilii* konterkarierte meine Versuche bleibende Gewebe-ablation zu erzeugen. Jedoch war es mir möglich, das Photopigment der Adultaugen signifikant zu reduzieren. In der Art geblendete Würmer konnten innert 72 Stunden ihre endogene circadiane „clock“ an einen invertierten Tag/Nacht-Rhythmus gewöhnen. Dieses Resultat lässt sich entweder durch nicht vollständige Blindheit, Unabhängigkeit der Lichtwahrnehmung von Adultaugen oder Kompensation in den Photorezeptorsysteme zur Gewöhnung der circadianen „clock“ erklären. Meine Entdeckung von bisher unbeschriebenen rhabdomeren Ocelli in älteren Stadien spricht für Unabhängigkeit oder Kompensation. Weiters konnte ich vier mutmaßliche „clock output genes“ klonen und mittels in-situ-Hybridisierung ihre jeweilige Expression im Wurm feststellen. Die Expression von *Pdu pdf*, ein Neuropeptid, konnte ich in mehreren Zellgruppen im zerebralen Ganglion nachweisen. Die Klonierung von *Pdu aanat*, *Pdu hiomt*, Enzyme der Melatonin Synthese, und ein N-terminales Genfragment von vermutlich *Pdu hiomt like*, dessen Funktion unklar ist, konnte ich erfolgreich abschliessen und ihre (Co-)Expression in einer gemeinsamen circum-oralen Struktur nachweisen. Zum Zweck des „phylogenetisches footprinting“ konnte ich zwei orthologe *c-opsin* Gene von *Nereis virens* klonen.

Zusammengefasst konnte ich mit Erfolg UV-Laser vermittelte Ablation von Adultaugen in mehreren Stadien von *Platynereis dumerilii* etablieren, während die Resultate mehrere Deutungen zulassen. Meine Entdeckung von bisher unbekannten rhabdomeren Ocelli in *Platynereis dumerilii* lässt auf eine Rolle dieser in circadianem „entrainment“ plausibel erscheinen. Erfolgreiche Klonierung und Bestimmung des zellulären Expressionsmusters von mehreren „clock output“-Genen schließen meine Studie ab.



Figure 1 *Platynereis dumerilii* during atoke and epitoke phase

Illustration adopted from [3]. The two worms on the top depict the mature epitoke animal during mating – this locomotor behaviour is widely referred to as “nuptial dance”. The lower two worms are exemplary for the atoke phase, one worm is in its tube surrounded by algae, the other worm illustrates the benthic life style of *Platynereis dumerilii*.

3 Introduction

3.1 *Platynereis dumerilii* as a model organism

Platynereis dumerilii is a marine polychaete belonging to the lophotrochozoan branch and together with the ecdysozoa they comprise the protostomia. Both together with the deuterostomia they make up the bilateria. Classical model organisms in molecular biology are rather ecdysozoa (*Drosophila melanogaster*, *C. elegans*) or deuterostomia (rat, mouse,...). As a model organism *Platynereis* being a representative of the marine lophotrochozoa provides insights into bilaterian evolution and relationships. Based on its genomic structure comparison of intron/exon organisation of 30 orthologue genes found among bilateria yielded that genomic structure is more similar to vertebrates than to insects (*D. melanogaster*, *C. elegans*) [7]. This in turn points to loss in introns in other invertebrate species and retention of introns in slow evolving species like humans. Also in this respect *Platynereis* resembles human genes more than e.g. ecdysozoan genes. From a more practical point of view and more important to my study *Platynereis* exhibits a series of biological phenomena like its lunar photoperiodic spawning behaviour and its regeneration capabilities. The organism is easy to culture in plastic boxes with natural and artificial saltwater. Its establishment as a laboratory culture started in 1955 by C. Hauenschild and since 2000 generations of inbred strains provide a consistent genomic background. Its generation time (3-4months) is comparable other model organisms.

Once in a lifetime it produces hundreds of offspring via external fertilisation which are thereby developmentally synchronized; although developmental dispersion within offspring from same parents commonly occurs. Due to its semelparous life form forward genetics is rather difficult. The larvae as well as the atoke worms are transparent, which makes them susceptible for microscopic approaches. Molecular biological standard techniques are already established, like immunological staining or WMISHs. Techniques [8] to interfere transiently (injection of dsRNA) with genexpression have been demonstrated to work in *Platynereis dumerilii* [9] and transgenesis is a topic heavily researched. Genomic BAC libraries and sequenced transcriptome already exist. The literature provides detailed data on anatomy and regeneration.

3.1.1 The or-mutant

The inbred strain used for the initial experiments was the RE-line. The strain is termed RE connoting for “red eye” due to the fact that it derived from the so called or-mutant [10, 11]. The trait is recessive and affects the worm only when inherited homozygous. The advantage of these mutants is that the pigment- and support cells fail to incorporate their optical dense red-brown pigment[12]. In general the pteridine pigment [13] synthesis is deficient on the systemic level. Thus the eye colour originates solely from the PRCs’ pigment giving the eyes an orange or faint red appearance. This appearance in fact is characteristic for all *rhodopsin* expressing cells. Discrimination of PRCs from other cells therefore is easy using standard Nomarski optics. Having a look is therefore sufficient to distinguish the orange coloured PRCs from pigment cells, thereby providing a clear identification of the eye’s light sensing units.

3.1.2 Life cycle

Platynereis dumerilii is a semelparous, marine organism that quickly dies after mating (Figure 1, upper two worms). The fertilized egg develops in a typical spiral cleavage and the embryo further differentiates (Figure 2). Life continues as a zooplanktonic trochophore larva that actively swims in a phototactic manner with its ciliary band. It further grows to an elongated

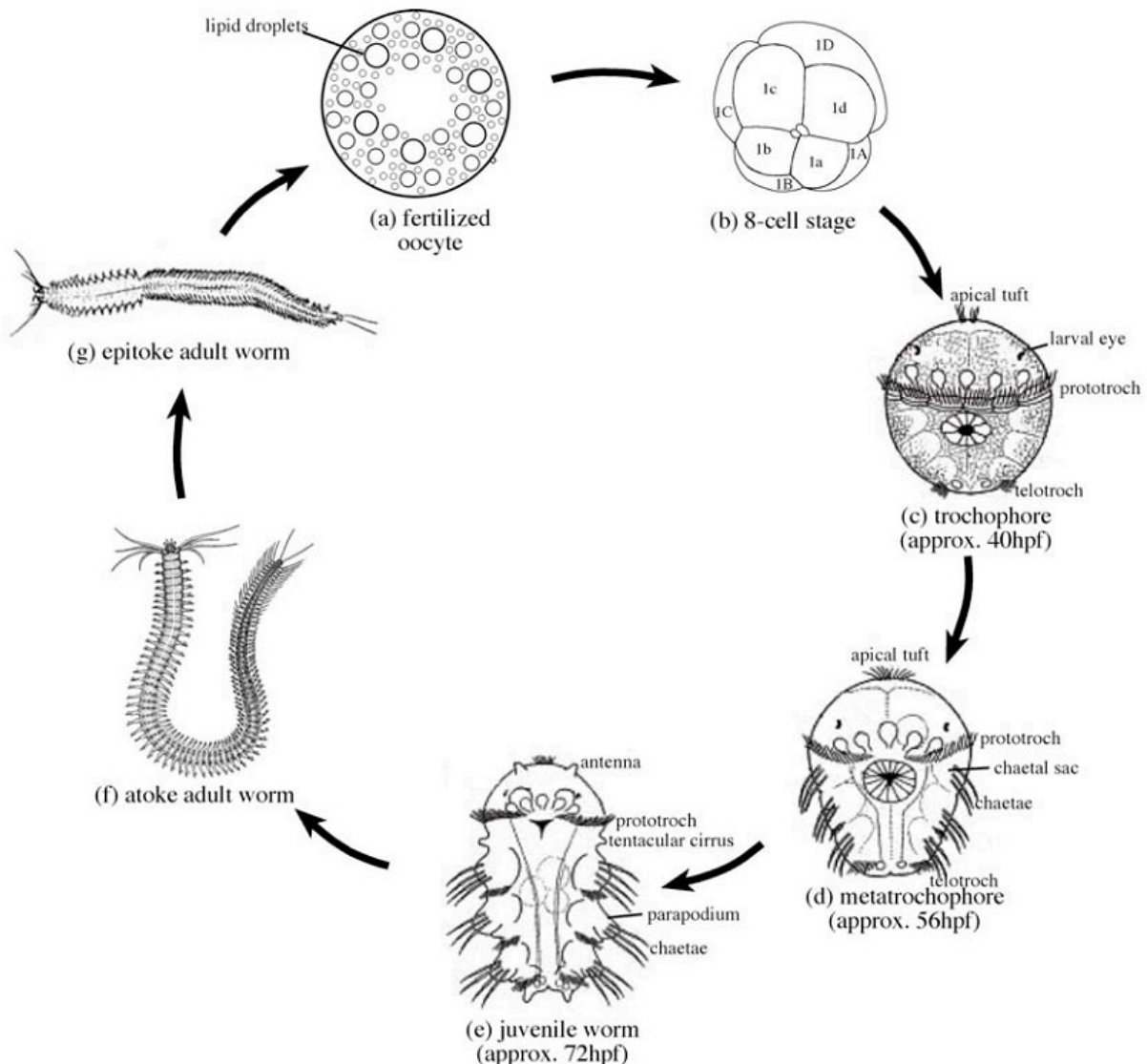


Figure 2 The life cycle of *Platynereis dumerilii*.

Adopted from [3]. Life starts from embryo with planktonic life style and changes to benthic when parapodia are developing. Metamorphosis marks transition from atoke to epitoke animal. Life ends quickly after reproduction. The time scale of developmental stages is valid for 18°C. For details see text.

juvenile animal (72hpf) with three parapodial segments bearing chaetae. It changes its life style to benthic (Figure 1, lower two worms) and elongates by forming new segments from the growth zone. 3-4 weeks after fertilisation cephalisation occurs when the anterior parapodia are transformed into tentacular cirri. Growth by forming new segments occurs till sexual maturation. This is considered as the end of the animal's atoke phase. It sexually matures typically after 3-4 months post fertilisation and develops into the epitoke heteronereis. This transformation includes loss of muscle cells, parapodia and addition of blood vessels. The gametes' colour confers the colouration of mature female and male worms (Figure 1, upper two worms) [14].

3.1.3 Photoreception in *Platynereis dumerilii*

3.1.3.1 Phototransduction motifs

Sensing light relies on a pigment, retinaldehyde (or derivate) that changes conformation (photoisomerisation) upon photon absorption and a protein, OPSIN, detecting this change (for review [15]). To increase sensitivity to light PRCs incorporate as many opsins as possible in their membrane. Photoreceptors exhibit two modes of surface enlargement in order to harvest light (Figure 3). The cell's apical surface is enlarged by various folds or microvilli which corresponds to rhabdomeric PRCs. The formation of a modified cilium corresponds to ciliary PRCs. Despite being seven-transmembrane GPCRs, employed photopigments also differ and are categorized into ciliary (*c-opsin*) and rhabdomeric opsin (*r-opsin*). This difference also concerns the downstream coupling to trimeric GTP binding protein (the C-terminus' aa sequence differs). In ciliary photoreceptors the C-OPSIN is coupled to G_{ai} (transducin) type G protein whereas rhabdomeric opsin is coupled to G_{aq} type G protein. Thus binding of the G protein α -subunits occurs to different effector proteins.

Light absorption activates C-OPSIN which in turn activates coupled G protein by GDP/GTP exchange. G_{ai} dissociates and binds to phosphodiesterase (PDE), active PDE hydrolyzes cyclic GMP. Decreased cGMP levels lead to closing of cyclic-nucleotide-gated channels (CNG channels), which otherwise are permanent open in darkness. These channels are non-selective cation channels with steady inward current depolarizing the cell at -30mV (dark membrane potential) in order to sustain constant glutamate release. When levels of cGMP are decreased and channels closed the cell gets hyperpolarized and reduces or stops synaptic transmission. The C-OPSIN phototransduction motif leads to hyperpolarization and stop of transmitter release [15].

For R-OPSINS phototransduction is not as clear although it is best studied in *Drosophila*. Light absorption activates R-OPSIN which leads to GDP/GTP exchange in the coupled G protein. The α -subunit activates phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositoltriphosphate (IP_3) and diacylglycerol (DAG). From here steps still need clarification. IP_3 seems to play a minor role as increase in Ca^{2+} levels is minimal, and IP_3 receptor mutants show no defects. DAG may convey downstream signalling by formation of metabolites like polyunsaturated fatty acids by the enzyme DAG lipase. Also

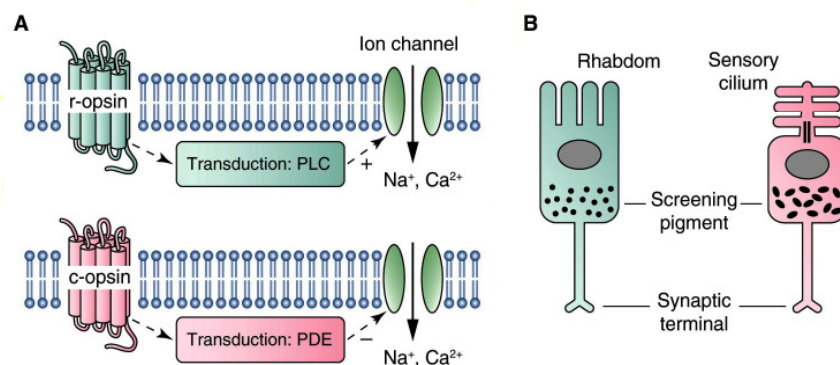


Figure 3 Phototransduction motifs and cell morphology

Adopted from [4].

- A) **R-OPSIN** phototransduction employs downstream PLC pathway and results in depolarisation. **C-OPSIN** phototransduction employs downstream PDE pathway and results in hyperpolarization.
- B) Different morphology of rhabdomeric and ciliary PRC

decreased PIP₂ might play a role. However the ultimate targets are the two transient-receptor-potential channels (TRP and TRPL) which open at the end of the signal cascade. Both are non-selective cation channels although TRP exhibits high selectivity for Ca²⁺. The R-OPSIN transduction motif leads to opening of cation channels and depolarisation of the cell and transmitter release [15].

3.1.3.2 Photoreceptors

3.1.3.2.1 Rhabdomeric photoreceptors

In *Platynereis dumerilii* both types of photoreceptors can be found. While rhabdomeric PRCs are implemented in the cerebral eyes [16], ciliary PRCs [17] are not associated with pigment cells and are not engaged for directional vision. It can be argued that rhabdomeric PRCs are for directional vision (or just vision) in contrast to ciliary PRCs which are for luminance detection. Directional seeing is the function of the eye, which has to be made up from a PRC with an associated pigment cell in its most basic form; hence PRCs without shading pigment cells are not regarded as eyes. The eyes in *Platynereis dumerilii* are so called pigment cup eyes due to their shape. The pigment cells form a cup like structure that shields the PRCs from backwards radiation. Light can only enter through the opening.

Platynereis' cerebral eyes (Figure 4) have been subject of extensive research in order to highlight evolution of eyes, exemplary for transition of simple structures to complex structures. Its larval eyes are very basic visual organs, consisting of only one shading pigment cell and one rhabdomeric PRC – which is the considered layout of a hypothesized “proto-eye”. At about 22 hpf a single pair of larval eyes appear in a lateral position of the head. They shift in a ventral direction during development and eventually end up at the base of the palps [18]. The larval eyes are termed inverse because the rhabdome of the PRC extends into an invagination of the pigment cell (Figure 4 A). Larval eyes mediate phototaxis in the trochophora, where they directly innervate ciliated cells responsible for swimming movements [19]. The larval eye is thought to decay between 3 -4dpf although there is no distinct proof for complete disappearance [20].

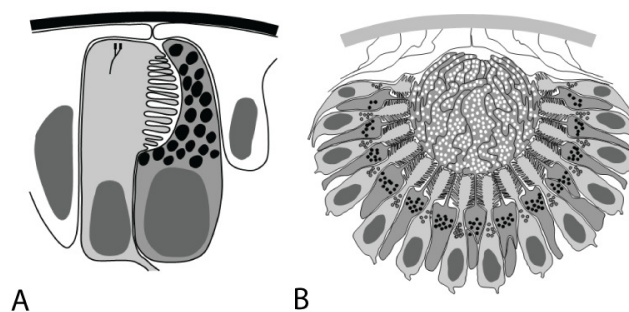


Figure 4 Cerebral eyes of *Platynereis dumerilii*.

A) the larval eye (24hpf) consists of one PRC and one SPC
B) the adult eye is made from multiple PRCs, SPCs and support cells. Adopted from [5].

It has been shown that the first morphological evidence for the adult eye in developing embryo appears at 53 hpf [21] in vicinity to the larval eyes. The adult eye splits into two on each side at about 72hpf and shift to the dorsal side of the head (Figure 5). They grow in size by mitotic division of its constituent cell types, pigment -, photoreceptive- and support cells throughout the atoke phase. During atoke-epitoke metamorphosis the eyes grow by solely by

extension of the cells. Therefore *Platynereis*' adult cerebral eyes have in contrast to the larval a multicellular layout and are not just a derivate of the larval eyes. They are termed everse because the photoreceptor cells project away from pigment cells into the cup towards incoming light (Figure 4.B) Although it is thought that they develop from an inverse layout to an everse during ontogeny [5]. Adult eyes have already simple optics [22]; they can widen or narrow the aperture ("pupil") and thus achieve light/dark adaptation. Support cells comprise the inner filling mass or vitreous body – its refractory index is not known, therefore it cannot be said if it acts as a lens.

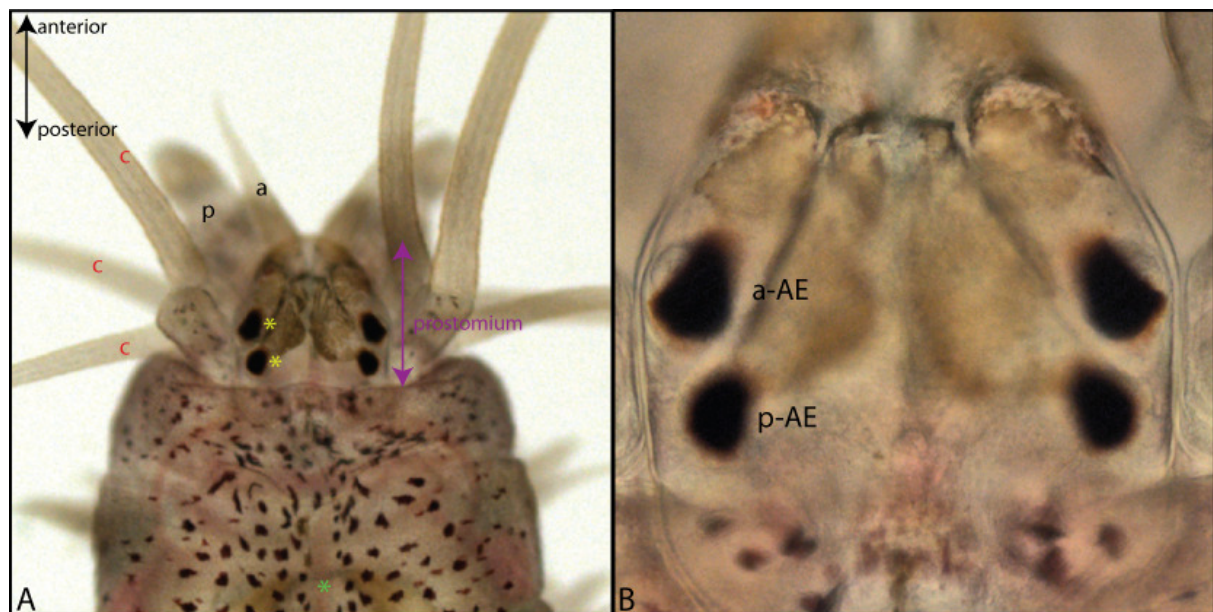


Figure 5 Head region and important anatomical landmarks in 2 month old *P. dumerilii*

- A) Dorsal view on the anterior end of the worm bearing two pairs of adult eyes, one pair of antennae, one pair of palpa and four pairs of tentacular cirri. a=antenna, c=cirri, p=palpa, yellow asterisk=adult eye, green asterisk=jaw
- B) Higher magnification of the head. The adult eye pair is divided in one anterior and one posterior eye. a-AE=anterior adult eye, p-AE=posterior adult eye.

3.1.3.2.2 Ciliary Photoreceptors

In addition to the rhabdomeric eyes there are ciliary light sensors found in *Platynereis*' brain. *Ciliary opsin* and *r-opsin* both are thought to evolve in parallel since gene duplication in a common ancestor. Unlike in vertebrates ciliary light sensors in *Platynereis dumerilii* are not implemented in the eyes. First by immunological staining for α -tubulin and then by ISH against *Pdu c-opsin* a paired structure in the dorsal part of the brain has been identified to be ciliary and photoreceptive [17]. Interestingly these ciliary PRCs contain an oscillator and are located within a cluster of clock gene expressing cells. Possibly they are an input stage and residence of the circadian clock. These features also account for the comparison with vertebrate, submammalian pinealocytes which also show direct light entrainability of their oscillator by their intrinsic C-OPSIN mediated light sensitivity. Whereas its rhabdomeric sister cell type lost this function and e.g. is only merely present as R-OPSIN phototransduction motif in retinal ganglion cells termed "melanopsin" [23] (see chapter 3.3.1 for details).

3.1.3.2.3 *Platynereis dumerilii*'s rhabdomeric PRCs and vertebrate retinal ganglion cells

The afore mentioned retinal ganglion cells are known to be involved in circadian entrainment by signalling to the suprachiasmatic nucleus [24]. Thus the question arises whether circadian entrainment is a reminiscent function of rhabdomeric PRCs that is still retained in vertebrates. The two rhabdomeric photoreceptor domains in *Platynereis dumerilii*, the larval and the adult cerebral eye, might be employed in circadian entrainment. The resemblance of the vertebrate retinal ganglion cell and the *Pdu* rhabdomeric PRC is reflected in the morphology and transcription factor cascade leading to their differentiation [17, 21]. Initially both *Pdu* larval and adult eye develop from *Pdu ath* (an orthologue of *atonal*) positive cells. The larval eyes express *pax6*, *brn3* and early *six1/2* in contrast to adult eyes. Adult eyes develop in absence of *pax6* expression but share early and continuous *six1/2* expression. In the respect of transcriptional fate the *Pdu* larval eye resembles the vertebrate retinal ganglion cell which expresses *ath*, *brn3* and *pax6* during differentiation stages. Vertebrate orthologues of *six1* and *six2* transcription factors are in contrast expressed in the late differentiating retina. In a morphological respect *Pdu* larval eyes and retinal ganglion cells resemble each other by sending out their axons connecting the eyes to the brain. Due to the similarities of both cell types the hypothesis of a similar function in regard to circadian entrainment is tempting and plausible. In this study the influence of the adult cerebral eye and not the larval was tested on circadian entrainment.

3.2 Biological Rhythms

The variety of biological rhythms is field of research of so called chronobiology. A biological rhythm can be seen as a temporal sequence of recurrent biological functions. A variety of species exhibit biological rhythms, from prokaryotes [25], that divide several times during the day but still are capable of measuring a twentyfour hour cycle, to specialized eukaryotes, that in the extreme case depolarizes 60 times and more within a minute. Rhythms in chronobiology are defined by their period (see Figure 6) rather than by frequency. Periods longer than twentyfour hours are called “infradian” and can match the duration of a year, then called “circannual”, or approximately a month alike the lunar cycle (29,5 days) or shorter, the semilunar cycle (14,8 days) reflected by tidal rhythms. Periods shorter than twentyfour hours are called “ultradian”, like the cycles of sleep [26] lasting a few minutes or the defecation behaviour of *C.elegans* lasting 45 seconds [27] or the human cardiac cycle lasting less than one second .Hence a variety of species or cells within a species show a variety of different biological rhythms. Although the most prominent rhythm is the circadian rhythm that approximately matches a day’s length. The fact that the circadian rhythm does not match exactly a day’s period already reveals its endogeny. Yet the crucial criterion for actual endogeny can only be revealed in a free running experiment. That is its oscillation continues in absence of the zeitgeber. If the circadian rhythm is in phase with the meteorological day it is called “diurnal” rhythm. A second feature of an oscillation despite its period is the amplitude. It must not dampen during the oscillation and be sufficient high to drive an output. In some cases it is also important for binary or discrete decisions[28] – e.g. when two cycles are competing and one wins over the other [29, 30]. Otherwise the amplitude plays only a minor role in characterisation of biological rhythms. Like every oscillation the phase is another important feature needed for description. The phase of the rhythm is determined by a “zeitgeber”. A zeitgeber is usually an external, environmental cue (e.g. astronomic events...). This is essential for a biological rhythm to coordinate intrinsic biological activity with the outside or extrinsic world by means of a zeitgeber. Temporal coordination of biological activity is the core function of a biological rhythm. Therefore rhythms are also characterized by their zeitgeber which adjusts the phase and the period (circadian: up to 29hours, intensity dependent [31]). If the biological rhythm matches the rhythm of the zeitgeber it is called synchronous. The process of synchronisation of endogenous rhythm with an environmental rhythm is referred to as “entrainment”.

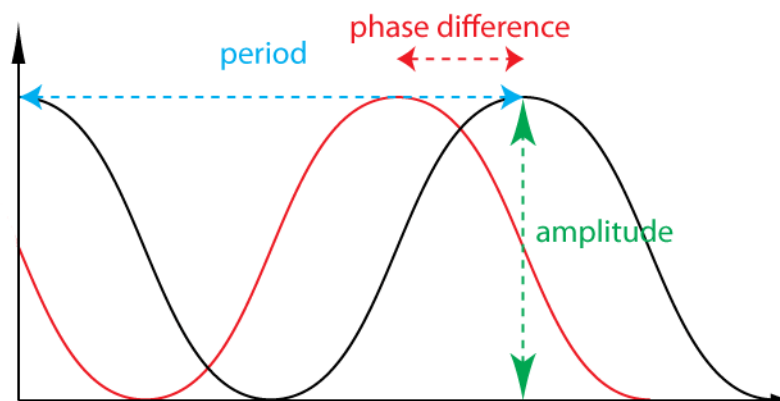


Figure 6 three basic characters for description of an oscillation
Time is plotted on the abscissa and signal strength/ concentration on the ordinate.

These basic characters (period, phase, amplitude) only describe an oscillation, but they do not reveal information on its causing mechanics. The underlying internal mechanism that enables those biological rhythms to resonate in consonance with environmental rhythms is called “clock”. Biological rhythms that do not adjust to environmental rhythms are also very abundant (e.g. cell cycle) but are not considered as clocks. In case of photoperiodicity the external light regimes are described by their light and dark period – “LD” means a light period is followed by a darkness period connoted by their ratio (e.g. 16:8 hours). “LL” means permanent light and “DD” permanent darkness respectively.

After all the concept of a biologic clock comprises three basic elements (Figure 7)[32]:

An input stage receives stimuli from the external zeitgeber and subsequently transfers the information to the next stage. The next stage is the oscillator that resonates with the period of the zeitgeber and again transfers temporal information to the next stage. The last stage are the output pathways that affect metabolism, physiology and behaviour. Interference on the first two stages, input and oscillator, allows insights into the nature of the clock’s mechanism. Manipulation of the output pathways yields information on their biological function but not on the clock per se. Some gene regulatory networks or protein interaction networks share at least one common concept of a clock but they lack another element (e.g. the input stage). Nevertheless the boundaries are blurry. Summed up a clock enables the anticipation of recurrent external events. It does so by comparing an endogenous temporal template (the oscillator) with external time cues.

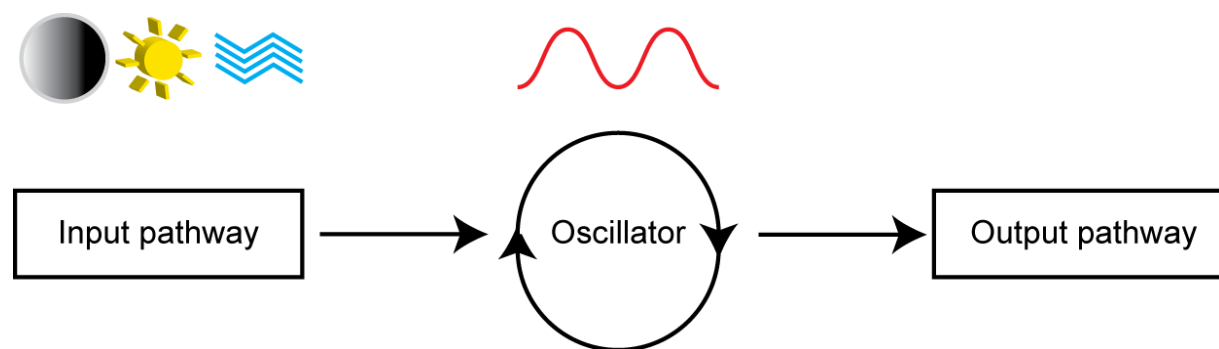


Figure 7 The three basic stages of a clock.

The signal flow happens from left to right. Temporal information enters the circuitry via the input. The temporal value is then integrated in the oscillator and modulated into an instructive signal to the output pathway. From the perspective of a hierarchy the order is not necessarily reflected in the signal flow cascade e.g. the circadian oscillator is self-sustained and cell-autonomous; therefore the other two stages were implemented to fit the oscillator. Symbols above the box depict recurrent phenomena serving as zeitgeber (moon, sun, tide). Symbol above the circle represents an oscillation.

3.2.1 The input stage

The input pathway is responsible for phase resetting of the endogenous clock. Phase resetting makes an oscillation entrainable otherwise it would be a permanent free running system. The mechanism behind this entrainment is the disturbance of the free running rhythm of an oscillator component by the zeitgeber. The disturbance leads to an adjustment in its concentration which in turn leads to phase reset. Recurrent exposure to a zeitgeber (=entrainment) leads to formation of a robust synchronized oscillation. As outlined periodic geophysical events provide a lot of recurrent stimuli (tidal, lunar, solar...) an endogenous rhythm can adjust to. In the most prominent clock, the circadian clock, the dominant stimulus for entrainment is light and its sensors are photoreceptors. Thus the photoreceptor must be linked to the clock's oscillator.

3.2.2 The oscillator stage

The oscillator is a prerequisite to formation of any clock and produces self-sustained rhythmicity. Therefore it is independent from an input in order to produce an undamped oscillation. To be useable in a clock its frequency must correlate to an external frequency. It has been demonstrated that consonance of endogenous and exogenous oscillation lead to increased fitness[33]. In a set of oscillators the dominant one is called “pacemaker” or sometimes “master clock” (e.g. SCN[34], sinoatrial node).

A model that has been extensively used to explain a basic oscillator is the *Goodwin oscillator* (Figure 8) [35].

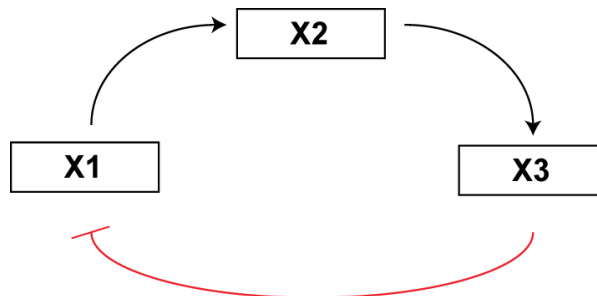


Figure 8 the Goodwin oscillator

It is a popular concept to model the mechanism of an oscillator. X1 induces X2. X2 in turn induces X3. Whereas X3 inhibits X1.

The basic oscillator comprises four conceptual elements [36] in order to achieve rhythmicity instead of homeostasis. Negative feedback and time delay are the essential components. Negative feedback is needed to reset the signal to its initial state or start respectively. Time delay is essential in order to produce an oscillating pattern otherwise reactions result in steady state equilibrium of the involved components.

First of all the X3 element negatively links to X1 which is the negative feedback. And it is called amplified because X1 amplifies X3 via X2-induction. All links between the components (X1, X2, X3) have a time delay due to protein synthesis (and degradation) and cellular transport (nucleus entry) or intermediates. When looking at the *Goodwin oscillator* (Figure 8) all experimental data from circadian clocks of cyanobacteria, drosophila melanogaster, neurospora crassa, birds and mice can be explained when simplified to its basic elements. X1 and X2 can be regarded as “positive elements” and X3 as “negative element”.

The input stage has either impact on positive elements or negative elements (in the following cases the negative elements are affected by the input). And it can either act by destabilizing (drosophila) or inducing (mammal) the elements. The physical substrates for the components (X1-3) are encoded by the so called “clock genes” and regulated on transcriptional and posttranslational level. Summarized a basic oscillator consists of three elements interconnected by positive and negative feedback.

3.2.3 The output stage

There is no exact definition of a clock output but it is very plausible that an output must be rhythmic as well. The output stages are often directly hardwired to the oscillator e.g. they share promoter motifs with components of the oscillator (Figure 9A). These are the so called “clock controlled genes” (ccg) or sometimes “first order clock controlled genes”. Another option involves indirect induction of genes via intermediates (D-element binding proteins) or slave oscillators (Figure 9B). Nevertheless, the affected genes are expressed in a periodic manner influencing physiology (e.g. body temperature, blood pressure...) and behaviour (sleep and wake, movement...). Up to 10% of mammalian mRNAs exhibit circadian rhythmicity [37]. The amount of circadian output genes is in the range of thousands [38, 39] relating many biological fields to chronobiology (e.g. chronopharmacology). Prominent examples of clock outputs are melatonin (in vertebrates) and PDF (in insects).

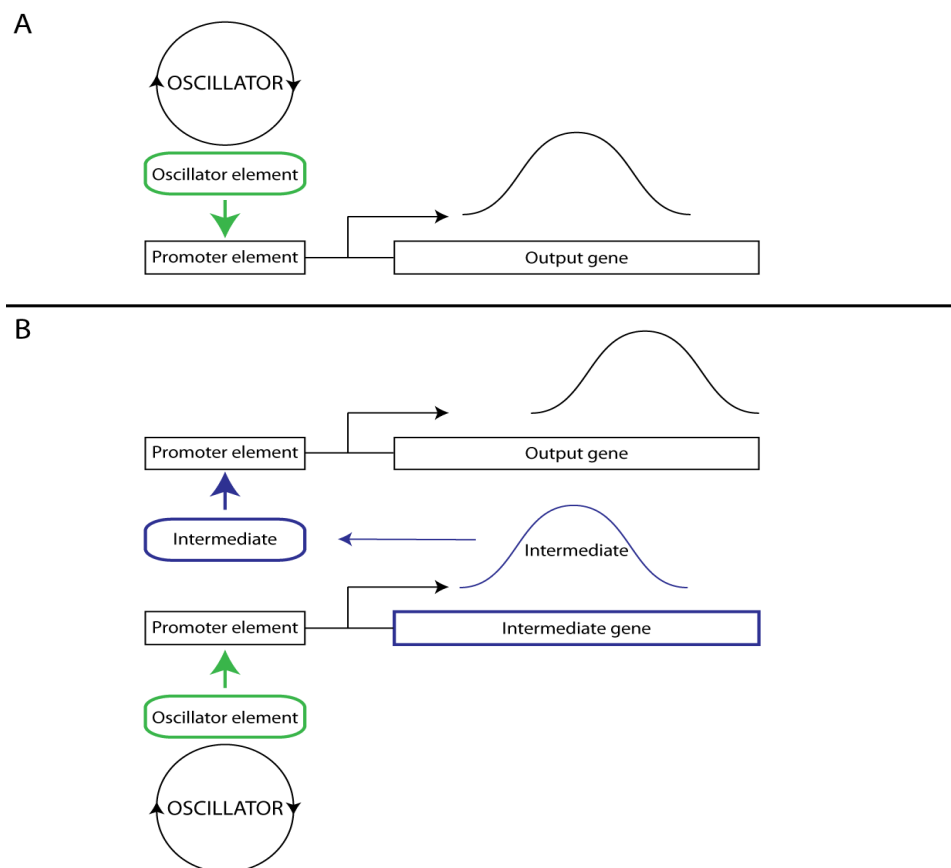


Figure 9 Mechanics for a clock output gene.

- A) The clock output is directly hardwired to the oscillator. An oscillator element induces output expression by binding to the promoter of the output gene.
- B) The clock output is indirectly induced by an intermediate that is directly hardwired to the clock's oscillator.

3.3 Circadian Clocks

3.3.1 Input stage in Mammals

The dominant external cue for entrainment of the mammalian circadian clock is light and it is perceived via photoreceptor cells in the retina of the eye. Recently it has been demonstrated that a few retinal ganglion cells are intrinsically photoreceptive and express melanopsin (a *r-opsin*) as chromophore [24]. This population is therefore called “intrinsically photosensitive retinal ganglion cells” (ipRGCs) or “melanopsin-expressing retinal ganglion cells” (mRGCs). They exhibit low sensitivity for light compared to rods or cones due to the low concentration of melanopsin in their plasma membrane. Also they have no enlarged membrane surface. These features are in accordance with the assessed low light sensitivity of the circadian input pathway [40]. The absorption spectrum of melanopsin (480-511nm, peak response 484nm) coincides with the wavelength most effective for photic entrainment [23]. The mechanism of phototransduction in ipRGCs is elusive up to date, but it might use the PLC pathway similar to invertebrate *r-opsins* [15]. Depolarization of the ipRGC after applied light pulses by an inward current has already been confirmed [41]. Alike the melanopsin-negative retinal ganglion cells they receive inputs from hundreds of other photoreceptors (rods and cones, which are ciliary photoreceptors) via bipolar- and amacrine cells. Owing to their endogenous MELANOPSIN and their innervations they are sensitive to a lot of different wavelengths and have an enlarged receptive field. Their axons form the retinohypothalamic tract (RHT) which project to special neurons forming the suprachiasmatic nucleus (SCN). Induced lesions of the RHT abolish light entrainability of the SCN [42]. The contributions of the individual photoreceptors (cone, rod, retinal ganglion cell) to signal to the SCN are hard to discern (~10-20% of the RHT are non-ipRGCs). If one system is abolished the others can functionally complement [43]. Although essential roles for rods and ipRGCs in circadian entrainment is the most recent notion [44], still conflicting data exists whether cones play a role [45]. But abolishing rod and cones leaves circadian entrainment intact, therefore they are not required as an afference. Triple mutants for cone/ rod vision and melanopsin ambient light sensing are not entrained by light [46].

The terminals of the RHT release glutamate (like cones and rods) as transmitter [47] which act on the SCN neurons. Glutamate has been shown to depolarize the SCN neurons thus being excitatory. The SCN neurons are the cellular substrates of the circadian pacemaker. Upon glutamate binding (to AMPA- and NMDA receptors) the photic input is conveyed to the oscillator and rapidly induces components of the negative feedback element. The light induction of clock genes in mammals largely relies on chromatin remodelling [48].

Downstream of the receptor signal cascades involving MAPs and CaMKs converge on the phosphorylation of cAMP-responsive element binding protein (CREB). As a result CREB protein is phosphorylated at Ser133 and Ser142 by CaMKII α [49] and converted to its active form. CREBp can now bind to cAMP response elements (CRE) regulating transcription of the negative elements (mPer1 and mPer2). It does so by recruiting histone acetyltransferases (HATs) to the promoter site. The periodic acetylation and deacetylation of H4 and H3 histones in the promoter region of mPer facilitates rhythmic expression of *mper1* and *mper2* [50]. The CRE dependent induction of gene transcription is not relying on other promoter binding events.

Summarized in mammals light induces the negative elements of the oscillator and photic entrainment is carried out.

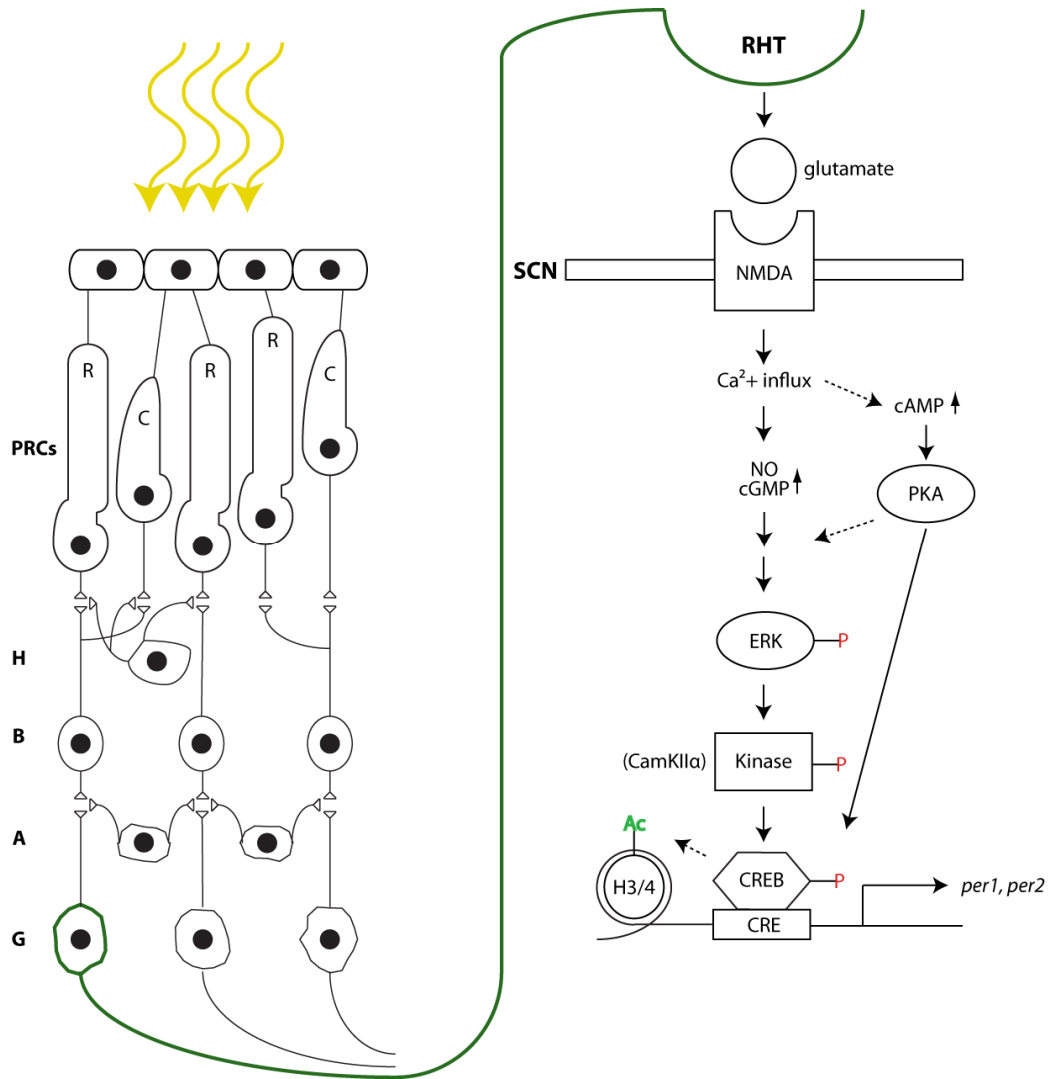


Figure 10 Signaltransduction at the circadian clock input stage of mammals.

Schematic adapted from [1] and [6], modified. Light is perceived by the ipRGC (green) via the r-opsin phototransduction motif. Its efference (green) projects to the SCN where the synaptic terminals release glutamate. NMDA receptor on the SCN neuron binds the transmitter and initiates an intracellular signal cascade that leads to phosphorylation of CREB that translocates into the nucleus and induces transcription of the negative oscillator elements (*per1*, *per2*) by binding to CRE. The left side represents the retinal cellular composition in the mammalian eye. The right side represents the signalling cascade inside a SCN neuron. R = Rods, C = Cones, photoreceptive cell = PRC, H = horizontal cell, B = bipolar cell, A = amacrine cell, G = ganglion cell, PKA = protein kinase A, P (red) = phosphate group, Ac (green) = acetyl group, ERK = Extracellular-signal regulated kinase, H3/H4 = histone 3 and 4. Yellow arrows on top left represent light stimulus.

3.3.2 Oscillator stage in Mammals

The mammalian oscillator resides in a heterogeneous brain structure termed suprachiasmatic nucleus because of its location above the optic chiasm. The molecular machinery producing the oscillation is contained in the neurons comprising the SCN[51]. The neurons are connected intercellular via electrical and chemical pathways which aids in synchrony and precision of oscillation [52]. The inherent oscillation is cell autonomous, which means it is not relying on external signals in order to sustain the oscillation. Each single neuron is an autonomous pacemaker. SCN neurons are able to sustain the rhythm for a longer time compared to pacemaker from different tissues [53]. The SCN rhythm overrides peripheral clocks [34] proving its dominance in a multi-oscillator-system. Another difference between the central pacemaker and slave oscillators is its light entrainability. Thus the morphological substrate for the oscillator is the SCN, the cellular substrates are the single neurons within the SCN and the molecular substrates are the clock genes.

Broken down into the basic elements of a Goodwin oscillator the clock genes can be categorized as follows:

- X1 vulgo positive elements: RORa
- X2 vulgo positive elements: CLOCK(constantly expressed), BMAL1
- X3 vulgo negative elements: REV-ERB α , mPER1, mPER2, mCRY1, mCRY2

Most mammalian clock genes have been identified by their homology to their insect counterparts in *Drosophila melanogaster*. BMAL and PER are highly conserved clock genes. Their orthologues are found within the circadian clock of *Platynereis dumerilii* (Zantke, J., unpublished) which is the reason to choose them for evaluation in my study.

Positive elements:

- BMAL1 dimerizes with CLOCK. It binds to E-boxes (enhancer element - CACGTG) in concert with CLOCK. Its promoter contains RORE (Retinoid-related Orphan Response Element) sequences.
- CLOCK is constantly expressed, dimerizes with BMAL1 and binds to E-boxes as heterodimer.
- RORa binds to RORE and initiates *bmal1* transcription. Its promoter is thought to contain an E-box.

Negative elements:

- mPER dimerizes with CRY. Its promoter contains E-boxes as well as CRE sequences. Therefore BMAL1/CLOCK dimer and phosphorylated CREB-P can induce transcription independently which is the cause for perturbations in the circadian rhythm. The perturbations are again the reason why the system is entrainable.
- mCRY's promoter contains E-boxes and is induced by BMAL1/CLOCK and dimerizes with PER. Photic induction and entrainability is independent of cryptochrome light sensing [54].
- REV-ERB α binds to RORE sequences as a monomer and inhibits transcription by competing with RORa.

The actual oscillation involves posttranslational control (see Figure 11) besides transcriptional control – positive and negative.

Posttranslational control is conducted via phosphorylation, degradation and cellular localisation. Protein kinases CK1 ϵ and CK1 δ are known to phosphorylate PER [55] as well as CRY and BMAL1[56]. Thereby controlling their stability (hyperphosphorylated mPER2 is

thought to be degraded by the proteasome) and cellular localisation (Figure 11). Simplified phosphorylation regulates the timepoint off nuclear entry (CLK/BMAL1)[56] or cytosolic retention (PER1) [57]. Phosphorylation further regulates protein (PER) stability by marking it for ubiquitination and further degradation [58].

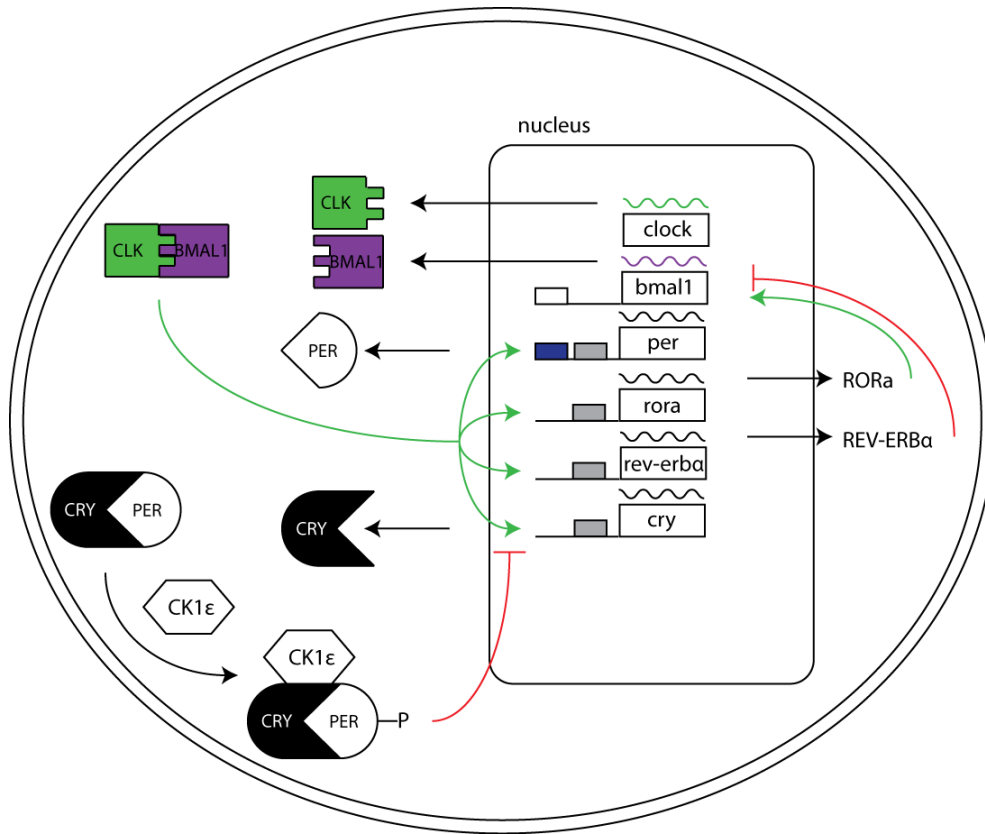


Figure 11 Model of translational control in a mammalian SCN neuron.

Adapted from [1] and modified. Green arrows represent positive feedback loops. Red lines represent negative feedback loops. Grey boxes next to the genes represent E-boxes inside the promoter whereas a blank box represents RORE sequences inside the promoter. Dark blue box represents the CRE site upstream of *per*. Clock is constantly expressed and dimerizing with BMAL1 to bind to E-boxes and initiate transcription of *cry*, *per*, *rev-erba* and *rora*. CRY and PER levels increase, form a complex with CK1ε and gets phosphorylated. In the nucleus they bind to CLOCK/BMAL1 dimers and inhibit further transcription. REV-ERBα and RORα are competing for the RORE sequence in the *bmal1* promoter. RORα initiates transcription from RORE sequences and REV-ERBα represses it.

3.3.3 Output stage

3.3.3.3 Melatonin as a clock output

Melatonin has been discovered as a lipophilic substance from an acetone extract of the bovine pineal in 1917. When fed to tadpoles their skin will lighten up and make them transparent with increasing concentration of the pineal extract. This effect is due to condensation of melanin granules in melanocytes; hence the substance's name melatonin [59]. Melatonin production in vertebrates is rhythmic and reflects the daily change of light and darkness. Melatonin levels peak at night and decline during the day, thereby signalling dark information. Melatonin levels only signal changes in light and darkness because it is independent from behaviour – diurnal, nocturnal or crepuscular species all produce melatonin at night. Melatonin is also found in bacteria, fungi, macroalgae, vesicular plants and in some invertebrates. Also here circadian regulation of melatonin levels is encountered quite often. Nocturnal maxima are found in many insects, *Periplaneta* [60], *Locusta*, *Bombyx mori*, *Gryllus bimaculatus*, in dinoflagellates, *Euglena*, in planarians, *Dugesia dorotocephala* and crustacean, *Procambarus clarkia* and gastropod, *Aplysia californica*. Circadian regulation also occurs in *Drosophila* with the exception that melatonin peaks during daytime [61] like in the crustacean, *Macrobrachium rosenbergii*. In *Bombyx mori* melatonin levels peak at night, are found in the head and decline in constant light [62]. Hence circadian regulation across species can be found but not necessarily with a scotophase peak. In *Enallagma civile* no daily fluctuations in melatonin levels occur, whereas *Ischnura verticalis* exhibits melatonin peaks during scotophase [63]. In fungi e.g. *Saccharomyces cerevisiae* melatonin levels are not related to dark or light environment but to growth and precursor availability [64]. The functions of melatonin in insects is open to question (especially the “darkness hormone” function), also its daily fluctuations are not related to light and darkness or even absent in many insects. This in turn means that melatonin does not always convey darkness information or temporal information but has different functions apart from a clock controlled output. Due to its chemical features and small size melatonin is able to cross a lipid bilayer [65] on its own and can exert effects that are intrinsic to the molecule itself. One of its very attractive chemical features is its ability to directly scavenge free radicals (ROS) which results in the formation of stable end-products. This is melatonin's most basic function, occurs in all species and it is thought to be its only function in lower organisms. Although it has to be pointed out that pharmacological relevant effects are observed at concentrations 10^6 times greater than the actual physiological concentration [66] and there are only few organisms that actually achieve concentrations high enough to render the antioxidant effect physiological relevant [67]. The antioxidant effect is indirectly enhanced by melatonin induction of antioxidative defense elements [68].

In mammals melatonin is excreted into the blood stream and into the cerebrospinal fluid via the third ventricle. It acts as an endocrine hormone on GPCRs, MT_1 and MT_2 expressed on target cells [69]. Melatonin relays circadian temporal information to peripheral clocks e.g. entrainment of the pituitary relies on melatonin sensing [70]. It also feeds back to the SCN and inhibits neuronal firing by hyperpolarising neurons [71]. In seasonal breeders it provides synchronization to the time of the year by measuring the changing photoperiod reflected by duration of melatonin synthesis [72, 73]. Administered melatonin delays reproductive maturation in pubertal rats. In rat pituitary inhibition of gonadotropin-releasing hormone (GnRH) induced release of luteinizing hormone (LH) is observed when melatonin is applied [74]. It also effects the secretion of prolactin in a seasonal fashion [75] and prenatally influences puberty onset in goat [76]. It represses gonadotropin releasing hormone

transcription [77]. In humans it regulates vasodilatation (MT₂) and constriction (MT₁) thereby influencing body temperature [78, 79]. Melatonin levels correlate with sleep propensity and melatonin administration facilitates sleep [79].

Summarized in vertebrates melatonin acts as seasonal and circadian timer influencing maturation, breeding and sleep, wake rhythms. These are considered the “classical” roles of melatonin. Although it has been known that memory formation is subject to diurnal variation, a recent finding uncovered a non-classical role in vertebrates where melatonin acts as suppressor of memory formation and learning [80]. Another non-classical role employs melatonin as an immunologic enhancer aiding the immune response [81, 82].

In non vertebrates the roles of melatonin are even less clear. As mentioned before it might act in a classical way in some species and fulfil different functions in others. Classical roles are thought to be related with nocturnal melatonin peaks or seasonal fluctuations and maturation/breeding. In the honeybee, *Apis mellifera*, melatonin reflects seasonal light fluctuation between summer and winter [83]. In Cockroach, *Periplaneta americana*, melatonin exerts a releasing effect on prothoracicotropic hormone (PTTH) on neurosecretory cells in the brain. PTTH in turn stimulates molting hormone ecdysone [84]. All these findings link non vertebrate melatonin to classical roles although supporting data is equivocal. The appearance of melatonin is thought to be a side effect of *aanat* and *hiomt* evolution. Initially AANAT activity only protected the photopigment retinaldehyde in PRCs from a non-enzymatic reaction. Two molecules pigment can be nucleophil attacked from arylalkylamines (e.g. generated from decarboxylation of amino acids or xenobiotics) and result in toxic bisretinyl-arylalkylamines. This leads to pigment loss and toxicity for the cell. Detoxification by AANAT is thought to be further enhanced by HIOMT and eventually melatonin was generated from serotonin as a side effect [85].

3.3.3.4 Melatonin Synthesis

In vertebrates the cellular substrates for melatonin synthesis are the pinealocytes. In lower vertebrates pinealocytes contain every element necessary to autonomously control temporal activity of AANAT. They have an input stage being a photoreceptor, an oscillator stage being the clock genes, and an output stage being AANAT. The photoreceptor entrains the clock and initiates photic turn off. The clock genes are in control of *aanat* transcription. In amphibians, reptiles, aves and fish the pinealocytes are directly light sensitive – in principle they are photoreceptor cells with a neuroendocrine function governed by light and intrinsic clock. They use PINOPSIN pigment, a CILIARY OPSIN phototransduction motif first identified in chicken [86]. In mammals pinealocytes are highly specialized and lost all light sensitivity and have no oscillating clock to measure time. The photoreception occurs in the retina, the clock resides in the SCN and the clock output in the pineal. Release of norepinephrin from afferent fibers and its binding to receptors on the pinealocytes stimulates melatonin synthesis.

In insects there are no pinealocytes. Melatonin is found in the brain (and retina and haemolymph) of cockroach and *aanat* expression was tracked to cells in the optic lobes in vicinity to pacemaker neurons and to a greater extent to pars intercerebralis, where clock genes are co-expressed with *aanat* [60].

Melatonin is not stored in cells but released directly when produced. Therefore the synthesis machinery directly regulates the levels. A clock controlled output resulting in rhythmic melatonin levels must therefore target the synthesizing enzymes. Most efficiently regulation concerns the first enzyme in the synthesis pathway.

The synthesis (Figure 12) starts from 5-Hydroxytryptamine which is N-acetylated by the highly specific arylalkylamine N-acetyltransferases (AA-NATs). Other N-Acetyltransferases (NATs) with broader substrate specificity can catalyse the same reaction. The resulting

molecule is N-acetylserotonin (NAS) which serves as substrate for hydroxyindole O-methyltransferase (HIOMT), the last enzyme in the synthesis pathway. HIOMT catalyzes the transfer of a methyl group to NAS generating the melatonin molecule. Also at this step other methyltransferases can functionally complement.

The real clock output controlled gene is *aanat* because melatonin is only a result from its activity. In order to conduct temporal control activity of AANAT is regulated in a clock dependent fashion and in a light dependent fashion. The regulation itself can be on a

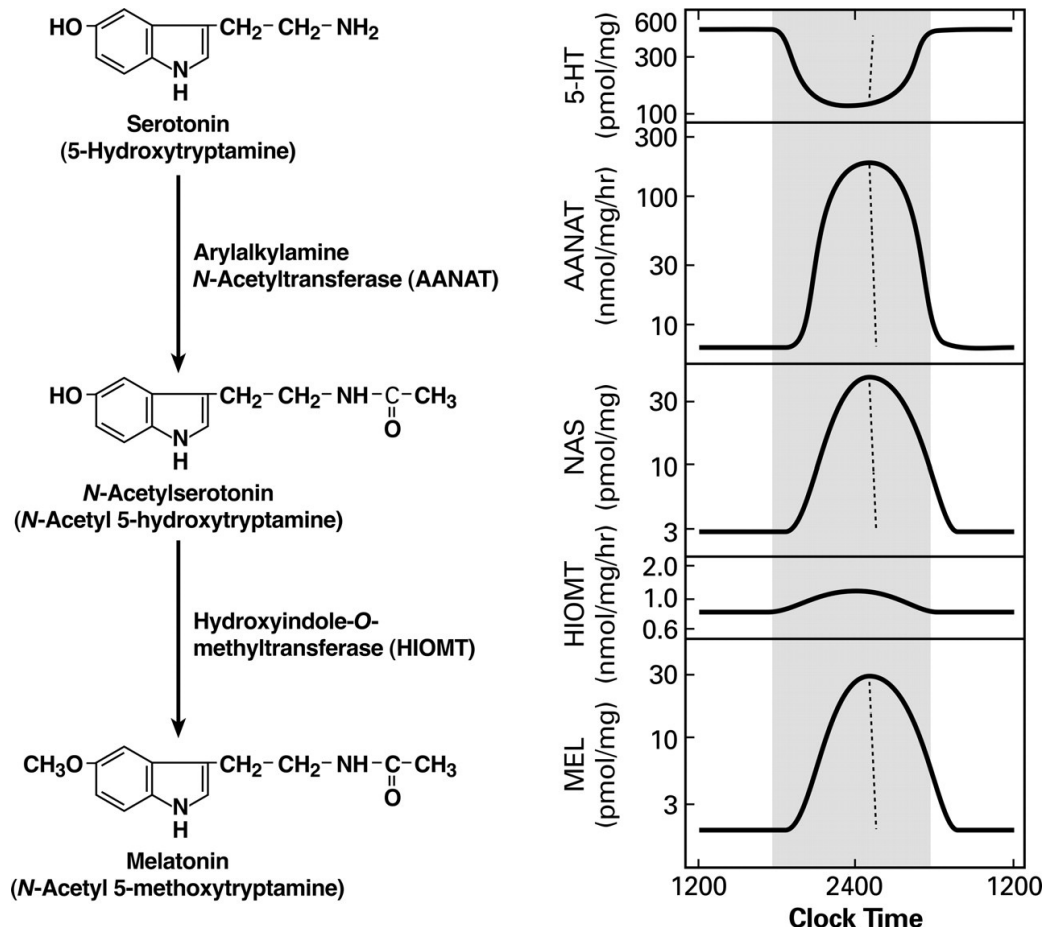


Figure 12 Synthesis of Melatonin from Serotonin

Adopted from [2]. Synthesis pathway for Melatonin involving AANAT and HIOMT in vertebrates. Graphs depict abundance of educts and product and enzyme activity over the day. Serotonin levels are the lowest when melatonin peaks at night.

transcriptional level or a posttranslational level. The light regulation involves a “turn off” mechanism of AANAT. The clock control involves either a transcriptional regulation or a posttranslational regulation.

Control in rodents:

Afferent nerve fibres release norepinephrine (NE), which binds to the α 1- and β 1-adrenergic receptors on the pinealocytes membrane. β 1-adrenergic receptor activates adenylatcyclase which elevates cAMP levels. High cAMP levels cause activation of protein kinase A (PKA) which in turn leads to phosphorylation of constantly expressed CREB at serine 133 (and maybe 142). The phosphorylated CREB (CREBp) binds to CRE sequences in the *aanat* promoter and induces transcription. CREBp also induces transcription of per and inducible cAMP early repressor (ICER) which is involved in termination of *aanat* transcription. PKA dependent phosphorylation on two sites of vertebrate AANAT is required for binding of two 14-3-3 proteins. 14-3-3 binding to AANATp determines stability and increases activity in vertebrates. Dephosphorylated AANAT is targeted for degradation via the proteasome.

α 1-adrenergic receptor activates protein kinase C (PKC) via phospholipase C (PLC) and elevated Ca^{2+} levels. PKC potentiates β 1-adrenergic effects on AANAT activity. Elevated Ca^{2+} levels activate MAPKs and downstream regulatory element antagonist modulator (DREAM). DREAM is transcriptional repressor acting on genes with downstream regulatory elements (DRE), *aanat* and *icer*. Termination of AANAT activity is thought to involve attenuated NE release from sympathetic fibers, dephosphorylation of CREBp, AANATp ICER negative feedback and DREAM repression[87].

In mammalian pinealocytes (except rodents) *aanat* is constantly expressed. AANAT levels and activity are regulated solely by posttranslational events. The principal signal transduction cascade is the same though. AANAT activity relies on phosphorylation and 14-3-3 binding. Unphosphorylated AANAT is quickly degraded by the proteasome and only upon NE stimulation AANATp and subsequently 14-3-3/AANATp complex is formed[88].

The turn off mechanism employs the proteasome pathway. Upon illumination cAMP levels decline quickly. 14-3-3/AANATp complex dissociates, AANAT is formed by the action of a phosphatase which counteracts decreasing PKA activity. AANAT is finally degraded.

In vertebrates with light sensing pinealocytes the clock is contained within the cell and *aanat* expression is governed by an E-box in its promoter.

Summarized there are differences in the light control whether pinealocytes are directly photoreceptor cells or lost light sensitivity. Whether photoreceptive or not AANAT control can be posttranslational (mammals except rodents) or transcriptional (rodents and sub-mammals).

3.3.3.5 The horizontal gene transfer hypothesis

Aanat and *hiomt* are repeatedly regarded as paradigmata for horizontal gene transfer (HGT). Horizontal gene transfer means the introduction of a gene directly from one organism into another unrelated species in contrast to vertical gene transfer. Literature suggests a strong distinction in vertebrate and non vertebrate AANATs [2, 89-91]. Primitive *aanat* homologues are found in bacteria, in fungi and in green algae. Due to this recent state of knowledge that despite its existence in vertebrates the *aanat* gene has not been found in any other eukaryotes than fungi and unicellular algae the notion of HGT from bacteria is plausible [90]. The earliest entry of a homologue *aanat* gene into the chordates is found in the cephalochordate *Amphioxus* ([89]). *Amphioxus aanat* exhibits rather bacterial gene structure in that it is lacking introns, which is aiding the HGT argument. An alternate explanation though would be loss of introns during evolution. The *Amphioxus aanat* also resembles the non-vertebrate homologue in lacking the regulatory domains of vertebrate *aanat*. It lacks a proline containing tripeptide in the catalytic domain seen in vertebrates. It lacks tissue specific expression to the retina and pineal seen in vertebrates. In *Amphioxus* 2 *aanat* orthologues are expressed throughout the body. It lacks the substrate specificity for arylalkylamines found in vertebrates. Thus there is no vertebrate homologue in *Amphioxus* [89] but 7 non-vertebrate homologues exist in its genome. Non-vertebrate *aanats* are thought to be involved in detoxification and biogenic amine inactivation. They are thought to have functions that do not include melatonin synthesis. The evolutionary gap between gram-positive bacteria and eukaryotic algae, fungi and chordates in *aanat* distribution can be explained by HGT events, although the lack of resolution in phylogenetic data might be the underlying cause for this pattern.

3.3.3.6 PDF as a clock output

Pigment dispersing hormone (PDH) was originally identified from epidermal cells of crustacean's eyestalks. It is a hormone or neuropeptide consisting of eighteen amino acids. When excreted from the sinus gland of the optic stalks it promotes dispersion of dark pigment in *Pandalus borealis* [92]. The dispersion is triggered by ambient light conditions. PDF's function is to adjust pigmentation in eyes of crustacean in a daily rhythm.

So far PDF is the best described circadian clock output in insects.

This homologue called PDF is also found to be expressed in a small subset of neurons in *Drosophila* brain. These neurons showed immunoreactivity to PDH antibody and were categorized as lateral ventral neurons (LN_v) due to their anatomical location. These 16 neurons are further divided into the small (sLN_v) and large (lLN_v) ventral lateral neurons. They are in turn part of the 150 neurons encompassing the pacemakers for daily locomotor behaviour. In comparison the immunostaining in crustaceans mark neurons in the brain, in the stomatogastric nervous system and as mentioned in the optic stalk. The PDH immunolabeled neurons in *Drosophila* express clock genes tested by cytological staining for *per* and *tim*. Interestingly the axon terminals of the H-B-eyelets project to the sLN_vs [93]. The sLN_vs are thought to drive daily rhythmic locomotor behaviour [94, 95]: morning activity ("M") but not evening activity ("E"). *Drosophila* is a crepuscular animal showing activity at dawn (driven by the "M" pacemaker) and dusk (driven by the "E" pacemaker). In this model of a dual oscillator the two pacemakers are organized into two cell populations ("M" cells and "E" cells) and coupling of phase information occurs in a master-slave relationship, where M-cells reset E-cells' oscillation daily [96]. As M-cells (sLN_v) express PDF it is argued that PDF is the signal conveying temporal information [97, 98].

The functional role of the lLN_v is still elusive but it is argued that they might affect the gating of photic input [99]. *Pdf* mutants lack an activity peak in the morning and the activity peak in the evening occurs phase advanced [100]. The free-running rhythm under constant conditions (DD) of mutants becomes arrhythmic in PER levels among the four sLN_vs. Similar effects can be observed in flies with genetic ablated LNs [101]. PDF acts on the PDF-receptor which is a GPCR and a null mutant for *pdf-r* displays the same behaviour observed in *pdf* mutants. Therefore LNs comprise a locomotor pacemaker. PDF levels are constant in wildtype but its release from axon terminals of the sLN_vs is orchestrated in a daily rhythmic manner. Mutants for *per* or *tim* abolish the rhythmic release from axonal terminals but again do not affect PDF levels. Null mutants of *clk* and *cyc* on the contrary affect transcription of *pdf* and protein levels in the sLN_vs although an E-box is absent within the *pdf* promoter [102].

Summarized PDF is an output because a) it is exocytosed in a rhythmic manner posttranslationally regulated by the clock genes *per* and *tim* b) its transcription is depending on the clock genes CLOCK and CYCLE. It can be argued that PDF synchronizes oscillation among the four sLN_vs because mutants show phase dispersion in these cells (a functional analogy to VIP in mammalian SCN). After all each cell is an autonomous pacemaker. Besides its presence in insects and crustaceans it has also been found in the cerebral ganglia of the gastropod, *Aplysia californica*, where it has been termed "cerebrin". It is thought to be linked to feeding related locomotor behaviour [103].

Thus PDF is so far characterized in insects, crustaceans and recently found in *C.elegans* [104] and as mentioned a gastropod.

3.4 Lunar Clock and Rhythms

3.4.1 Lunar light controlled synchronised reproduction

The assumption of existence of a lunar rhythm derives from spawning behaviour of various invertebrate marine species [105]. As for these the prevalent reproductive act comprises external fertilisation it has to be ensured that semen and egg will encounter each other. In the vast volume of the sea gametes are rapidly diluted. Therefore mating has to be conducted in local and temporal vicinity. Maturation of gonads and eggs and sperms has to be completed when mating. The crude temporal coordination of the reproduction is correlated with the phase of the moon in diverse marine species.

Palola viridis, the palolo worm is a famous example for a lunar driven reproduction cycle. Its epitoke body part (mainly consisting of gonads) separates from the atoke part of the animal at the last quarter of the moon. The epitoke parts swim up at night and spawn into the sea whereas the atoke parts stay in the reefs [106].

In *Clunio marinus* the timing of ecdysis and the preceding metamorphosis can be synchronized by an artificial moon in the laboratory. Although a population inherits the rhythmicity from the parental generation, it is able to entrain to a new lunar cycle [107]. Shading pigments of larval ocelli in *Clunio* larvae exhibit lunar clock controlled transparency changes to result in a more sensitive photoreceptor in dim light [108].

Acropora millepora, a coral, spawns around full moon. An event that is depending on the ambient light perception by cryptochromes [109].

Many nereid species have a reproductive behaviour that is temporally synchronized with the lunar cycle. *Nereis succinea* has a spawning peak around new moon. *Nereis virens* shows a semilunar swarming maximum. Spawning of *Nereis diversicolor* is regulated by temperature additionally to a lunar cycle synchronisation. The annelid, *Eunice fucata* swarms around the last quarter of the moon.

Also chordates exhibit lunar spawning, an event frequently observed in reef fish [110]. Thus it can be said that many different marine species, from lophotrochozoa, to chordates, adapted and synchronized their reproductive behaviour to a lunar time scale.

3.4.2 *Platynereis dumerilii*, a model to study lunar periodicity

In *Platynereis dumerilii* populations lunar driven spawning rhythms could be confirmed. In absence of other geophysical events like tides the dependence of synchronous spawning on moonlight could be assessed. Dim light during the night mimicking the moon is sufficient to entrain *Platynereis* in culture [111]. Worms will trigger metamorphosis to its mature form according to the artificial moon and spawn nearly immediately after maturation is complete. Spawning peaks commonly occur 9-13 days after full moon [112] (Figure 13). The endogeny of the lunar clock is proven by discontinuation of the artificial moon after 3 months of entrainment and persistence of periodic spawning for another three months [112]. Also decreased moon light intensity (from natural 0,2 to 0,02 lux) was still sufficient to entrain animals although maxima and minima are not as prominent. This indicates a high sensitivity for light input to the lunar clock, because even those low intensities suffice for entrainment. In constant conditions (LL or DD) spawning becomes randomly which again indicates that moon light is required for spawning synchrony, but is not required for the maturation per se. The maturation is thought to be controlled by an endocrine factor produced in the prostomium in a region between the eyes. The region could be tracked down by transplantation assays. As the

endocrine factor decreases metamorphosis starts. Thus lunar clock appears to act on the maturation by means of a still unidentified superimposed endocrine factor. Up to date not a single element of the lunar oscillator is known, despite the fact that lunar periodicity is a widely observed phenomenon among marine species and even might be conserved across species

Thus identification of lunar light as the major zeitgeber for synchronized spawning behaviour in *Platynereis dumerilii* was uncovered [111, 113]. A more elaborate issue was to uncover the organic/cellular substrate that enables platynereis to receive this photic input and coordinate accordingly in time. Transplantation experiments suggested the zeitgeber sensing organs were located in the prostomium. Cerebral adult eyes were reported to not be required for lunar light driven maturation by repeatedly destroying them with an electric epilation needle [114]. Their morphological characterisation was also subject of early investigation [22].

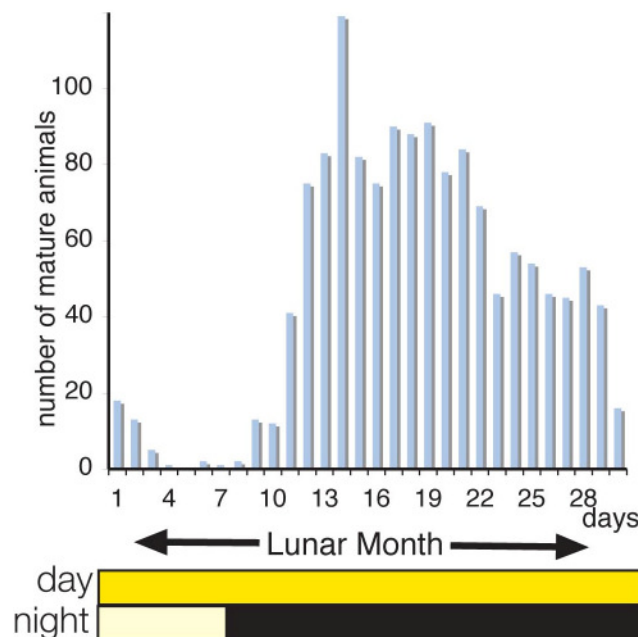


Figure 13 Spawning frequency of *Platynereis dumerilii*. Adopted from (J.Zantke, unpublished). Number of mature worms increases one week after nocturnal illumination and has a peak about 9-14 days after full moon (around new moon). A minimum of matures can be observed during full moon.

The reproduction is fine tuned by the circadian rhythm because spawning occurs at night when animals start to leave their tubes. The process is again fine tuned by release of pheromones which triggers change in locomotion and gametes release. Thus it appears release of gametes is temporally fine scaled by pheromones, locomotor behaviour (leaving of tubes and swimming at night) by the circadian clock and more crude the maturation by the endogenous lunar clock. Thus interplay between both oscillators seems likely in order to produce synchronous spawning. The evidence for photoperiodicity of the *Platynereis*' spawning is reproduced in our laboratory by the simple fact that keeping the animals in two different rooms with two different moon phases results in two different spawning frequencies.

3.5 Laserablation

Laser assisted photodestruction has already been put to use to selectively destroy cells or intracellular structures since thirty years[115]. Therefore it is an established method for some of the common model organisms like *C. elegans*[116] and *Drosophila melanogaster*[117-119]. This technique also helped to gain insights in atypical organisms[120]. So far it has been confirmed that the method causes apoptosis in irradiated cells and thereby killing them [121]. Or if higher laser powers are applied the targeted cells are volatilized at the cost of dealing damage to surrounding tissue. More importantly this approach was already successfully adopted for *Platynereis dumerilii* larvae [19]. In this study it was demonstrated that larvae exhibit phototactic behaviour and insights into mechanism were gained by selective destruction of the larval eye. The larval eyes of the trochophora being the input stage were destroyed by a laser.

I used a very similar approach to address a different question. Significant differences compared to their experimental setup and needs are in my case obviously the focus on the post-trochophoral cerebral eyes instead (Figure 5B, anterior and posterior adult eyes), the employment of worms much older than 52 hpf and finally the requirement for stably and permanently destroyed eyes. Thus the experimental setup had to be tested and aligned for my application case, especially considering the requested long term effects. The actual intention was to obtain permanently blind animals by means of UV-laser destruction.

3.6 Aim of study

Lunar and circadian rhythmicity have to be phased to external photic cues in order to exhibit synchronous rhythmicity. By means of ambient and/or directional photosensation both clocks might be entrained to oscillate in phase to the zeitgeber. The period for both lunar and circadian oscillations is of course different, a reflection of the zeitgeber's period. It is argued that rhabdomeric PRCs comprise the visual system, enabling the animal to see directional. Whereas other light sensory celltypes implement the function of ambient light perception. Thus the question arises which system, the visual system represented by the rhabdomeric PRCs or the ambient light receptive system represented by the ciliary PRCs, transduces photic stimuli to each of the two clocks. Hence the next question follows whether one clock is independent of the other on the input stage, or do they both rely on the same sensory cells. Summed up two different zeitgeber result in two different oscillation periods and might be perceived by two different sensory systems. To test which of the sister photoreceptor cells[122] are responsible for carrying out the analogue function of light perception in clock entrainment, a straight forward assay is installed. By UV-Laser mediated destruction of cerebral adult eyes their input to circadian and lunar clock is disrupted. To test the effect of this disruption entrainment to a new light regime is assessed. The assessment is carried out by a molecular readout of clock genes' expression over time.

To test if the endogenous clock can be separated from environmental photic cues by means of destruction of rhabdomeric PRCs an enforced 12 hours shift in the 24 hour LD rhythm (new moon) is superimposed. If the intrinsic circadian clock continues to express the clock genes aligned to the former rhythm, it would be a "free-running" rhythm. This means that it failed to adapt to the 12 hour phase shifted LD entrainment, because the external photic zeitgeber is ignored. That in turn would mean that the circadian clock failed to adapt, because new external information could not be integrated. It could not be integrated because stimulus transduction was non-functional, as intended. Thus the induced blindness would lead to loss

of entrainability to extrinsic light stimuli if adult eyes are required for entrainment and no other photoreceptor can complement. This inability can commonly be observed in blind humans [123]. On the other hand if the clock could attune to the newly applied entrainment in absence of rhabdomeric PRCs, it would mean that they are not required for the circadian clock circuitry. To prove the actual absence of rhabdomeric PRCs in the treated animals the expression of *Pdu r-opsin* had to be measured.

4 Materials

4.1 Technical equipment

APPLIED BIOSYSTEMS Step-One-Plus cycler
LEICA LMD 6500
ZEISS Stemi 2000
ZEISS Axioplan 2
ZEISS LSM-510 Meta
HANNA INSTRUMENTS pH210 Microprocessor pH meter
HERAEUS PICO 17 centrifuge
BIORAD Gene Pulser
BIORAD Electroporation cuvettes, 2mm
BIORAD Vacuum Blotter 785
INVITROGEN Safe Imager blue-light transilluminator
STRATAGENE UV Stratalinker 1800

4.2 Software

Axiovision 40 V4.8.1.0, ZEISS
LMD Software V 6.7, LEICA
CLC Main Workbench 5.6, CLC Bio
Oligo 6.8, Molecular Biology Insights
Photoshop CS3, Adobe
ClustalX 2.0.12
StepOne Software 2.1
LSM Software 4.2, ZEISS
ImageJ 1.43
MAFFT 6.717
PhyML 3.0
Excel 2007, Microsoft
MyHits (<http://myhits.isb-sib.ch/cgi-bin/index>)

4.3 Kits

AMBION T7 MAXIscript Kit
CLONETECH SMART RACE cDNA Amplification Kit
INVITROGEN RadPrime DNA Labeling System
PROMEGA pGEM-T Easy Vector System
QIAGEN QIAquick PCR Purification Kit
QIAGEN Plasmid Maxi Kit
QIAGEN QIAfilter Plasmid Maxi Kit
QIAGEN QuantiTect Reverse Transcription Kit
ROCHE Transcriptor High Fidelity cDNA Synthesis Sample Kit

4.4 Markers

Riboruler RNA Ladder, High Range (FERMENTAS)
2-log DNA ladder (NEB)

4.5 Vectors

pGEM-T easy (PROMEGA)

4.6 Chemicals

10x PBS	70g NaCl, 62,4g Na ₂ HPO ₄ ·2H ₂ O, 3,4g KH ₂ PO ₄ , pH7.4
20xSSC	3M NaCl (175,32g/l) , 0,3M Trisodium citrate (88,23g/l)
50xTAE	242g Tris base, 57,1ml acetic acid, 100ml 0.5M EDTA, pH 8.5
KSW	artificial sea water
NSW	natural sea water imported from the north sea
staining buffer	100mM TrisCl pH 9.5, 100mM NaCl, 50mM MgCl ₂ , 0,1%Tween20
denaturing solution	0,5M NaOH, 1,5M NaCl
neutralisation solution	1,5M NaCl, 1M Tris-HCl
10xMOPS	200mM MOPS, 80mM NaAcetate, 10mM EDTA, pH 7.0
1xTNT	0,15M NaCl, 0,1M Tris pH7.5, 0,1% Tween20
1xTNB	TNT, 1% DIG blocking reagent (ROCHE)
mQH ₂ O	millipore milli q water

4.7 Media

SOC	10mM NaCl, 20g Trypton, 5g Yeast extract, 2,5mM KCl 10mM MgCl ₂ , 10mM MgSO ₄ , 20mM Glucose
LB	20g NaCL, 20g Trypton, 10g Yeast extract fill up to 2L with ddH ₂ O, autoclave
LB+AMP	LB + ampicillin (50mg/ml)
LB agar	7,5g agar powder to 500ml LB, autoclave

4.8 Animal culture and strains

4.8.1 Bacteria

One shot Top 10 Cells (INVITROGEN)

One shot Top 10 electrocomp Cells (INVITROGEN)

JM109, *endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (r_k^- , m_k^+), *relA1*, *supE44*, $\Delta(lac-proAB)$, [F' *traD36*, *proAB*, *laqI*^q Δ M15]

4.8.2 Worm culture

Animals were kept in translucent plastic boxes in a 1:1 mixture of imported natural sea water (source was the North Sea) and artificial salt water. A constant temperature of 18°C and a regulated light-dark cycle of 16 hours of artificial light and 8 hours of complete darkness provided consistent conditions. Full Moon was mimicked[113] by interrupting the constant light-dark cycle. Therefore dim light of known intensity (1-10 lux) was turned on instead of the 8 hour darkness phase for the period of 7 days. Full Moon was followed by 21 days of regular light-dark rhythm. For practical reasons there were two rooms with 14 days shifted moon-phase. To realize special light conditions optical sealed boxes were installed with adjustable light source on the inside. In my experiments the focus was on the inbred lines termed B321XXX or REXXX, both with documented lineage. If the experiment did not demand defined lineage (e.g. WMISH) wildtype strains were used whose inbred status was not clear.

Worms were fed two times a week with a diet of spinach leaves and algae (platymonas).



Figure 14 shelving system for worm culture

4.8.3 Primers

Primers were ordered from SIGMA ALDRICH.

primers	sequence	gene
RACE		
aan 5'Race In	AGTAAAGCAATCCGTTTCACTCCGC	aanat
aan 5'Race Out	CTTTCTTGACCATGAACAATCGATG	aanat
aan 3'Race In	ACAAGGGCCGCTTCGGAGAAAT	aanat
aan 3'Race Out	GGGTACTTTGAAAGCGCTGGAATG	aanat
hiomt 5'race In	AGCATAAATGATTGGGCTGATGGGACTC	hiomt
hiomt 5'race Out	CCACCTCATTAACCCATCCTTTTAAGTACC	hiomt
hiomt 3'race In	TCACGCAAGTCCAAATTCATCGTACGC	hiomt
hiomt 3'race Out	GGGAAGGAATATCGCCACCTGCTGG	hiomt
RACE_UPM_long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
RACE_UPM_short	CTAATACGACTCACTATAGGGC	RACE
RACE_NUP	AAGCAGTGGTATCAACGCAGAGT	RACE
degenerated PCR		
annelidCopsinU1	GCNGCITAYYTITTYTTYAT	c-opsin
annelidCopsinL1	AAYTGYTTRTTCATRAAIACRTADAT	c-opsin
annelidCopsinL2	NGCRTAIGGIGTCCAIGC	c-opsin
annelidCopsinL3	CCARTCDATISWRCACCA	c-opsin
annelidCopsinU3	GSNCCIYTIBTIGGITGG	c-opsin
annelidCopsinU2	TAYYTNRCIGTIGYIMGICCITT	c-opsin
annelidCopsinU4	TGGTGYWSIATHGAYTGG	c-opsin

PCR		
aan up 1	TTCTGGAACAGTTGAACTTTAAAAGC	aanat
aan up 2	AAGCTGTGAGAGTGTTTCAGGC	aanat
aan low 1	AGACATACAAAATAATAATCTATTGTGC	aanat
aan low 2	GTAAATGCATTTACAAATCGTATTGC	aanat
hiomt up1 new	GTTTGGCAGCATGCTGAACATGGC	hiomt
hiomt up2 new	GTGGCCTTCCCCGCACATATCACC	hiomt
hiomt low1 new	TTGATTATGTCTTGAAATGTCACAGC	hiomt
Pdu hiomt lo3	TTCTCGTGTCTATCGACCACAAATG	hiomt
Pdu hiomstup2	CACCTTCATCATGACATTATGTGC	hiomt
Pdu hiomstup1	GAGAAATGTCTGAATGATCACAAGG	hiomt
Pdu hiomtlikelo1	CATCAGTRCCAACRTGAAATGAGTGG	hiomtlike
Pdu hiomtlikelo2	CCTTTKGGAGTAACCAATACCACGCC	hiomtlike
Pdu hiomtlikeup3	GGCTCTAAAATTAATCTCCGTTTATC	hiomtlike
Pdu pdfup1	GACCCACAACAAGTTGAGAAAGTCAGC	pdf
Pdu pdfup2	CAGTGTTTCATCTGTACAGTG TTC	pdf
Pdu pdflo1	TTTTTCTATTCAAAGGCTTG GTTATTGC	pdf
Pdu pdflo2	GCGGATGGGTCTGTTCCAGGTCG	pdf
Pdu aanatup1	CAGAGGGAAGCACCACAGTTGTG	aanat
Pdu aanatup2	GGGTACTTTGAAAGCGCTGGAATG	aanat
Pdu aanatlo1	GGTGCTACAAATATCCTTCTCTGTCTC	aanat
Pdu aanatlo2	CCACAAACTGGAACCCGGCTTTTG	aanat
qPCR		

pduc5_L1	CCTATTGACATGGACGAAGATG	cdc5
pduc5_R1	TTCCCTGTGTGTTTCGCAAG	cdc6
Pdu_period_L2	GGTCAACATGAAGTCGTACAGG	period
Pdu_period_R2	CACTGGTTTTTCGGCTCAAG	period
Pdu_bmalL3	TCCGATTTATCTCCACGAGAA	bmal
Pdu-bmalR3	TCCGTCTTTACAGGCAGCA	bmal
Pdu_rops1_L1	ATGTGATCACCAACCCCTA	r-opsin
Pdu_rops1_R1	TGATGAAGGCCCTCTTCTTG	r-opsin
Pdu_rops1_L2	GCCGAGATCGACAAACACTT	r-opsin
Pdu_rops1_R2	GGCTTTTGGTTTGGGTTTG	r-opsin

5 Methods

5.1 Microscopy

5.1.1 Confocal laser microscopy and z-stacks

A feature of NBT/BCIP precipitate was made use of [124] to reflect certain wavelengths (630-640nm) of a confocal laser scanning microscope. This technique is termed “confocal reflection microscopy”. The specimens were excited with a 633nm laser and reflection around the excitation wavelength was detected with ZEISS LSM-510 Meta. Pictures were obtained with a 40x oil immersion objective. Microscope adjustments and laser settings as well as picture administration were carried out with ZEISS LSM software. Picture processing such as 3D reconstruction was carried out with ImageJ 1.43. Specimens were embedded in 87%glycerol or ProLong Gold Antifade Reagent (INVITROGEN), which has the advantage of solidifying over 24 -48 hours thereby inhibiting involuntary movement of the specimens and preserving the fluorescent probe.

Slides were prepared by creating a central chamber flanked by layers of tape of the same height. Animals were embedded between those tape layers to prevent the specimen from being crushed between slide and coverslip on top.

5.1.2 DIC microscopy

For DIC microscopy Köhler illumination was set up using a ZEISS Axioplan 2. A 40x Oil immersion objective was used. Pictures were obtained with Axiovision 40 V4.8.1.0 (ZEISS). Animals were mounted as described above.

5.2 *UV-laser ablation*

5.2.1 Mounting

Depending on their size and age multiple or single worms were mounted on a glass slide. Layers of tape flanking the slide's middle area formed a chamber where the worm was either incubated in a 1:1 dilution of 0,35 M MgCl_2 and KSW or in a 1:4 dilution of 0.7M MgCl_2 and KSW. A coverslip sealed the chamber on top.

The paralyzing solution (KSW, 0,175M MgCl_2) effectively inhibited movements of the worms. Immediately after the procedure the paralyzing solution was washed off as it inhibits neuromuscular transmission [125] and kills the animal at higher concentrations and long exposure. Thus the photoablation procedure was performed as quickly as possible to decrease exposure time (usually between 5-15min) depending on the number of worms mounted.

5.2.2 Ablation

Microdissection device was LEICA LMD 6500 and control software "LMD SOFTWARE V6.7".

The procedure was carried out intravitally with very conservative settings opting for destruction of PRCs without doing damage to surrounding tissue. The success was monitored over the following days by monitoring the presence or reappearance of PRCs' pigment. Settings were determined experimentally. Used offset value was 150 for all conditions. Offset value influenced the vertical position of the laserbeam focus in relation to optics' focus and once aligned therefore never was changed. Speed of the servo motors was adjusted to 22 for all conditions. The settings were as follows:

For 2-4 weeks old animals the used objective was 40x dry. Aperture of laser was set to 1 to prevent scattering of the beam and hitting off target. Laser power was set to 0 to prevent damage to off target tissue especially in vertical plane. Up to five worms were mounted simultaneously.

For 4-8 weeks old specimen the 40x dry objective was used, the laser power was adjusted to 8 and aperture to 1. Up to five worms were mounted simultaneously.

For 3 month old worms laser power was set to 20-23. The 20x dry objective came to use. Aperture of the laser was increased to 10-12. Only one worm was mounted at a time.

Worms were dismounted and put into KSW without being fed for 3 days to circumvent infectious contamination of the water. After those three days they were regularly fed with algae. Ablated worms were kept apart from other worms in plastic boxes.

5.3 *Circadian clock phase shifting*

The test group treatment comprised two ablation trials at the intervals of seven days as usual. The treatment of the negative control group comprised randomly irradiating the prostomium with the same laser energy and exposure duration as the actual test group. The positive control group consisted of untreated worms of same age as the other two groups. Upon completion of treatment worms were phase shifted exactly at six different time points (9am, 12pm, 4pm, 9pm, 12am, and 4am). 3 groups of 6 individual worms were shifted per time point. The total size of 18 worms for each time point was determined by the statistical need to have at least three biological samples (Table 1). The exposure to the inverted light regime was arranged to 72 hours. Meaning three complete shifted LD cycles were imposed on the test subjects, a

duration which proved to work to shift other species [126]. After this period the worms were decapitated at the designated time point. RNA from heads was extracted and abundance of transcripts of interest was measured via qPCR.

5.3.1 Experimental Setup

The illumination scheme over the 24 hours was LD 16:8 hours in both entrainments.

Considering my results the requirements for the experiment were as follows:

- pre-mature worms have to be around 3 months old to reach a sufficient size for decapitation
- worms have to undergo two ablation trials to ensure *Pdu r-opsin* absence
- food deprivation for the duration of the light entrainment to prevent non-photic entrainment and infection.
- subject treated animals to 180° inverted LD phase as the test group
- subject untreated animals to 180° inverted LD phase as a positive control
- Measure *Pdu r-opsin* decrement in the final test group and its references

entrained 3 months	+/-12hours →	phase inverted 72 hours
timepoint 9am		timepoint 9pm
sample I 6 worms		sample I 6 worms
sample II 6 worms		sample II 6 worms
sample III 6 worms		sample III 6 worms
timepoint 12pm	→	timepoint 12am
sample I 6 worms		sample I 6 worms
sample II 6 worms		sample II 6 worms
sample III 6 worms		sample III 6 worms
timepoint 4pm	→	timepoint 4am
sample I 6 worms		sample I 6 worms
sample II 6 worms		sample II 6 worms
sample III 6 worms		sample III 6 worms
timepoint 9pm	→	timepoint 9am
sample I 6 worms		sample I 6 worms
sample II 6 worms		sample II 6 worms
sample III 6 worms		sample III 6 worms
timepoint 12am	→	timepoint 12pm
sample I 6 worms		sample I 6 worms
sample II 6 worms		sample II 6 worms
sample III 6 worms		sample III 6 worms
timepoint 4am	→	timepoint 4pm
sample I 6 worms		sample I 6 worms
sample II 6 worms		sample II 6 worms
sample III 6 worms		sample III 6 worms

Table 1 Schematics of shifting procedure.

5.4 Whole Mount In Situ Hybridisation

This technique[127] was used to monitor gene expression in situ i.e. to locate the site of the related mRNA on the cellular level of whole animals. The fundamental principle of the procedure is to visualize the mRNA indirectly by detection of tagged hybridized antisense RNA molecules with an enzyme linked antibody. Animals' stage typically varied between 48

hpf and 14dpf when fixed. Slight modifications of the protocol's instructions were put in place (Benjamin Backfisch, Katharina Schipany, personal communication) to obtain better staining. Proteinase K digestion times have been minimally shortened as well as the usage of a further diluted proteinase K solution, 50µl (20µg/µl) of PK in 40ml 1xPTW (1xPBS, 0,1% Tween20).

48 – 72hpf embryos were digested 45 seconds, 3-7dpf embryos were digested <2min, 8-14dpf embryos were digested for 2min 45 seconds.

5.4.1 Fixation

Animals of desired stage were gathered by pouring them onto a small meshed net at the end of a 50ml falcon tube. Retained animals were transferred onto a smaller sized net at the end of a modified 1,5ml eppendorf tube by rinsing with KSW. Finally animals were flushed into a 2ml tube by rinsing them with the fixative (4%PFA, 2xPTW). Animals were incubated in the cross linking reagent for 1 hour shaking at room temperature or up to two days at 4°C. As PFA preserves the specimen via crosslinking of proteins the fixation still requires a permeabilization step. When fixation time elapsed animals were permeabilized by exchanging the PFA/PTW solution with 100% MeOH to provide sufficient probe penetration. Worms were washed three times with 100% MeOH for 5 minutes at room temperature whilst shaking. Pure methanol was exchanged once more before specimens were stored at -20°C until put to use.

5.4.2 WMISH DAY 1

Following steps were carried out at ambient temperature and on a shaker if not explicitly revoked

Specimen were transferred onto nets submerged in 100% MeOH

Rehydrated in 75%MeOH/1xPTW

Rehydrated in 50%MeOH/1xPTW

Rehydrated in 25%MeOH/1xPTW

Washed in 1xPTW for 5 minutes

Washed in 1xPTW for 5 minutes

Digestion with PK without shaking

Washed shortly in 1xPTW/glycine (2mg/ml)

Washed shortly in 1xPTW/glycine (2mg/ml)

Fixation in 4%PFA/1xPTW for 20 min

Washed in 1xPTW for 5 x 5 minutes

Following steps were performed in a water bath heated to 65°C

Specimen were transferred to 2ml eppendorf tubes

Prehybridized in 1ml of Hyb-mix for 1-2 hours at 65°C

Denatured 4-10µl probe in 200µl Hyb-mix at 80°C for 10minutes

Removal of prehybridisation solution and addition of probe to specimen

Hybridisation o/n

5.4.3 WMISH DAY 2

Following steps were performed in a water bath heated to 65°C

Washed embryos 2 x 20min in 1ml of 50%formamide/2xSSCT (2xSSC, 0,1% Tween 20)

Washed embryos 15min in 1ml 2xSSCT
Washed embryos 2 x 20 min in 1ml 0,2xSSCT

Following steps were performed at ambient temperature on a shaker

Blocked embryos in 1ml block (5% sheep serum in 1xPTW)
Incubated embryos in 200µl antibody (Anti-DIG-AP, F_{ab} fragments 1:2000 in 2,5% sheepserum/1xPTW) for 1-2 hours or o/n at 4°C
Washed 4 x 10min in 1ml 1xPTW
Washed o/n in 1ml 1xPTW

5.4.4 WMISH DAY 3

Washed embryos 2 x 5min in 1,5ml staining buffer to equilibrate
Transferred embryos to 6 well plate
Added 1ml of SB+NBT+BCIP (NBT 4,5µl, BCIP 3,5µl per ml SB)

Embryos were stained protected from light and depending on gene and temperature of up to two days. Reaction was stopped by washing in 3 x 5min with 3ml of 1xPTW. Specimen were occasionally postfixed in 4%PFA/1xPTW o/n and afterwards washed 3 x 5min in PTW. Solution was aspirated and exchanged with 87% glycerol for further storage (4°C).

5.4.5 Double fluorescent WMISH

This technique resembles in principle the standard WHISH protocol. Differences are the fluorescein tagged probes instead of DIG labelling, thus the primary antibody is against fluorescein. The ab is conjugated with a horse-radish peroxidase(Anti-fluorescein-POD, Fab fragments, ROCHE) that in turn catalyzes deposition of a substrate. In detail the enzyme produces reactive tyramide radicals that bind fluorescein labelled tyramid and thus confine the fluorophor to the site of reaction. To circumvent the problem of unspecific reflection with the confocal reflection microscopy technique described above this approach was carried out. Primary antibody was diluted 1:50 in 1xTNB. The minor changes compared to the standard WMISH protocol start from WMISH DAY 2:

5.4.5.7 WMISH DAY 2

Following steps were performed in a water bath heated to 65°C

Washed embryos 2 x 20min in 1ml of 50%formamide/2xSSCT (2xSSC, 0,1% Tween 20)
Washed embryos 15min in 1ml 2xSSCT
Washed embryos 2 x 20 min in 1ml 0,2xSSCT

Following steps were performed at ambient temperature on a shaker

Washed embryos with TNT for 2 x 5 minutes
Block embryos in 1x TNB for 1-2hours
Incubated embryos with 300µl of primary antibody dilution
Washed 6 x 5min in 1xTNT
Equilibrated in 100µl of 1x Fluorescein Amplification Reagent (PerkinElmer)

Diluted Fluorescein Fluorophore Tyramide 1:25 in 1x Fluorescein Amplification Reagent (= staining solution)

Added 25µl staining solution

Following steps were performed protected from light. Stained in darkness up to 2-4 hours. When staining was complete, proceeded:

Washed 3 x 5min in 1xTNT

Incubated in 1% H₂O₂ for 20 minutes

Washed 4 x 5min in 1xTNT

Embryos were transferred to a 6 well plate and solution was exchanged with glycerol. Stored protected from light at 4°C.

5.4.6 Probe generation

The prospective RNA probe molecule has to be antisense in order to hybridize with the coding mRNA counterpart. Thus linearization strategy of pGem-T easy containing needed gene fragments inside its MCS was chosen accordingly. 2µg plasmid was digested in compliance with restriction strategy. Linearized plasmid was separated on a gel and eluted. 4µl of eluted linear vector was then loaded onto another DNA gel to determine the amount in comparison to loaded marker (2-log DNA ladder) of known concentrations. A maximum volume of 12,5µl for the template should suffice to contain 1µg of DNA. Depending on concentration of eluted DNA the rest of the 12,5µl is filled up with ddH₂O. 0,5µl RNasin (RNase inhibitor, PROMEGA), 2µl DIG-UTP Mix 10mM(ROCHE) or 2µl Fluorescein-UTP 10mM (ROCHE), 2µl transcription buffer (NEB), 2µl of DTT (ROCHE) and 1µl of polymerase (T7 or SP6, NEB) were added to start the in vitro transcription. The reaction was incubated at 37°C for 4 hours. The DNA template was digested with DNaseI(NEB) for 15min at 37°C. The reaction was stopped and the transcribed RNA probe purified by using RNeasy Mini Kit (instructions for RNA Clean Up in manufacturer's protocol). RNA was taken up in 50µl Rnase free water and an aliquot was subjected to a test gel after mixing it with 4µl RNA loading solution (AMBION) and denaturing the test sample for 10min at 80°C. If a band of estimated size was detected 75µl of Hyb-buffer was added to the probe and the probe was stored at -20°C.

5.4.7 Antibody staining

As the name indicates in contrast to WMSIH this technique uses primary antibodies to detect an epitope of interest. In my case the epitope of interest was the acetylated tubulin and the used primary antibody was the anti-acetylated tubulin antibody (mouse). As indicated by the name the anti-acetylated tubulin ab labelled microtubules of axons, thereby providing an anatomical landmark under the microscope. For detection of the primary antibody a second antibody coupled with a fluorophore was used (anti-mouse ab, alexa 488 or Cy5). The anti-acetylated tubulin antibody was diluted 1:200 in 1xPTW, 1xTNB or in 2,5%Block. Secondary antibodies were used in a 1.250 dilution in 1xPTW. 300µl of antibody dilution was used per sample. In principle there were some options to choose an entry point for this technique to combine with the standard WMISH protocol or the double fluorescent WMISH protocol.

Prior to the last post-fixation in the standard WMISH protocol and before stored in glycerol in the double fluorescent WMISH protocol embryos were transferred into an eppendorf tube. 300µl of primary antibody dilution was added and incubated on a shaker for 2hours at room

temperature. 5 x 5 minutes washing steps with 1xPTW or 1xTNT followed. 300µl of secondary antibody dilution was added and incubated on a shaker for two hours at ambient temperature. 5 x 5 minutes washing steps with 1xPTW or 1x TNT followed. Animals were transferred to a 6 well plate. Solution was aspirated and exchanged with 87% glycerol for further storage(4°C).

When detecting the DIG tagged hybridized antisense probe the additional ab was also added to the 2,5%Block solution. The specimens were incubated for 1-2hours with the antibodies at room temperature whilst rocking. Embryos are washed 5 x 5min in 1xPTW on a shaker. The secondary antibody solution was added and specimen incubated another 2 hours at room temperature whilst rocking. The 5 x 5min washing procedure in 1x PTW was repeated to get rid of unbound excess ab. Animals were relocated to a 6 well plate. Solution was aspirated and exchanged with 87% glycerol for further storage (4°C).

5.5 Cloning

5.5.1 cDNA synthesis

As source to clone genes and extend existing fragments served cDNA, generated by a different method for each purpose.

First strand cDNA to clone genes was generated by using the ROCHE Transcriptor High Fidelity cDNA Synthesis Kit. 200ng or 1µg of RNA extracted from either adult heads (pooled new moon and full moon samples) or from larvae (pooled stages: 48hpf, 96hpf, 7dpf) was used as template for retrotranscription. The primer of choice was the provided anchored-oligo(dT)₁₈ primer. The procedure was carried out according to manufacturer's protocol, with the modification of lengthening the incubation time to 1 hour at 50°C.

RACE ready cDNA was synthesized using CLONETECH SMART RACE cDNA Amplification Kit. 200ng of RNA extracted from adult heads served as template for retrotranscription. As RT enzyme SuperScript III Reverse Transcriptase (INVITROGEN) came to use. All steps were performed according to manufacturer's protocol, except the incubation time was doubled to 3 hours at 42°C.

5.5.2 Agarose gel and gel elution

Amplicons from PCR and DNA in general were separated on a 1%, 1.5% or 2% TAE-agarose gel depending on the estimated size of the product. The gel was stained by adding a 1:10000 dilution of sybr safe DNA gel stain to the agarose gel if DNA was to be isolated from the gel. Otherwise the gel was stained by incubation in an EtBr(1:10000)/1xTAE bath. Fragments are cut out on a Safe Imager transilluminator. DNA was eluted by first separating solid agarose from dissolved nucleic acids. This was achieved by filtering the liquid through tightly packed silanized glass wool. Therefore the bottom of a 0,5ml eppendorf tube was perforated with a syringe, glass wool was stuffed inside tightly to cover the hole and the cut out agarose piece was placed inside the tube. The liquid was filtered through the glass wool and the hole into a 1,5ml tube by centrifugation (5000rpm, 5min). The obtained DNA solution was then further purified by QIAquick PCR Purification Kit according to the manufacturer's protocol.

5.5.3 Ligation

Taq generated amplicons from PCR are ligated into pGem-T easy. 1,5µ of purified amplicon is mixed with 0,5µl of pGem-T easy vector, 2,5µl of rapid ligation buffer and 0,5µl of T4 DNA ligase. The reaction was either performed for 1 hour at ambient temperature or o/n at 4°C.

5.5.4 Transformation

5.5.4.8 Chemical

For each ligation a 50µl aliquot of competent bacteria was used. Thawed shortly on ice and mixed with 2µl of ligation reaction. Further incubated for 20min on ice and heat shocked at 42°C for 45 seconds and immediately placed on ice for another 2 minutes. 350µl of SOC or LB was added to each transformation and was then put on a thermoshaker at 37°C and 300-400rpm for 45-60min. Therefor allowing the bacteria to transcribe the episomal resistance gene and start replication. Because of unknown transformation efficiency one time 120µl and the other time 60µl of the bacterial cultures are streaked out on selective agar.

5.5.4.9 Electroporation

For each ligation 25µl of competent bacteria was used and mixed with 2µl of ligation mix on ice. The electroporation cuvettes had 2mm spacing. The settings on the electroporation device were 200Ω, 2,4 kilovolts and 25 Farad capacitance. After the electric shock 250µl of SOC were added to the bacteria, which were then incubated at 37°C for at least 45min. Again two different amounts(120µl and 60µl) of each transformation were plated on selective agar.

E.coli were grown 8-16hours at 37°C.

5.6 Identification of clones

5.6.1 Colony PCR

To identify recombinant clones a colony PCR was carried out. A suitable amount of colonies were picked and added to the PCR mix.

PCR conditions:

Single reaction mix	
buffer 10x	2µl
MgCL ₂ (25mM)	2µl
dNTPs(5mM)	0,5µl
forward primer (5µM)	1µl
reverse primer (5µM)	1µl
FirePol DNA polymerase (SBD)	0,5µl
ddH ₂ O	11µl
total volume	18µl

PCR program:

temperature	time
-------------	------

automatic hotstart	94°C	2 min	} 28 cycles
Denaturation	94°C	30 sec	
Anneal	60°C	30 sec	
elongation	72°C	4min 30 sec	
final elongation	72°C	10 min	
Hold	10°C	∞	

5.6.2 Miniprep

Once the transgenic bacteria were identified via colony PCR, 5ml of selective LB are inoculated with each of the candidate colonies. The bacteria were grown at 37°C for 8 to 12 hours. 2ml of the cell suspension are transferred to an eppendorf tube and were harvested by centrifugation at 5000 rpm for 8 minutes. Supernatant was aspirated and cells were lysed by addition of 200µl of P1 buffer and 200µl of P2 solution. The mixture was incubated at room temperature. The alkaline lysis reaction was stopped after 5 minutes by neutralizing with 200µl of P3 solution. The whole suspension was centrifuged at 14000rpm for 10min. Supernatant was transferred to a fresh tube and mixed with 450µl of isopropanol to precipitate the DNA. The tube was centrifuged at 14000rpm for 10 minutes. Without disturbing the pellet supernatant was sucked off. A second washing step at 14000rpm for 3minutes with 1ml of 70% ethanol reduced contaminants in the DNA-pellet. Supernatant was aspirated and the pellet air dried. Once dry, precipitated DNA was taken up by resuspending in 50µl of ddH₂O. DNA concentration was measured with a Nanodrop photometer.

5.6.3 Test digests

According to chosen restriction strategy the length of inserts was determined primarily by EcoRI Fast Digest (FERMENTAS) digest whereas the homogeneousness of the clones was determined by digesting with polycutter HinfI (NEB).

Conditions for a single reaction mix:

plasmid	2,5µl
buffer 10x	1µl
enzyme	0,5µl
ddH ₂ O	6µl
total volume	10µl

The reaction was incubated for 1 hour at 37°C and separated on a 1% agarose gel in the case of EcoRI digest and 2% agarose gel in case of HinfI digestion. Gels were stained in an EtBr-TAE bath (60µl EtBr in 600ml of 1xTAE).

5.6.4 Sequencing

Sequencing of DNA was performed by AGOWA Genomics

5.7 Cloning of novel gene fragments

New gene fragments are either obtained by degenerated PCR or by SMART RACE.

5.7.1 Degenerated PCR

The primer design included sites of conserved primary sequence among orthologue genes. The redundancy of the genetic code to encode via different codons for a single amino acid was then used and the base triplets therefore variegated. The design itself was carried out with Oligo 6.8, a primer design computer program for macintosh. As template for the PCR served pooled first strand cDNA from *Nereis virens*. Proper amplicons were obtained in first and nested reactions.

Single reaction mix	
Template	4µl
10xbuffer	5µl
dNTPs (5mM)	1µl
forward primer (100mM)	1,5µl
reverse pprimer (100mM)	1,5µl
Hot Star Taq	0,25µl
ddH2O	36,75µl
total volume	50µl

PCR program		
automatic hotstart	95°C	5min
denaturing	94°C	1min
annealing	X - 5°C	2min
elongation	72°C	4min
denaturing	94°C	1min
annealing	X°C	2min
elongation	72°C	4min
final elongation	72°C	10min
hold	10°C	∞

} 5 cycles

} 35 cycles

10µl of the PCR were analysed on a 1,5% Agarose/Metaphor-agarose TAE gel (1:1). 40µl of the reactions that yielded an amplicon of the estimated size were than again separated on a 1,5% Agarose/Metaphor-agarose TAE gel with additional Sybr safe DNA gel stain. Bands of right size were cut out and eluted from the gel. Eluted fragments were cloned into pGem-T easy and transformed into *e.coli*. The bacterial amplified recombinant vector was prepped and digested with EcoRI and HinfI to measure size of insert and homogeneousness of the clones. Candidate clones were chosen by the results of these digestions and sent to AGOWA for DNA sequencing.

5.7.2 Smart RACE

To elongate known gene fragments a RACE was performed in the direction of the 5' - as well as to the 3' end of mRNA. Sites for primer anneal were chosen with regard to have at least >100nt of known sequence for labelled probe hybridization. As template in the first PCR served 3µl of either 5' - or 3' RACE-Ready first strand cDNA, whereas in a second nested reaction 3µl of the first PCR. Thus amplicons were obtained in two reactions, in a first PCR with an upstream gene specific and an universal counter primer (UPM) and in a second reaction with a further downstream gene specific primer and a nested universal counter primer (NUP). The exerted PCR program equals the degenerated PCR program above.

Single reaction mix	
template	3µl
genespecific primer (5µM)	2,5µl
UPM/NUP(CLONETECH)	5µl
HotStar Taq (Qiagen)	0,2µl
10x buffer (Qiagen)	5µl
dNTPS (5mM)	2,5µl
ddH ₂ O	31,8µl
finale volume	50µl

10µl of products were separated on a 1% agarose/metaphor agarose gel and the gels southern blotted. The filters were hybridized under low stringency conditions with a radiolabeled probe. Fragments that yielded a positive signal were separated on a 1% agarose/metaphor gel, eluted and cloned into pGem-T easy. The vector was transformed into bacteria and the transgene bacteria were filter lifted. Those filters again were hybridized under high stringency conditions (65°C) with a radiolabeled DNA probe. Thereby identified positive colonies were picked and prepped. The prepped plasmids are subjected to test digests: an EcoRI-digest to determine the size of the insert and a HinfI-digest to check for similarity of the clones. Candidate plasmids were then sent for sequencing.

5.8 Blots and Filter lifts

5.8.1 Southern blot

DNA gels were stained with EtBr and documented prior to blot. DNA was made single stranded by incubating the gel in denaturing solution (0,5M NaOH, 1,5M NaCl) for 20min. DNA was blotted onto a nylon membrane (peqlab, poresize 0,45µm) by capillary transfer o/n. Transfermedium was the liquid contained in the gel itself. The blot was assembled as follows. The gel bottom up was the undermost layer covered by the nylon membrane of the same size as the gel. Two layers of whatman 3MM blotting paper on top of the membrane followed by a pile of green paper towels stacked to a height of approximately 5-7cm. To prevent leakage between the layers the pile was compacted by weighing it down. After the o/n capillary transfer the blot was disassembled and the position of the loading slots of the gel was marked. UV-crosslinking (STRATAGENE UV Stratalinker set to auto-crosslink) immobilized the

DNA by creating covalent bonds between nucleic bases and amine groups on the surface of the membrane. Treated that way the membrane was stored at ambient temperature and protected from light until further use.

5.8.2 Northern blot

RNA gels were documented before performing the vacuum blot. The vacuum transfer was carried out with a BIORAD vacuum blotter according to manufacturer's protocol. Transfer conditions were 5 inches of Hg negative pressure for approximately 90 minutes. A nylon membrane (peqlab, poresize 0,45µm) was used. Positions of the gel loading slots were marked on the membrane and the RNA was covalently crosslinked via UV exposure. The membrane was kept protected from light at ambient temperature until further use.

5.8.3 Filter lifts

To screen whole agar plates of bacteria for recombinant clones at once filter lifts were performed. Transformed bacteria were grown at 37°C for 8-16hours. When grown to proper density and size the colonies were partly transferred onto membranes (Whatman BA-S 85 Optitran, 0.45 µm, 82 mm, circles). The filters were put onto the surface of the agar plates thereby partly adsorbing the colonies in a replica pattern. To mark the orientation two cuts were affixed to the filters and the agar. After 2-10 minutes the membranes were detached from agar plates and face up soaked with denaturing solution (0,5M NaOH, 1,5M NaCl) for 5min to lyse cells and denature DNA. Subsequent incubation for 2 minutes with neutralisation solution(1,5M NaCl, 0,5M TrisCl pH 7,4) followed. Another incubation with 2xSSC for 2 minutes was carried out. Membranes were allowed to air dry before subjected to UV-crosslinking. The membranes were stored light protected at room temperature until further use. Agar plates were stocked at 4°C after putting them at 37°C for 1-2h to boost bacterial growth.

5.9 Probe hybridization

5.9.1 DNA probe

Gene fragments had to be at least 100 base pairs long for high stringency hybridization at 65°C. Fragments were cut out from plasmid and purified by elution from a DNA gel. Radioactive labeled probes were synthesized by random priming with the Radprime DNA labeling kit (INVITROGEN) according to manufacturer's protocol. As a radioactive source served of $\alpha^{32}\text{P}$ dCTP (5µl with the activity of 50µCi per reaction) ordered from Hartman Analytic GmbH. Radiolabeled probes were purified from non-incorporated nucleotides with the help of illustra Sephadex G-50(GE Healthcare) columns pursuant to manufacturer's protocol. Membranes were rinsed in 2xSSC prior to hybridisation. Used buffer was Amersham Rapid-hyb buffer. Hybridisation was performed in compliance to the instructions of Rapid-hyb buffer manual for DNA probes. Temperature was set to 65°C and hybridisation time to 2 hours. Washed membranes were wrapped and mounted into an exposure cassette and radioactive signals detected by a Kodak BioMax film.

5.9.2 RNA probe

Gene fragments had to be transcribed antisense from the T7 promoter of pGem-T easy vector. Plasmid linearization strategy was chosen accordingly. Linearized vector was separated on gel and eluted. Probe synthesis was carried out with T7 MAXIscrip kit (AMBION). All steps were performed in compliance with the manufacturer's protocol. As a radioactive source served of $\alpha^{32}\text{P}$ UTP (5 μl with the activity of 50 μCi per reaction) ordered from Hartman Analytic GmbH. As above labelled probes were purified with illustra Sephadex G-50(GE Healthcare) columns. Membranes were rinsed in 2xSSC prior to hybridisation. Used buffer was ULTRAhyb Ultrasensitive Hybridisation buffer from AMBION. Hybridisation was performed in compliance to the instructions of ULTRAhyb buffer manual for RNA probes. Hybridisation time was extended to o/n and the temperature set to 68°C. Washed membranes were wrapped with saran wrap and mounted into an exposure cassette and radioactive signals detected by a phosphor screen.

5.10 RNA Extraction

Two methods were utilized to isolate RNA from living tissue. In principle trizol extraction was used for high amounts of RNA[128]from larvae whereas RNeasy Mini kit was used for RNA isolation from adult heads.

Heads were cut under the binocular microscope. The cut was placed right behind the head and before the first segment with parapodia. Heads of six average sized worms were cut sequentially, put into a safe lock eppendorf tube and immediately frozen in liquid nitrogen. Cut adult heads were used in frozen condition (kept in liquid nitrogen) without allowing them to thaw until disruption and homogenisation.

Larvae were harvested with the help of a small meshed net A modified 50ml falcon tube with one end open and the other end covered by a net served as a harvesting device. One to two well grown batches of larvae were poured through the modified falcon tube and washed two times by flushing with KSW. The net together with the retained larvae was detached from the tube, put into a 2ml safe lock eppendorf tube and frozen in liquid N₂.

5.10.1 RNeasy Mini kit(QIAGEN)

Manufacturer's protocol for purification of total RNA from animal tissue was carried out using a pestle for disruption. RNA was eluted from the column with 50 μl of RNase free H₂O. RNA concentration was measured with the nanodrop photometer. RNA was stored at -80°C until further use. RNA molecules smaller than 200 nucleotides in length were lost due to the purification method.

5.10.2 Trizol extraction

Starting with 200 μl of trizol (TRI reagent AMBION) and filling up to 1ml larvae and net were homogenized by gently resuspending. The reaction was incubated for 5minutes at room temperature. Centrifugation for 10 minutes at 12000 rpm and 4°C separated the phases. The aqueous supernatant was transferred to a new tube and 0,2ml CHCl₃ added. The emulsion was shaken by hand and incubated another 2-5 minutes at ambient temperature. Centrifugation for 10 minutes at 12000 rpm and 4°C separated the phases. Aqueous supernatant was transferred to a fresh tube and extracted with 1ml of phenol:chloroform:isoamylalcohol 25:24:1. To mix the emulsion it was recurringly shaken by hand. Centrifugation for 5 minutes at 12000rpm and 4°C. Aqueous supernatant was again transferred to a fresh tube and once again extracted

with 1ml of chloroform. Aqueous phase was detached and mixed in a fresh tube with 0,5ml of isopropanol. Formation of precipitate was boosted by incubation at -20°C for 30 minutes. Solution was centrifuged for 15minutes at 14000rpm and 4°C. Supernatant was aspirated and pellet washed with 0,5ml of -20°C cold 70% EtOH. RNA pellet was spun down for 5min at maximum speed. EtOH was removed and the pelled allowed to air dry before taken up in adequate amount of RNase free H₂O. RNA concentration was measured with nanodrop photometer and the samples were stored at -80°C.

5.11 Denaturing RNA Gel and genomic digest

5.11.1 Genomic digest

Total RNA from Trizol extraction was measured and the needed amount was digested with 2µl DNaseI (NEB). Therefore required amount of RNA is filled up to 32µl of mQH₂O, mixed with 5µl 10x DNase I reaction buffer (NEB) and 0,5µl RNasin (RNase inhibitor, PROMEGA). The reaction was incubated for 30 min. at 37°C. Afterwards the reaction is diluted with 150µl of mQH₂O. A subsequent Phenol/Chloroform extraction was followed by a Chloroform extraction. The RNA was precipitated following the standard ethanol precipitation protocol. 1/10 volume of 3M NaOAc pH 5,5 is added together with 2,5 volumes of absolute ethanol. RNA pellet was taken up in suitable volume of RNase free water.

5.11.2 Denaturing RNA gel

A formaldehyde gel was used to separate molecules by size. 3.2g of agarose was dissolved in 195g of mQH₂O by heating. When cooled down to 60°C 27ml of 10xMOPS were added together with 49ml of 37% formaldehyde. The solution was mixed carefully and poured into the gel chamber. The gel was stored under the fume hood until solidified. Gel was moved to 4°C cold room and let run in 1x MOPS for 10-20min at 80V before loading the samples. Samples were mixed at least 1:1 with RNA loading buffer (AMBION) and heated up to 74°C for 15 min, then immediately chilled on ice. Samples were spun down, mixed with 1/10 volume EtBr and loaded onto RNA gel. The gel was set to run for 13-16h at 80V. After the run the gel was documented.

5.12 Quantitative PCR

5.12.1 cDNA synthesis

As starting material heads from premature adult worms were cut in replicates of three times six heads per time point. As mentioned above RNA was extracted with the RNeasy Mini Kit. cDNA to measure abundance of transcripts in qPCR was synthesized using QIAGEN QuantiTect Reverse Transcription Kit. For each of the replicates 400ng of RNA served as template for the reverse transcription reaction. Every step was carried out in compliance with manufacturer's protocol except for little adjustments due to practical reasons (Enrique Arboleda, personal communication). The incubation time at 42°C of the genomic DNA elimination reaction was extended to 5min and the reverse transcription was extended to 20 minutes at 42°C. The final RT-reaction was diluted by adding 40µl of H₂O and was then put to immediate use or stored at -20°C.

5.12.2 qPCR Run and Analysis

Each of the biological replicates was run in two technical replicates on the same PCR plate. The total reaction volume was 20µl comprising 5µl cDNA template, 3µl H₂O, 10µl POWER SYBR Green PCR Master Mix (Applied Biosystems), 1µl of forward primer (10µM) and finally 1µl reverse primer (10µM). To test for contamination –rt controls were included i.e. reverse transcription reactions where the reverse transcriptase was absent from the beginning. A complete run for 2 hours was performed including a melting curve profile to check for aberrant amplicons. As an internal control for normalization of transcription level served the house keeping gene *cdc5*. Obtained data was analysed using Microsoft Excel 2007. Error bars correspond to +/-standard error $\sqrt{Var(x)}$

To test for significance between two populations, p-values were calculated using an unpaired student's t-test.

6 Results

6.1 Photodestruction of rhabdomeric photoreceptor cells in *P. dumerilii*

6.1.1 RE strain - 1 month post fertilisation

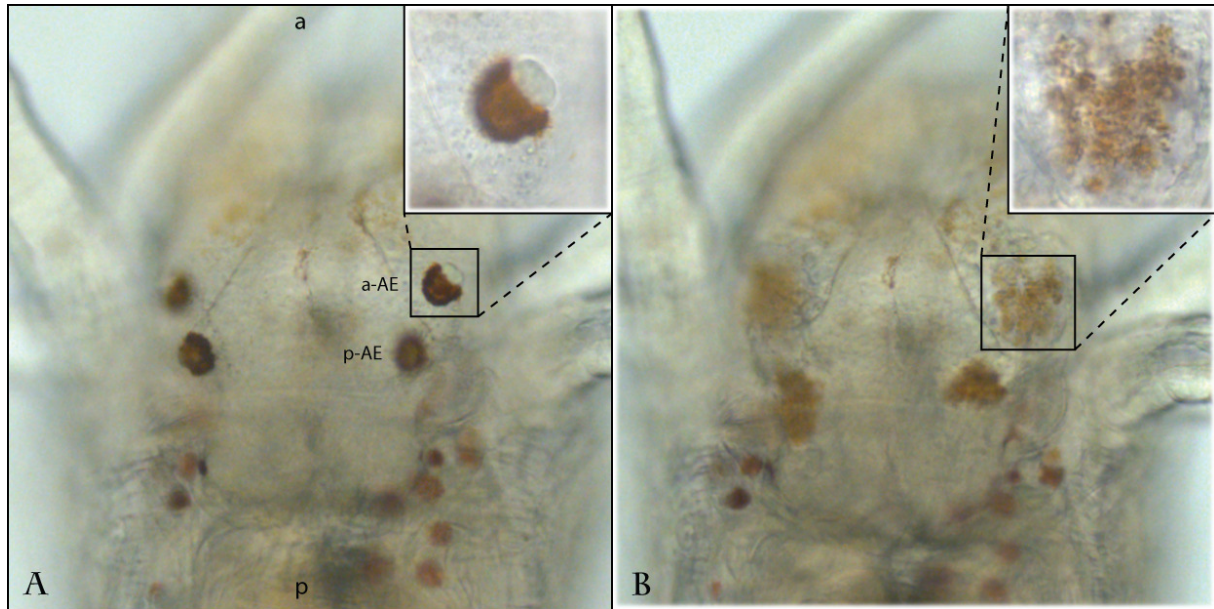


Figure 15 pictures show a 40dpf old worm before and after the procedure under a 20x dry objective. Higher magnifications the eyes or the remainders of the eyes respectively are shown with a 40x dry objective which was the used operating magnification for laser ablation. A) Head before ablation. B) Head after ablation. The eyes' orange colour was easy to detected and a good indication for rhabdomeric PRCs. The completion of the operation was determined by the fragmented and dispersed appearance of the eye. a-AE=anterior adult eye, p-AE=posterior adult eye, a=posterior, p=anterior

Worms at the age of 30-40 dpf were subjected to ablation (Figure 15) (settings and condition of the ablation are denoted in the methods section 5.2). The condition of the worms was checked daily by eye. The first step for improvement was to omit mineral oil which was intended to seal the viewing chamber thereby preventing evaporation. As soon as the oil made contact with the specimen its survival was critical and caused epidermal irritations (Figure 16), which could be clearly seen under the microscope. The skin of the animal lost its integrity (red arrows, higher magnifications, Figure 16). Attempts to wash off the oil did not prove to help. Getting rid of this step in the mounting and dismounting procedure also speeded up the whole process. After >7 days appearance orange coloured structures were detected upon the surviving worms at the location of the adult eyes (Figure 17A,B). The monitored structures were likely to grow ectopic, asymmetrically and in slight aberrant shapes (Figure 17). Also more than two of these structures could be observed (Figure 17B)

These results led to the demand for additional rounds of photodestruction of appeared structures. Therefore worms were scheduled for continuative trials to look for the effectiveness of the method.

Figure 16 Sideeffects of ablation

Dorsal view on 49dpf old worm. 9 days after 1st ablation. The ragged appearance of worm's epidermis under transmission light from a dorsal view. Iridescence effect is caused by contact of the skin with mineral oil. Higher magnifications on the right show skin irritations (red arrow) at a parapodial segment and at the pygidium.

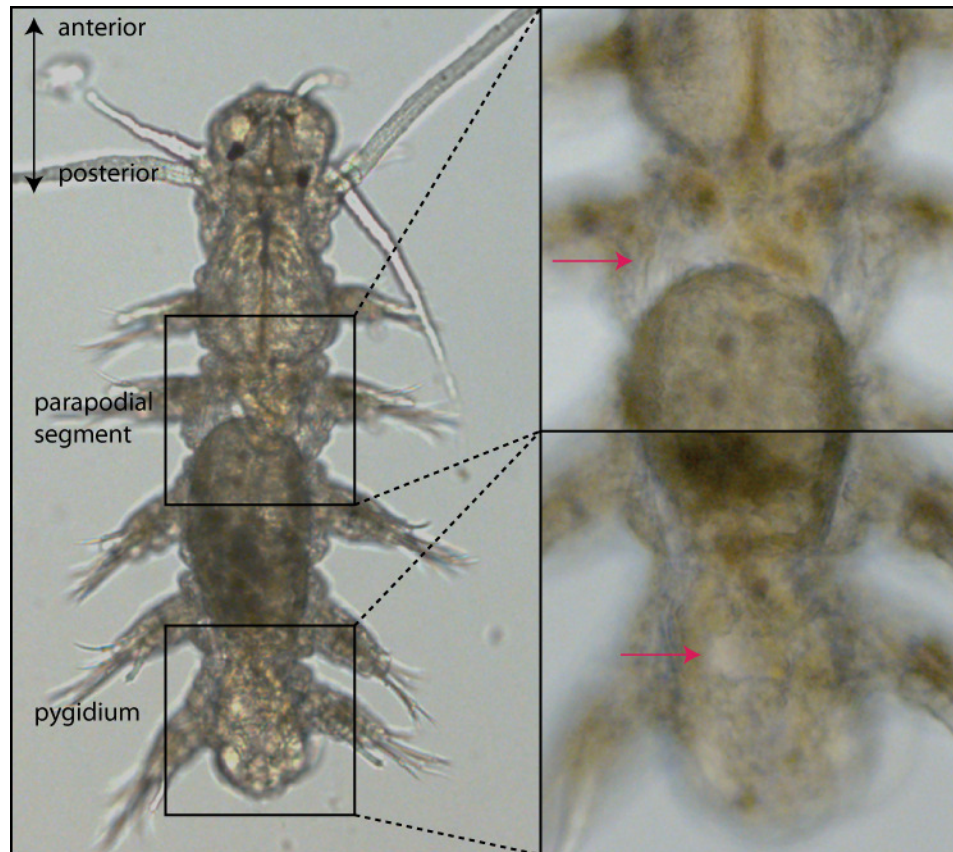


Figure 17 Appearance of

- A) Dorsal view on twice ablated 54dpf old worm. 7 days after 2nd ablation. Orange coloured structures at the location of the adult eyes.
- B) Dorsal view of another twice ablated worm of same age and condition like in A). Orange coloured structures also in places different from the adult eyes' position.
- C) Lateral view of 54dpf worm. 7 days after 2nd ablation. Eyes are clearly reduced in size. Eyes are not joined but still divided in anterior and posterior.

Yellow asteriks indicate the position of the described structures.

A limiting problem of the experiment was the low survival rate. Additional trials of photodestruction were performed in a weekly interval, giving the worms a maximum regeneration time of 7 days. Up to three trials were performed. But none of the specimens survived past three times of laser destruction of their PRCs.

6.1.2 RE strain – 14 days post fertilisation

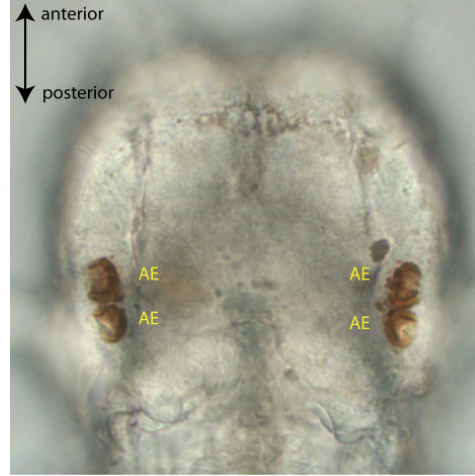


Figure 18 eyes of a 14 dpf old worm

Dorsal view on the head of a 14dpf old worm. The eyes (yellow AE) show typical pigment cup appearance. AE= adult eye

Based on these previous observations strategy changed with the intention to intercept the capacity for regrowth by switching to younger 14dpf worms. At the stage of 14dpf the worm has already proper developing adult eyes but still of small size (Figure 18). The four juvenile eyes have a typical pigment cup eye appearance. The progenitor cell therefore has not given rise to that many PRCs by mitotic division. Thus the ratio of stem cells to adult post –mitotic PRCs is higher compared to the eyes of older worms. Upon laser irradiation of the eye tissue the probability to hit a stem cell is increased compared to older stages.

Slight improvements were achieved the fundamental problem of regeneration of eyes could not be overcome due to low survival rate. Only with high number of specimen a small fraction of three times ablated worms could be achieved (Figure 19) but most of them they died within 3-4 days. The spawning behaviour of treated animals showed no abnormalities because when further cultured they matured regularly according to the phase of the moon. A problem that could not be dealt with was the regeneration capacity of the eyes. Amongst survivors all worms could regrow their eyes after a period of 14 days. As shown in previous cases the regenerating eyes grew sometimes ectopic and in aberrant shapes (Figure 19).

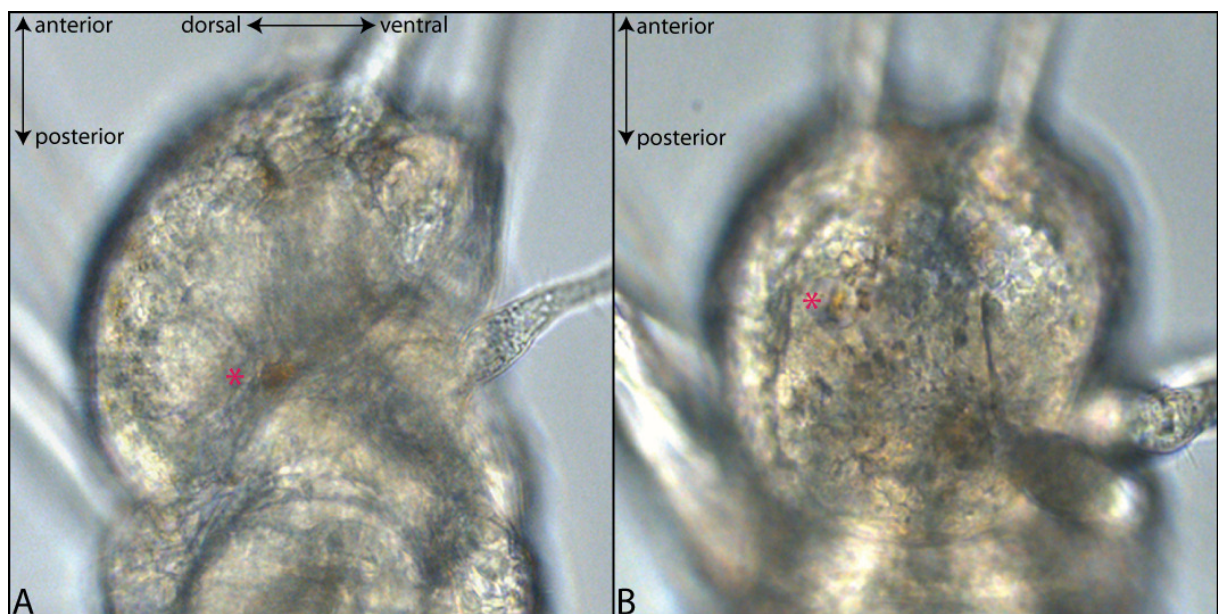


Figure 19 triple ablated worm. RE line, 35dpf. 7 days after the 3rd ablation.. A) lateral view B) dorsal view. There seem to be dispersed remainders of mentioned orange coloured cellular structures from dorsal view and an ectopic one in the lateral view. Position of the assumed remainders is indicated by a red asterisk.

6.1.3 B321 strain – 14 days post fertilisation

Due to the limited survival conditions of the used RE-line it was replaced with the B321 strain, which promised favourable viability. On the downside of falling back to B321 worm culture was the fact that PRCs can not be distinguished simply by the colour because the optical denser chromophore from the pigment cells are masking their appearance. To continue the experiment with the new strain I used 14dpf old worms for the first ablation trial and subjected them to subsequent procedures in a weekly interval as done before with the or-mutants. Also further improvements were carried out by using KSW instead of NSW, which has the advantage of being filtered, thus being cleaner and less contagious in comparison. Earliest feeding was restricted to 3 days after the ablation. This food deprivation was intended to reduce possible contaminants in the water or to avoid infection respectively. To evaluate the risk the time being in paralyzing solution poses two groups were compared. In the first group only two worms were mounted and subjected to the ablation, which speeded up the whole process. In the second group five worms per slide were mounted and the procedure took longer than compared to the first group. Ablations were performed as usual with both but they were kept separate. Surviving worms were counted 7 days after the ablation.

	1.ablation	Survivors After 7 days	2.ablation	Survivors After 7 days	3.ablation	Survivors After 7 days
Worms mounted	Age: 14dpf	Age: 21dpf		Age: 28dpf		Age: 35dpf
2	130 worms	84 worms	84 worms	33 worms	33 worms	0 worms
Survival-probability		65 %		25 %		-
Worms mounted	Age: 14dpf	Age: 21dpf		Age: 28dpf		Age: 35dpf
5	360 worms	230 worms	230 worm	104 worms	104 worms	3 worms
Survival-probability		64 %		29 %		<1 %

The time being in paralysing solution did not seem to make an impact on the survival rate. The time savings were in the range of 10-15 min. Therefore no further effort was made to minimize the duration of the whole procedure. The statistic only included the B321 strain. For the RE strain I did not evaluate survivors. Therefore no comparison between the two strains can be drawn.

Due to the low survival rate after 3 trials of ablations the procedure was not opted for more than two trials, or preferentially only a single trial. Otherwise the experiment would not be feasible. This requirement was of course conflicting with the need for more trials to reduce rhabdomeric PRCs. Thus it became more apparent to have a look into the actual distribution of *Pdu r-opsin* in the course of the ablations trials.

6.1.4 WMISH against rhabdomeric opsin

If there were eyes' rhabdomeric PRCs left after ablation, they have to express the according *Pdu r-opsin* as the GPCR on the first stage of photo transduction. Therefore a WMISH against *Pdu r-opsin* was scheduled to test whether these observed cells are indeed of this type. Simultaneously a co-immunostaining against anti-acetylated α -tubulin (a-a- α -t) should shed

light on the innervations of these cells. Another aspect that would be covered in the same instant by the immuno-staining is the off target damage due to the laserablation. If the connectivity of nerves in the adjacent tissue is impaired, off-target damage has been inflicted. Therefore two sets of confocal scans of the fluorescent labelled axons were compared. On one hand unaltered worms as a positive reference on the other hand a series of ablated worms as the test samples.

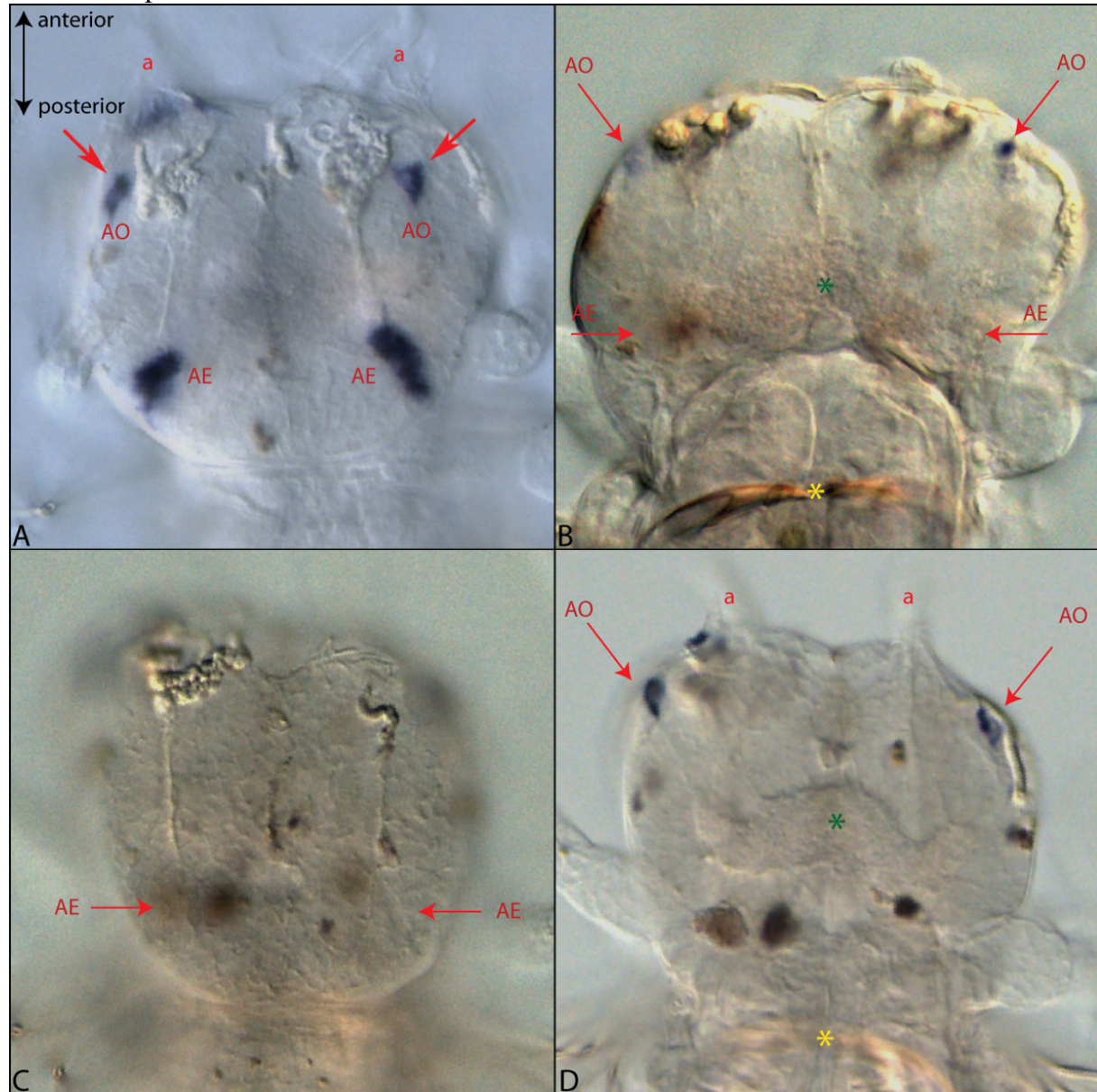


Figure 20 WMISH against Pdu rhabdomeric opsin.

A) dorsal view on a 28 dpf untreated worm. Strong signals come from the location of the adult eyes. Additional expression domains in the anterior part of the prostomium are marked by red arrows, dorsal and lateral from the antennae.

B) dorsal view on 21 dpf 1x ablated worm. No *r-opsin* staining is visible at the location of the treated adult eyes. Expression in the anterior ocelli is present.

C,D) dorsal view on 28 dpf 2x ablated specimen. No *r-opsin* transcripts are detectable at the focus level of the adult eyes.

D) *Pdu r-opsin* is expressed in a further ventral plane in the anterior part of the head in vicinity to the palps (D). The neuropil shows no obvious abnormalities due to laser inflicted damage in the DIC channel. a= antenna, AE= adult eyes, AO= anterior ocelli, green asterisk= neuropil, yellow asterisk= jaw

The worms were fixed 7 days after the ablation procedure. The positive control (28dpf) showed strong and robust staining at the location of the adult eyes and for an anterior domain. Staining of a paired anterior domain was found ventrally to the adult eyes and dorsolaterally to the antennae in the anterior part of the prostomium (Figure 20A). In one time ablated worms (21dpf) no signal from the position of the dorsal adult eyes was detected in most of the treated worms. In a more ventral plane in the anterior prostomium a signal from these anterior domains (anterior ocelli) was robustly detected (Figure 20 B). In two times ablated worms none of the examined specimen showed a signal at the location of the adult eyes (Figure 20 C). In the anterior prostomium dorsolaterally to the antennae again robust staining coming from anterior ocelli (AO) was detected (Figure 20 D).

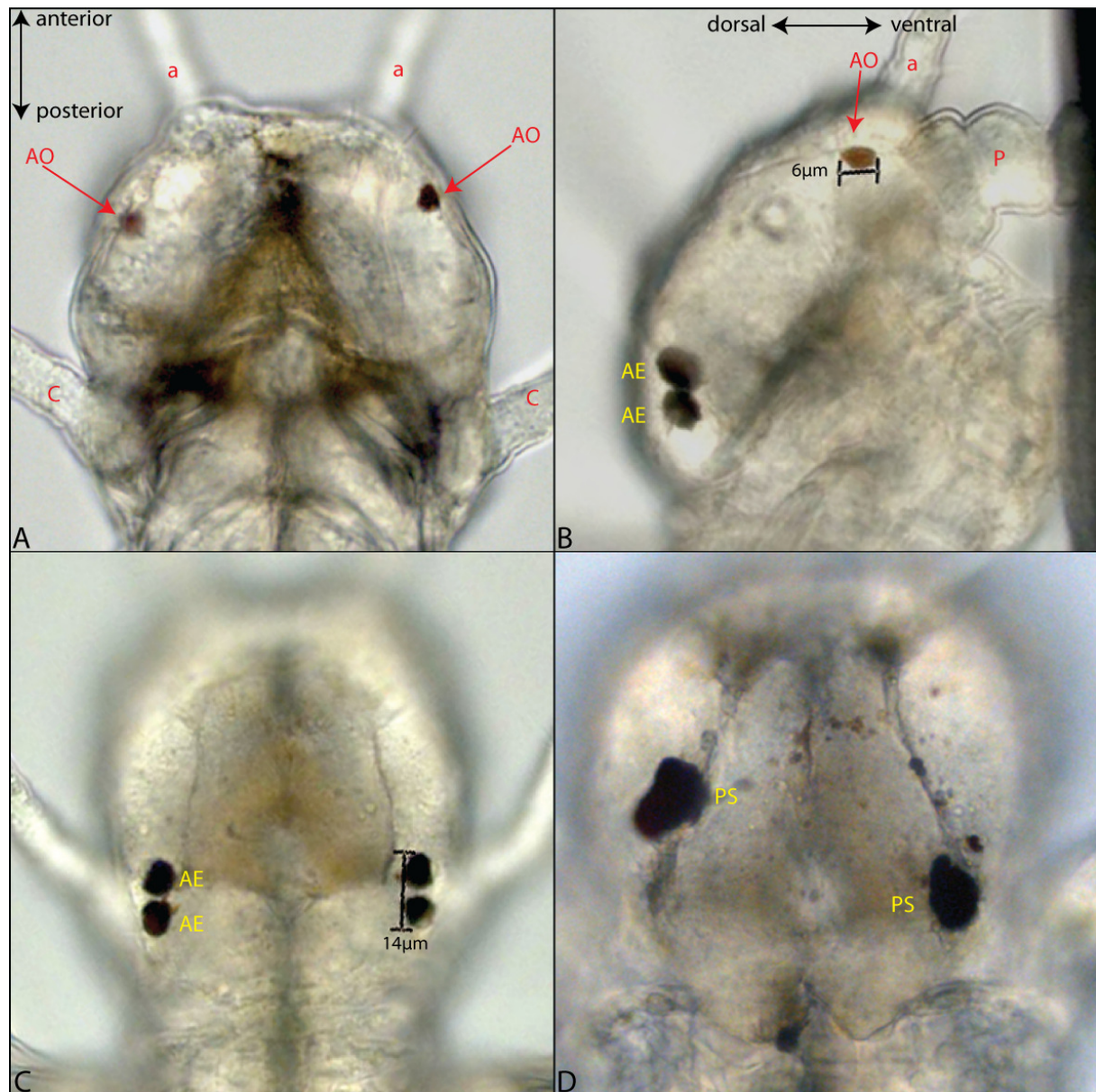


Figure 21 Location of 2nd *r-opsin* expression domain and reappearance of pigmented structures in B321.

- A) dorsal view on head of 28dpf old worm. Anterior ocelli are marked by red arrows. Anterior ocelli are found in a lateral and anterior part of the head, posterior from the antennae.
 - B) Lateral view on 28dpf old worm. AO is located dorsally to the palpa and laterally to the antennae and shifts in the direction of the palpa. Bar indicates the size or length (6µm) of the domain
 - C) Dorsal view on adult eyes of 28dpf old animal. Bar indicates the size of the eye pair (14µm) in comparison with the anterior ocelli in A) and B).
 - D) Dorsal view on 28dpf worm. 14 days after 1st ablation pigmented structures regrow in aberrant shape..
- A= antennae, AE= adult eyes, c= cirrus, AO= anterior ocelli, p= palpa, PS= pigmented structure

Yet regrowing pigmented structures were detectable (Figure 21D) after ten to fourteen days at the location of the adult eyes in both single and twice ablated animals. The additional expression domain of *Pdu r-opsin* led also to the need to ablate them in subsequent trials. This in turn also led to the prerequisite to identify those domains reliably in older stages. These anterior ocelli were spotted without need for labelling in 1 month old worms at the designated location (Figure 21 A, B). The spotted ocelli shifted ventrally in the direction of the palpa. Three months old worms were also stained for *Pdu r-opsin* but due to technical problems with the WMISH no readout could be derived. Due to this the position of anterior ocelli in 3 month old worms could not be reliably identified.

6.1.5 Laser Scanning Confocal Microscopy

The confocal laser scans of immuno-stained WMISH affirmed the location of the *Pdu r-opsin* expressing domains by providing an axonal landscape. As mentioned before, LSCM z-projections of treated versus untreated worms were compared to address the question for off-target harm to subjacent tissue. As usual animals had a period of seven days to regenerate after the ablation trials to regenerate, before they were fixed.

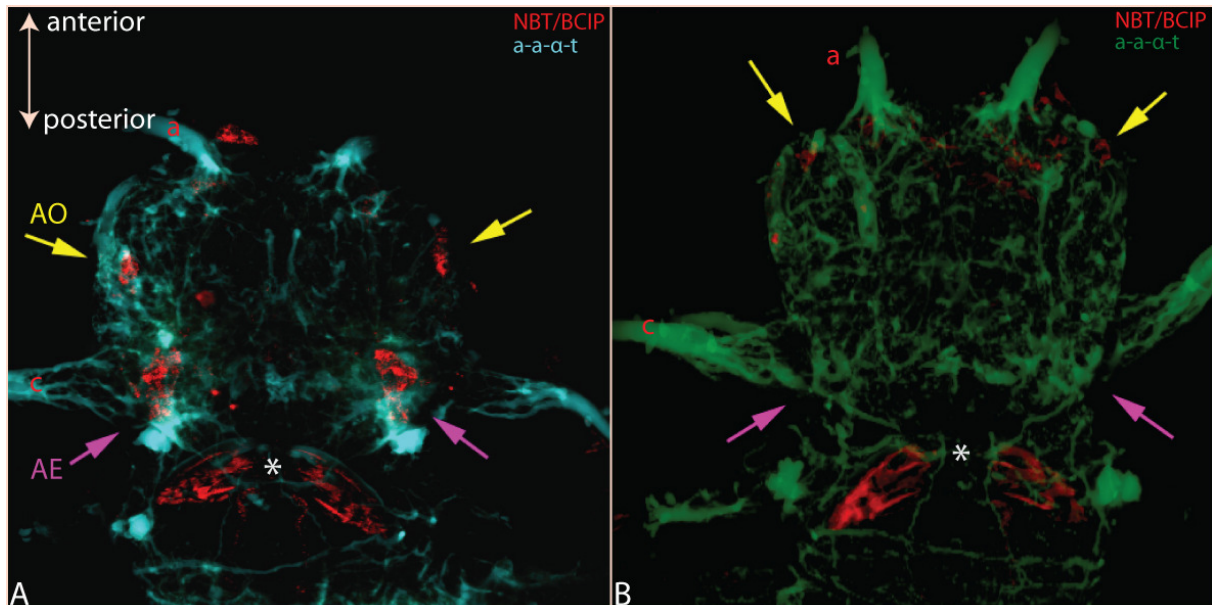


Figure 22 Locating expression domains.

Z-projections of confocal laserscans:

- A) dorsal view on prostomium of 28dpf old, untreated worm. Adult eyes as well as the second *r-opsin* domain can be identified reliably.
- B) dorsal view on prostomium of 28dpf old worm, twice ablated, fixed one week after procedure. No *Pdu r-opsin* staining detectable at the former position of the adult eyes. Anterior located *r-opsin* transcripts can be detected.

Green or turquoise colour both correspond to immunolabeling (alexa 488) of anti acetylated α -tubulin (a-a- α -t). Red colour ideally corresponds to laser reflection (630-640nm) by NBT/BCIP precipitate to visualize *Pdu r-opsin* staining. Unspecific reflections are obtained by the worm's jaws as well as from appendices adjacent to the antennae and unspecific sources. Yellow arrows point towards anterior *r-opsin* expressing AO. Purple arrows point towards the location of the adult eyes. White asterisk indicates position of the jaw. a= antennae, AE= adult eyes, AO= anterior ocelli

Projections of *Pdu r-opsin* reflections showed a strong signal at the location of the adult eyes as well as for the anterior ocelli in case of the positive control (Figure 22 A). The acetylated α -tubulin staining was restricted to superficial levels of the worm due to technical reasons with the WMISH. When looked at the twice ablated specimen (Figure 22 B) a signal deriving from the adult eyes was absent. The reflection from the AO was detected. Noise from unspecific reflection at the same area was masking real staining. Unspecific reflections came from the jaws and dust in both specimens. When looked into the neural ensemble underneath the adult eye no obvious damage due to objectionable laser penetration could be located (Figure 23). Despite the miscellaneous efficacy of the neuronal labelling due to differing probe penetration subjacent nerval structures seemed preserved in all scans of ablated worms.

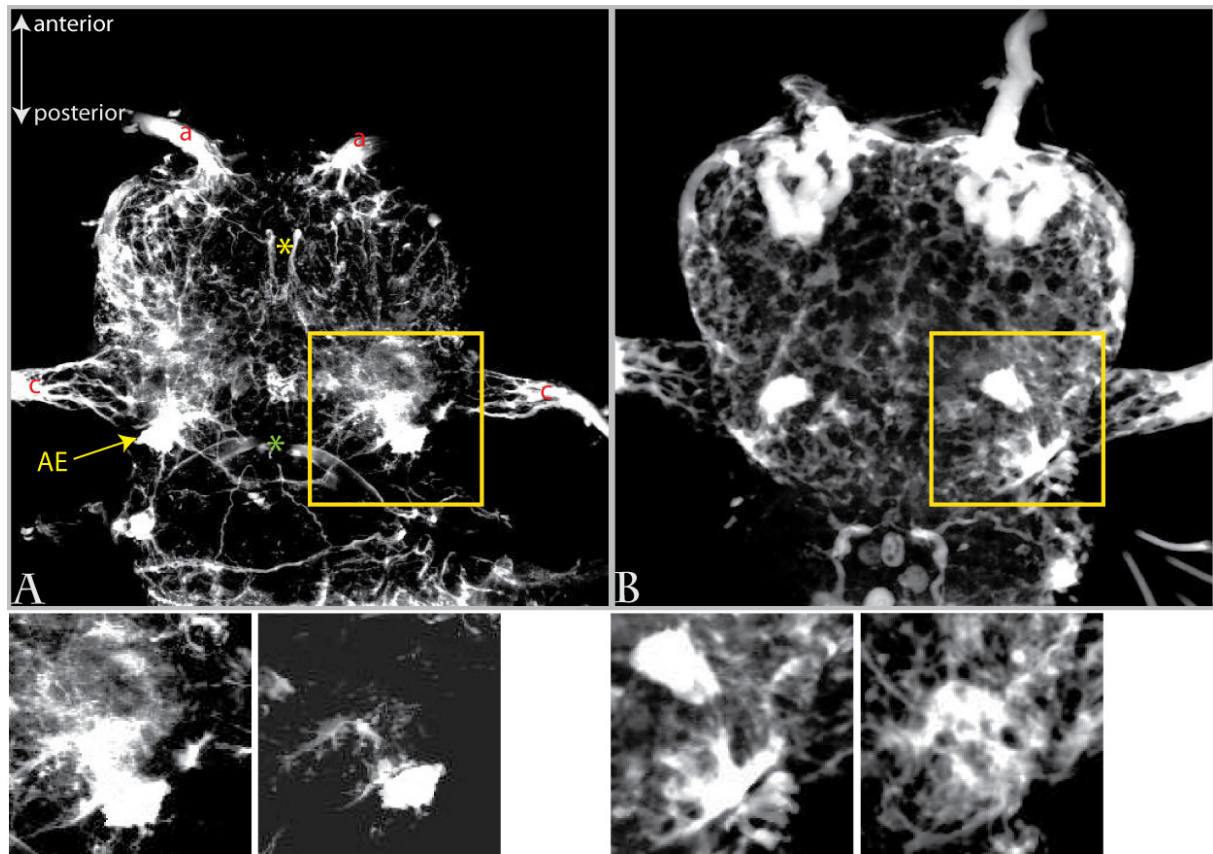


Figure 23 comparison of neural framework

z-projections of immuno-labeled anti-acetylated α -tubulin (alexa 488). Dorsal view on prostomium of 28dpf old specimens.

- A) Untreated sample. The yellow box enframes neurons located underneath and projecting from the adult eyes. The preservation of the prominent neuronal scaffold is a good indicator of presumable off-target damage. Thus deep tissue penetration of the probe is not mandatory because nerval projections radiate from the surface, where the eyes are located. Higher magnifications show a close-up of the respective, framed region of the same specimen, but from differently stringent scans.
- B) Single ablated worm. Tissue does not seem to be impaired. Prominent neuronal structure appears conserved and resembles positive reference. Notably the staining itself did not work equally well. Left magnification shows a detail of the pictured single ablated worm, right box shows a higher magnification of the respective area of a twice ablated animal.

A=antennae, AO= anterior ocelli, c=cirri, green asterisk= position of the jaw

6.1.6 Involvement of Pdu adult eyes in the Lunar and Circadian Clock

Since the involvement of adult eyes in Pdu circadian clock has also not been reported yet, I focused my analyses on the characterization of putative Pdu clock gene transcripts. Therefore the quantitative expression of two highly conserved *Platynereis*' clock genes orthologues (*Pdu bmal*, *Pdu period*) over 24 hours was monitored by Q-PCR.

6.1.6.10 Positive control

The positive control was tested for *Platynereis*' *period* expression and *bmal* expression. The abundance of *Pdu period* transcript throughout 24 hours (Figure 26) showed an oscillation with a maximum peak at 4 pm and a minimum from 24, 4, and 9 am for the 12 hours shifted control. Before the phase shift (Figure 26) a peak maximum in *period* expression level was detected at 4am and a minimum between noon and 9pm..

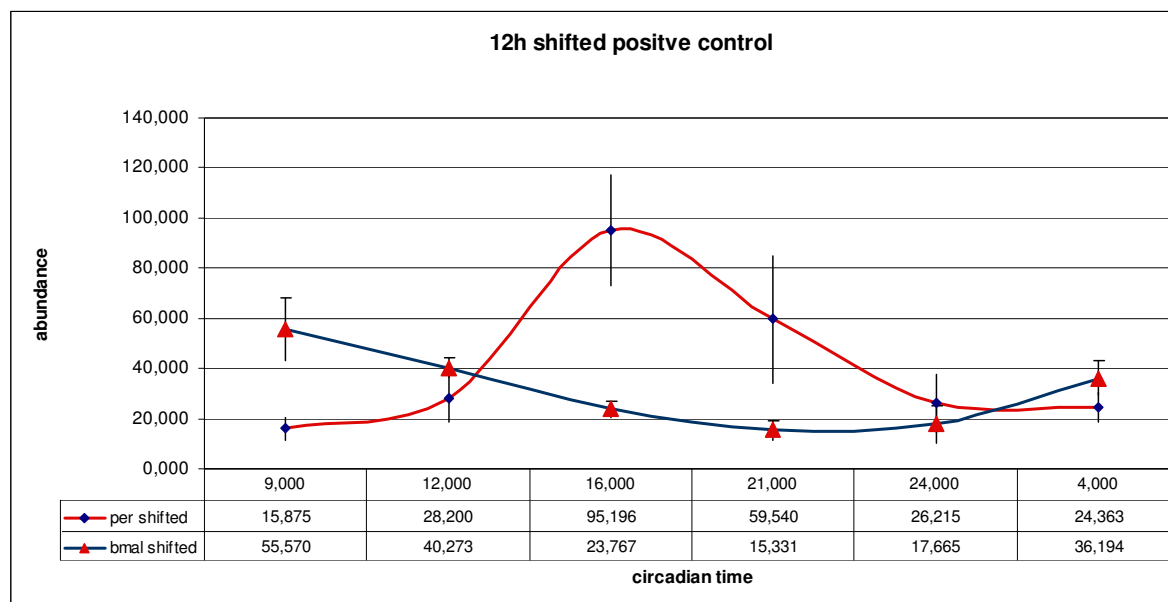


Figure 24 Circadian relative expression of *Pdu period* and *bmal* in 12 hours shifted positive control. *Period* transcript abundance over 24 hours is coloured in red. Numbers in the row of the “PER” chart legend correspond to relative expression. *Bmal* transcript abundance over 24 hours is coloured in blue. Numbers in the row of the “BMAL” chart legend correspond to relative expression. Error bars are +/- SEM.

For *Pdu bmal* mRNA abundance (Figure 25) shifted positive control showed a maximum peak at about 9 am and a minimum between 4pm and midnight. The *Pdu bmal* abundance for the positive control before the LD shift showed a maximum peak at 9pm (Figure 27).

The dotted and the continuous lines in Figure 26 and Figure 25 corresponding to clock gene expression in normal LD and 12 hours phase shifted LD respectively showed 180° phase inversion. Both show no error bars because they only have an illustrative function.

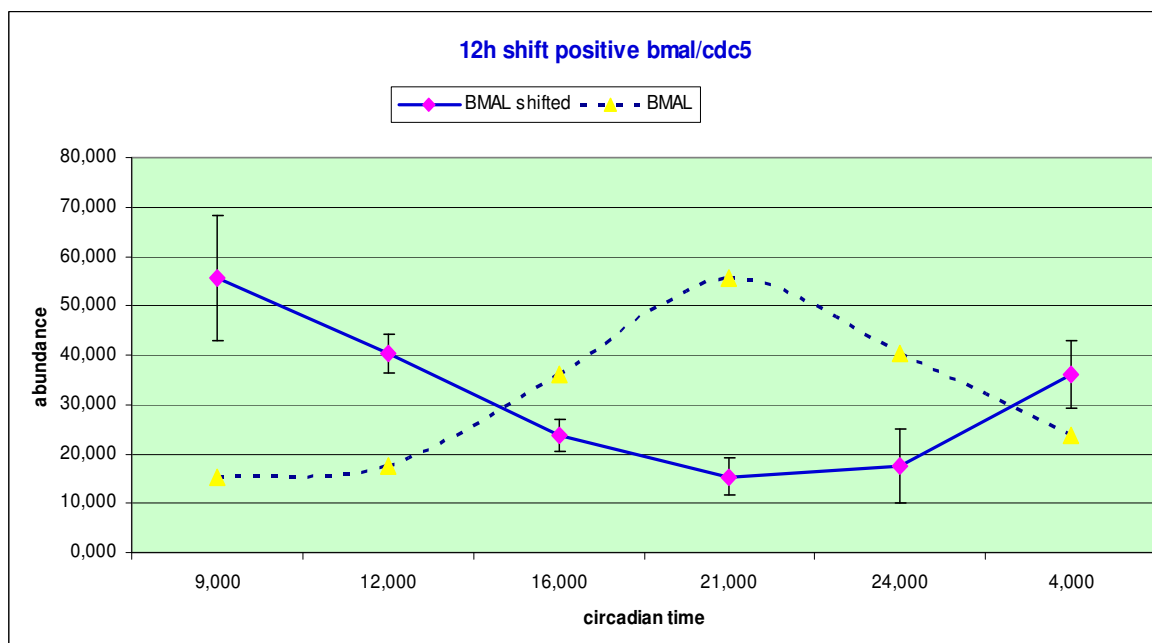


Figure 25 circadian relative expression levels of *Pdu bmal* before and after shifting

The dotted blue line corresponds to the relative abundance mRNA before the shift of the positive control (n=1). The continuous line corresponds to relative expression from shifted positive control (n=3). Error bars are +/- SEM.

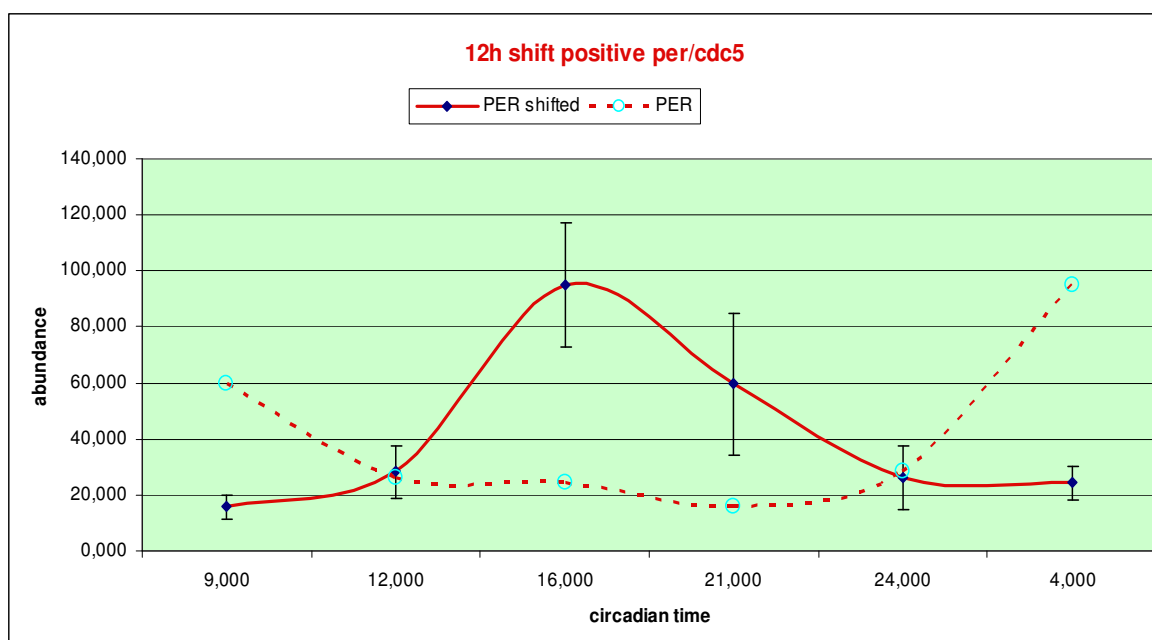


Figure 26 circadian relative expression levels of *Pdu period* before and after shifting

The dotted red line corresponds to abundance of period transcripts before the shift (n=1). The continuous red line corresponds to *period* expression in the shifted positive control group (n=3). Error bars are +/- SEM.

6.1.6.11 Test group

The expression of *Pdu period* and *bmal* was measured over 24 hours in the 12 hours shifted, blinded animals. A maximum in period abundance was measured around 4pm and a minimum between 4am and 9am. The levels of *bmal* had their peak at about 9am and reached a minimum around 9pm.

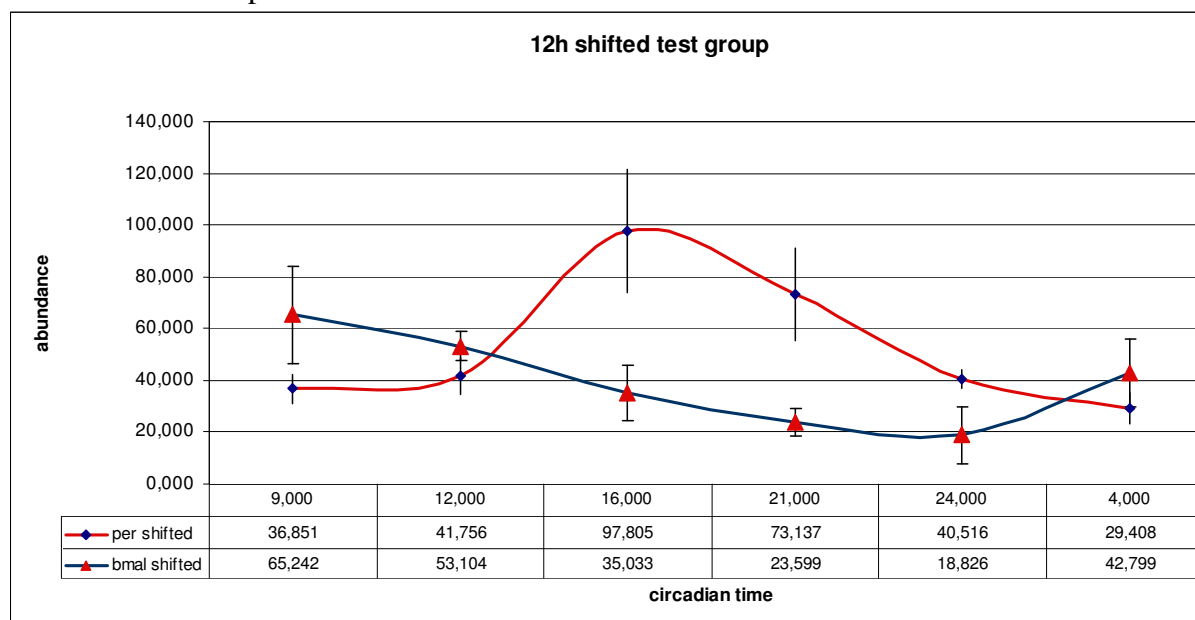


Figure 27 Circadian relative expression of *Pdu period* and *Pdu bmal* in 12 hours shifted test group.

Pdu period transcript abundance over 24 hours is coloured in red. Numbers in the row of the “PER” chart legend correspond to relative expression.

Pdu bmal transcript abundance over 24 hours is coloured in blue. Numbers in the row of the “BMAL” chart legend correspond to relative expression. Error bars are +/- SEM.

6.1.7 Rhabdomeric opsin expression

Circadian expression of *Pdu r-opsin* in positive control (Figure 29) and test group (Figure 28) was measured.

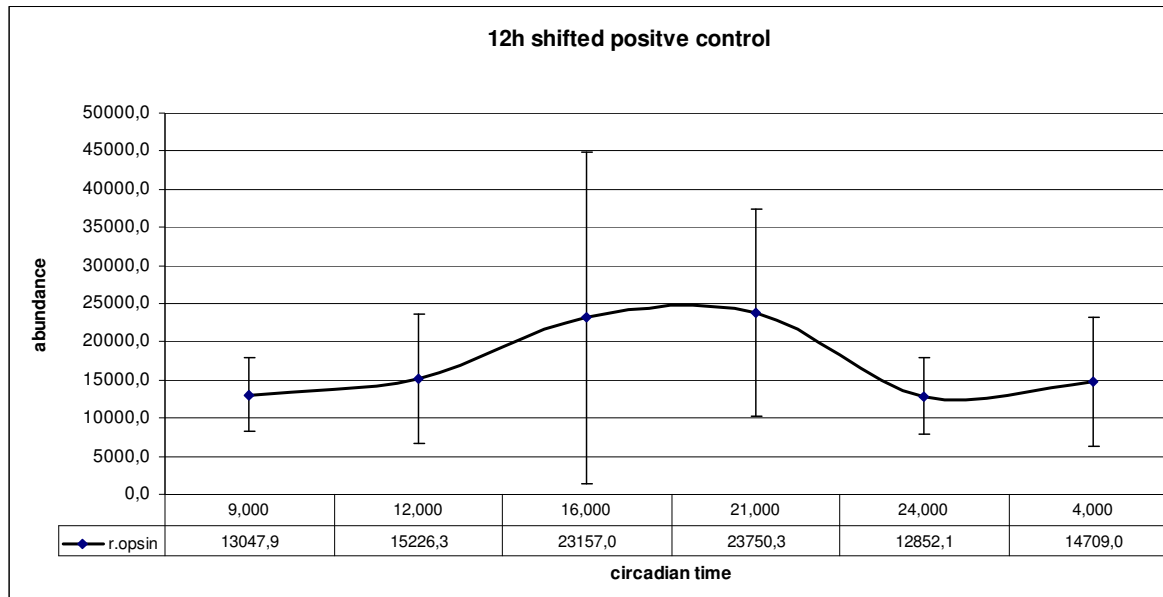


Figure 29 Circadian expression of *Pdu r-opsin* in 12 hours shifted positive control.

Pdu r-opsin levels throughout 24 hours after a 72 hours entrainment to an inverted LD cycle represented by the continuous black line. Error bars are +/- SEM.

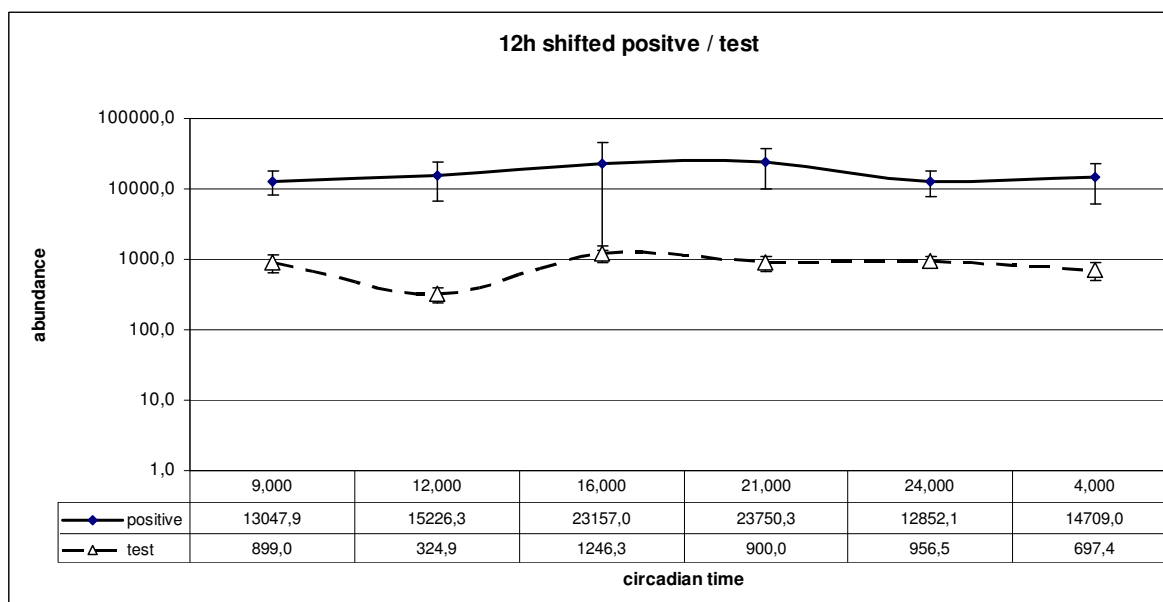


Figure 28 Circadian expression of *Pdu r-opsin* in 12 hours shifted test group and positive control.

Expression of *Pdu r-opsin* in shifted test group is represented by the dashed line whereas the continuous line represents the shifted positive control group. Error bars are +/- SEM. The ordinate is scaled logarithmic.

Expression of *Pdu r-opsin* had no obvious peak or minimum throughout 24 hours in the positive control. The test group showed no maximum or minimum in *Pdu r-opsin* expression (Figure 28). As the graph (Figure 28) indicates *Pdu r-opsin* levels in ablated animals were significantly reduced compared to untreated animals ($P = 0,00025$). *Pdu r-opsin* was highly different among biological replicates (Figure 30).

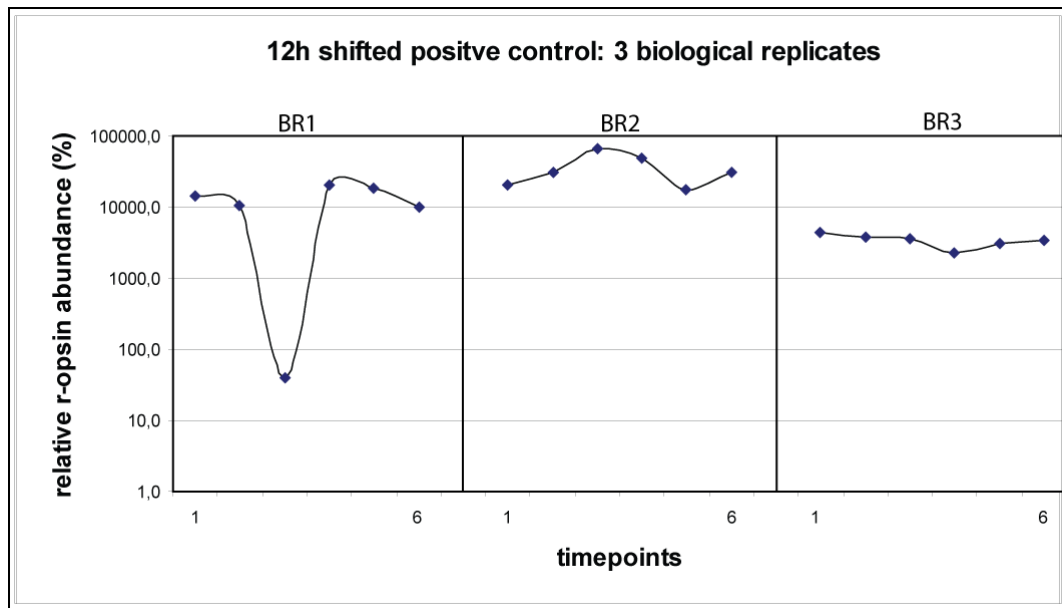


Figure 30 *Pdu r-opsin* expression from 3 single biological replicates in 12 hour shifted positive control.

6.2 Labelling of ciliary photoreceptor cells

To identify ciliary PRCs and to create a reliable detection method labelling of those cells via fluorescence is the aim of these experiment.

In the case of *Pdu c-opsin* its enhancer region was intended to be identified via phylogenetic footprinting[129, 130]. A fluorescent marker gene under the control of the *Pdu c-opsin 1* and *c-opsin 2* specific enhancer was the intended final result. My project was to obtain the orthologue upstream sequence of both from *Nereis virens*, a nereidid.

6.2.1 Cloning

Degenerated upstream primer “annelidCopsinU3” and downstream primer “annelidCopsinL1” yielded two 416bps long fragments (working name “7A 3” and “4c B1”). They proofed to be different in restriction fragment length polymorphisms (Figure 31) and thus were sequenced. Initially three clones exhibited a different pattern but only two of them were positive sequenced for *Pdu c-opsin*.

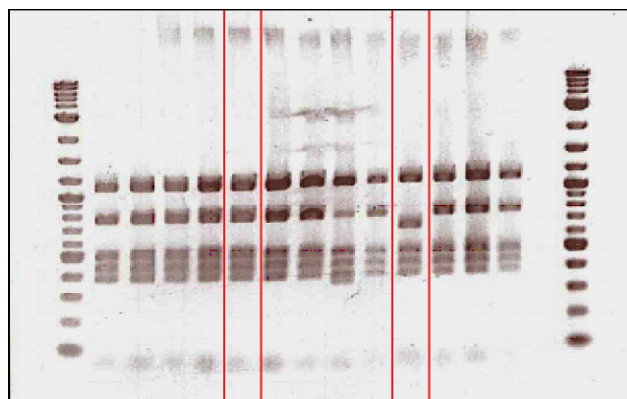


Figure 31 *Hinfi* digestion of *c-opsin* clones

Red boxes enframe both U3L1 amplicons with different restriction pattern. In fact three different restriction patterns were detected.

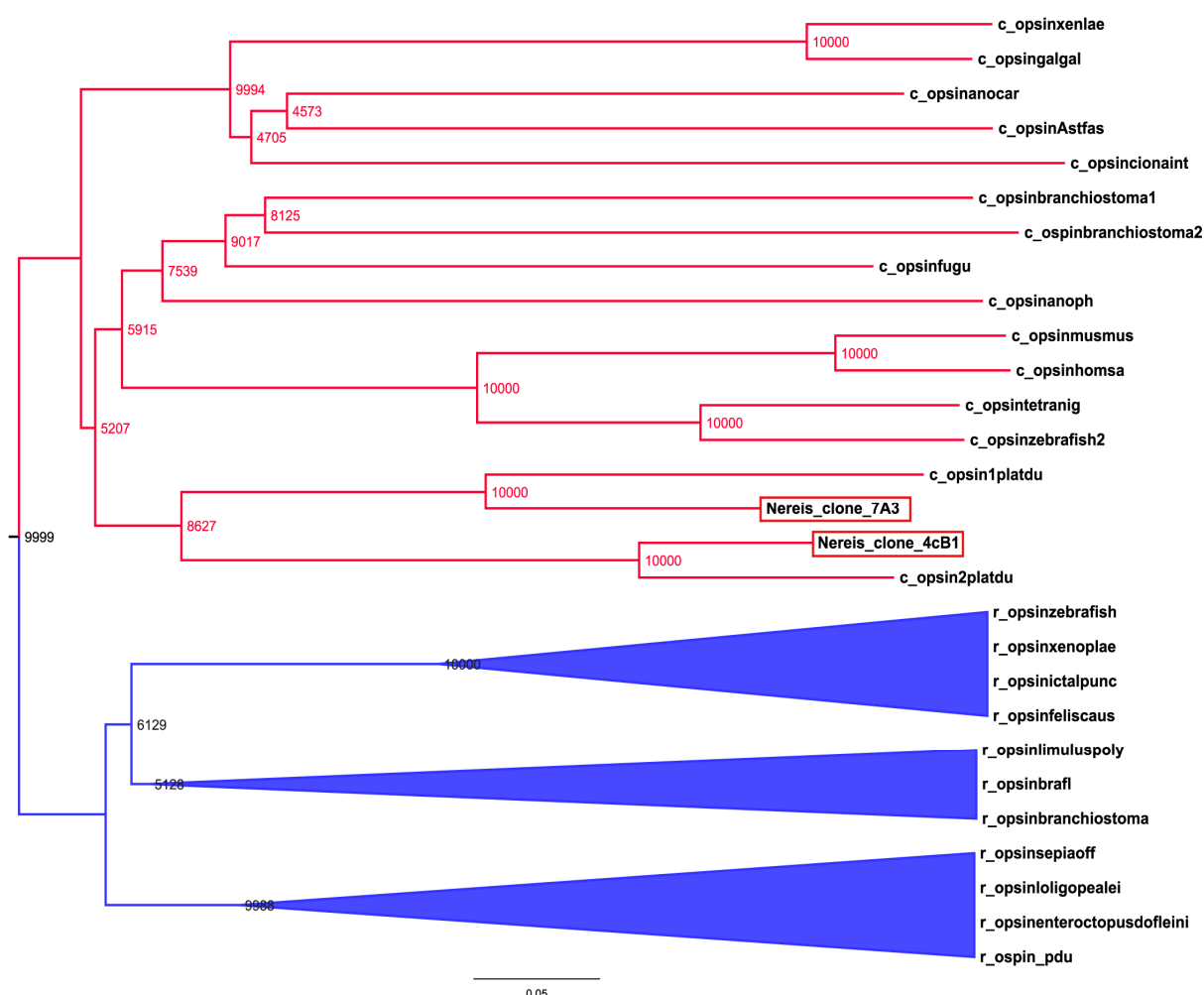


Figure 32 phylogenetic tree (NJ) of orthologue c-opsins and r-opsin

Alignment based on the aa sequences, tree generation via neighbour joining method. r-opsin in blue, c-opsin in red colour. Numbers at branches are bootstrap values. Cloned *Nereis virens* c-opsins (in red frame) cluster together with its *Platynereis dumerilii* orthologues.

The obtained sequences for my clones were translated into primary sequence and aligned with orthologues based on their amino acid sequence.

First of all the phylogenetic tree (Figure 32) shows a major division into two *opsin* orthologies – R-OPSINs and C-OPSINs. My two C-OPSINs clones in the phylogenetic tree locate to the same branch as the C-OPSIN orthologues 1 and 2 from *Platynereis*'. More specific, clone "7A3" clusters with *Pdu* C-OPSIN1 and clone "4cB1" clusters together with *Pdu* C-OPSIN 2. Both lophotrochozoan C-OPSINs, from *Nereis virens* and from *Platynereis dumerilii*, cluster together with chordate C-OPSINs. Therefore the restriction pattern showed a difference and the location of the clones in the phylogenetic tree also showed a difference. The next step in the workflow comprised hybridisation of radiolabeled *Nereis virens* c-opsin probes with a *Nereis virens* BAC library to identified *N. virens* c-opsin containing clones. Due to supplier-related problems with the ordered BAC filters no positive clones could be obtained during my attendance.

6.3 Possible output-targets of the circadian and lunar clock

6.3.1 AANAT

6.3.1.12 CLONING

Upstream primer “aan U2” and downstream primer “aan L2” yielded a 273 bps long fragment. To extend the gene fragment 5’ and 3’ Races were performed. 3’RACE yielded 627bps- 660bps fragments with counterprimer “aan 3’Race In” and “NUP”. 5’ Race yielded 517bps - 527bps fragments with counter primer “aan 5’Race Out” and “NUP” Both 5’ and 3’ RACEs together gave an assembled contig of 933 bps length.

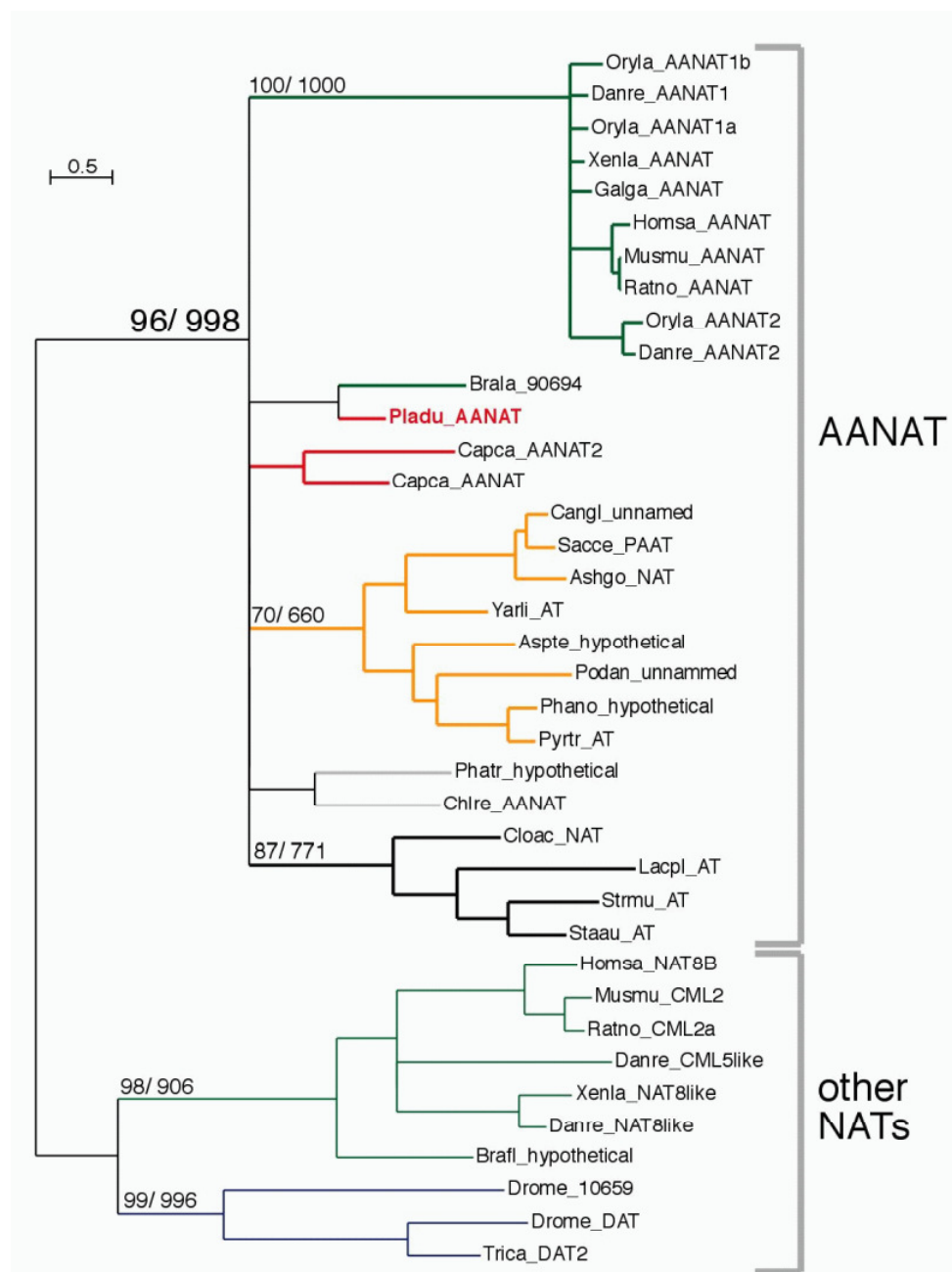


Figure 33 Phylogenetic tree of AANATs using aa sequences (adopted from Zantke, J., unpublished). Numbers at nodes are bootstrap values. Red branches are lophotrochozoan species. *Pdu* AANAT is enframed in red box. Insects AANATS were selected as an outgroup, because they are dopamine N-acetyltransferases. Lophotrochozoan branches are in red.. See text for details.

Pdu AANAT (Figure 33) clusters together with *Branchiostoma floridae*, which is at the base of the chordates and near *Capitella telata*, which is another annelid. Importantly it does not cluster to the insect orthologues, which are so called dopamine N-acyltransferases (DATs). Two lophotrochozoan AANATs cluster in vicinity to a true AANAT from a cephalochordate. Fungal, vertebrate and lophotrochozoan AANATS cluster apart from bacteria. Insects are an own group of AANATs or rather so called DATs, therefore they comprise an outgroup together with other NATs.

6.3.1.13 EXPRESSION

Working RNA antisense probes have been transcribed from templates derived from both, 5' RACE product (517bps) and 3' RACE product (660bps).

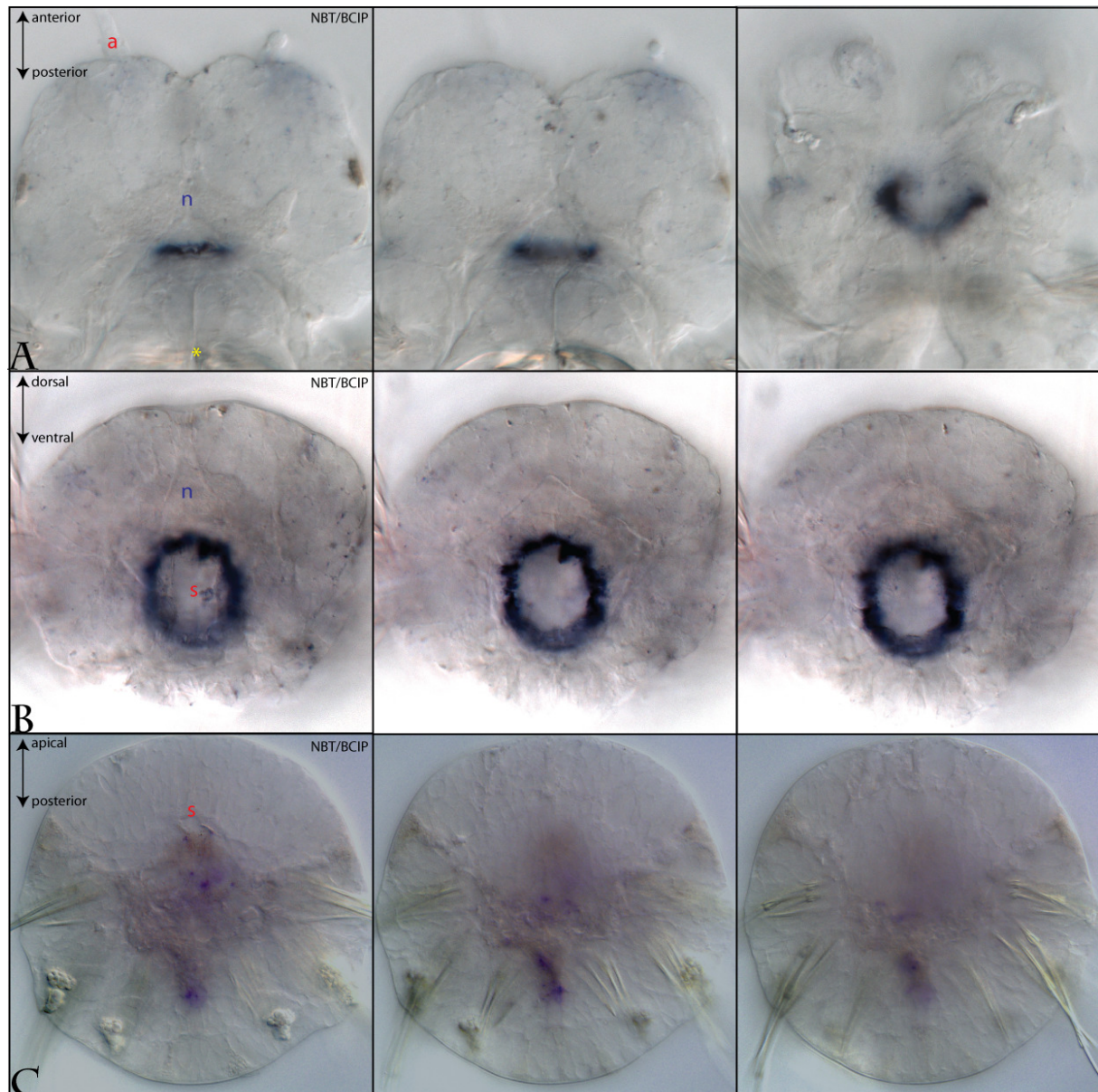


Figure 34 WMISH against *aanat* in 14 dpf and 60hpf larvae . Worms fixed at 9pm.

anterior “a”, posterior “p”, dorsal “d”, ventral “v”.

A) Dorsal views on worm. Focussing from dorsal to ventral planes, from left to right.

B) Apical views on worm. Focussing along anterior-posterior axis, from left to right.

C) Ventral view on Metatrochophora.. Focussing from ventral to dorsal from left to right. For details see text.

A= antenna, n= neuropil, s= stomodaeum, yellow asterisk indicates position of jaws

Pdu aanat expression (Figure 34 A, B) in 14dpf old worms was found in the peristomal region and restricted to a circle like structure around the stomodaeum. The stained structure is continuous and oval in an orthogonal view. At the medial posterior border of the brain the staining starts dorsally and then shifts ventrally and more posterior, to the left and the right of the stomodaeum, encircling it. The dorsal curvature of the staining is at the ventral border of the cerebral ganglion, the ventral curvature is just more ventrally to the mouth opening. This circum-oral expression is located anterior to the pharyngeal cleft and just adjacent to the stomodaeum.

In 60hpf larvae expression domains are found in a peristomal region as well as in parts of metastomium in the neurogenic region posterior to the stomodaeum.

6.3.2 HIOMT

6.3.2.14 CLONING

Upstream primer “hiomt up2 new” and downstream primer “Pdu hiomt lo3” gave a 396bp long fragment. RACE in both directions were carried out. Counter primer “hiomt 5'race In” together with NUP gave positive fragments of 1300bp length. 3'RACE with primer “hiomt 3'race Out” gave 390 and 395bp long fragments.

Reamplified 1,3 kb fragment was obtained from cDNA with “hiomt up1 new” and “hiomt low1 new” primer pair.

6.3.2.15 EXPRESSION

Interestingly *hiomt* gene fragments obtained from both RACEs were different compared to assembled contig from ESTs of *Platynereis*' transcriptome (Figure 35). The difference was restricted to the N-Terminus, due to extended sequence information on the 5' end. A HIS rich region could be identified at the N-Terminus in my RACE clones: 48-136 aa:

H₂N---

HRKRHDHSGNHHKEGHHHGHGHHHHNRHHHEPPLSPTRKLNIEEEEPHFAQQAHG
HPSHLHGHLSPHKHGHGHHHGHGHPGLIDSHALH---COOH

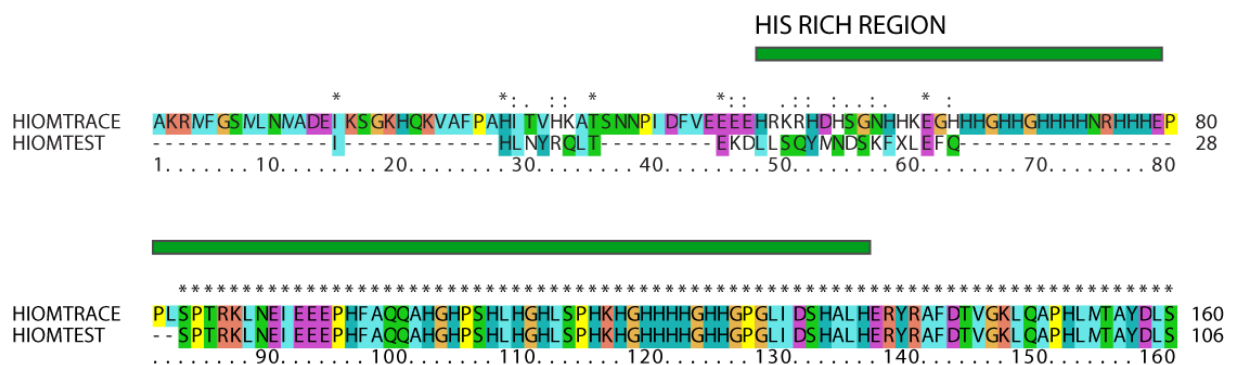


Figure 35 Alignment of N-termini of translated hiomt-sequences.

HIOMTRACE termed primary sequence corresponds to translated 5' end, nucleotide sequence derived from RACE.

HIOMTEST termed primary sequence corresponds to translated 5' end, nucleotide sequence from ESTs.

But it has to be mentioned that other *Pdu hiomt* clones did not exhibit this HIS rich region encoded by the 5' end (Figure 36).

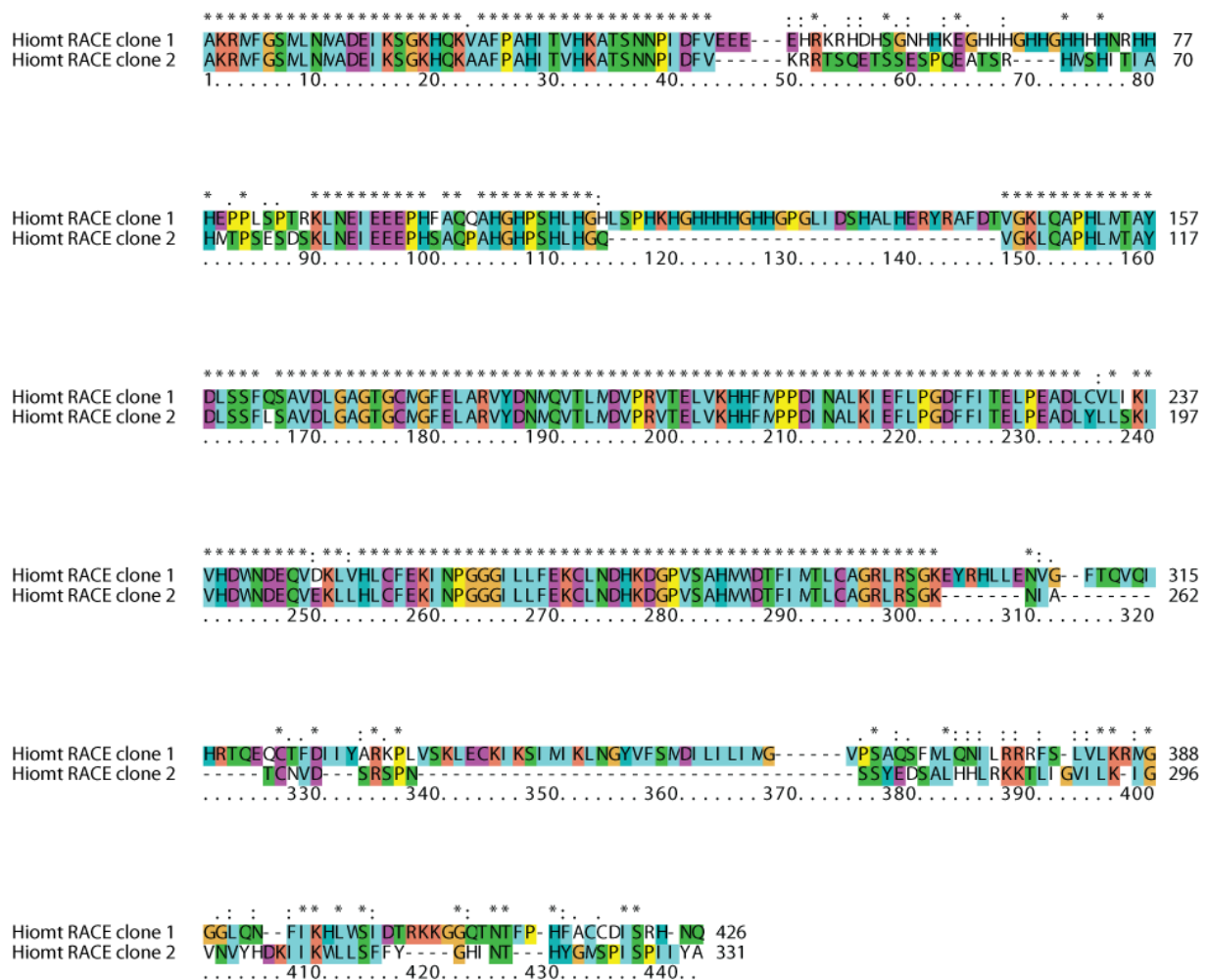


Figure 36 Alignment of two different Hiomt RACE clones.

Primary sequences are derived from translated RACE sequences. The HIS-rich region was not found consistently in all clones. Hiomt RACE clone 1 exhibits an N-terminal HIS-stretch that cannot be found in the other clone, HIOMT RACE clone 2.

I translated the contig of my cloned *hiomt* gene fragments from RACEs and looked into the phylogenetic relationship (Figure 37). The *Pdu* HIOMT clusters together with the orthologue from *Lottia gigantea*, a gastropode. The orthologues from the annelids *Capitella capitata* and *Helobdella robusta* also cluster together. These four HIOMT orthologues in turn, as members of the lophotrochozoa, cluster at the base of the chordates. Bacterial and fungal representatives of HIOMT cluster apart into two branches.

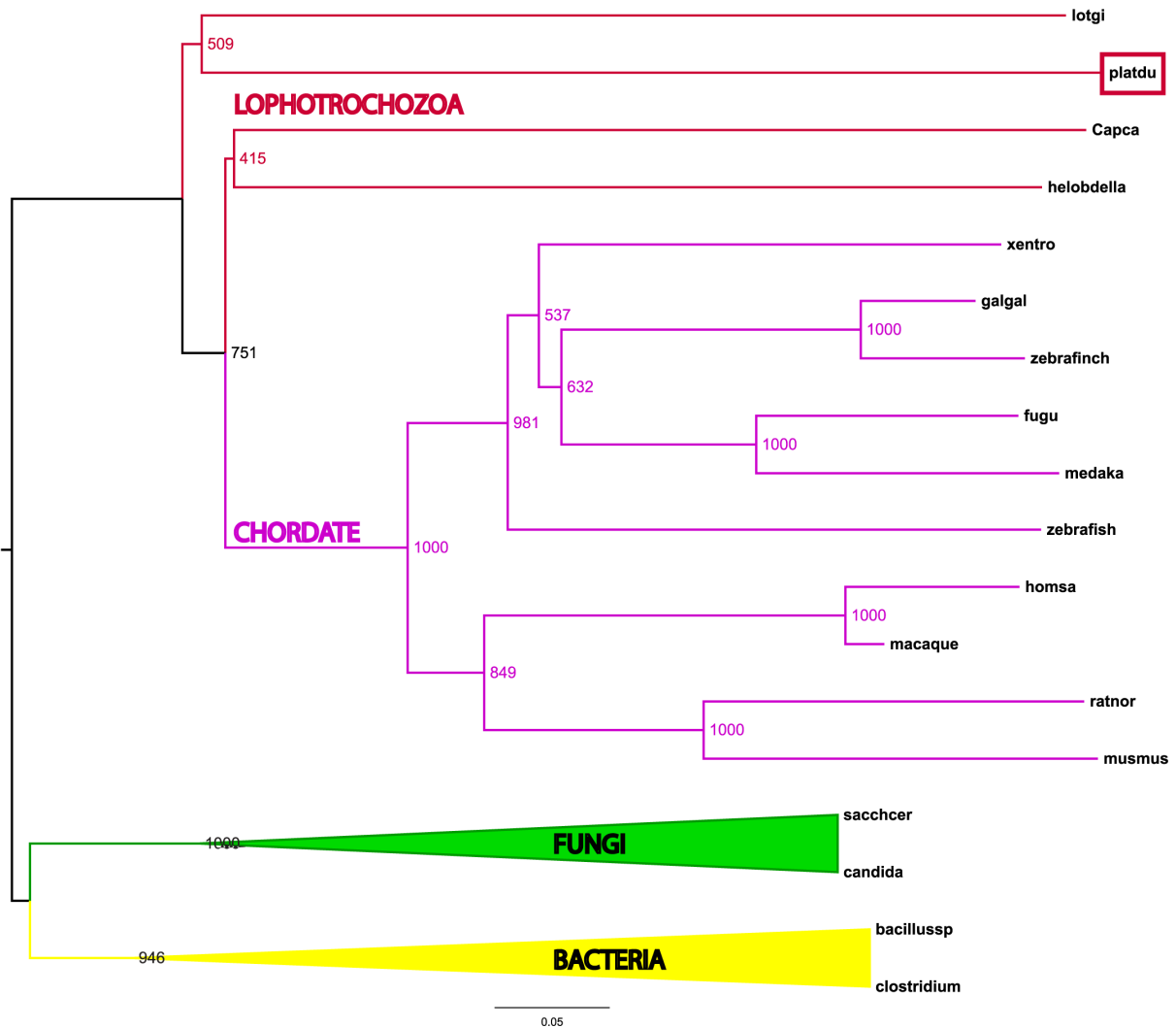


Figure 37 Phylogenetic tree for *Pdu hiomt*.

Alignment is based on aa sequences and tree is generated with NJ algorithm. Numbers at branches are bootstrap values. *Pdu hiomt* is enframed with a red box. For details see text.

From reamplified *hiomt* contig I transcribed an antisense riboprobe of approximately 1,2 kb length and looked into the cytological expression pattern (Figure 38). A robust staining was found in peristomal region or just on the posterior border of the prostomium. The expression pattern of *hiomt* in the 14dpf old worm follows the same anatomical landmarks like the *aanat* expression. The dorsal end of the circum-oral structure seems only weakly joined but still continuous. Dorsally it starts medially on the posterior border of the cerebral ganglia and dorsally from the stomodaeum. The structure projects to its ventral and more posterior curvature, lateral symmetrically encircling the mouth opening. Therefore the ventral part of the structure is more posterior but ventrally adjacent to the stomodaeum. The whole circle like structure is just anterior to the pharyngeal cleft and very close to the stomodaeum.

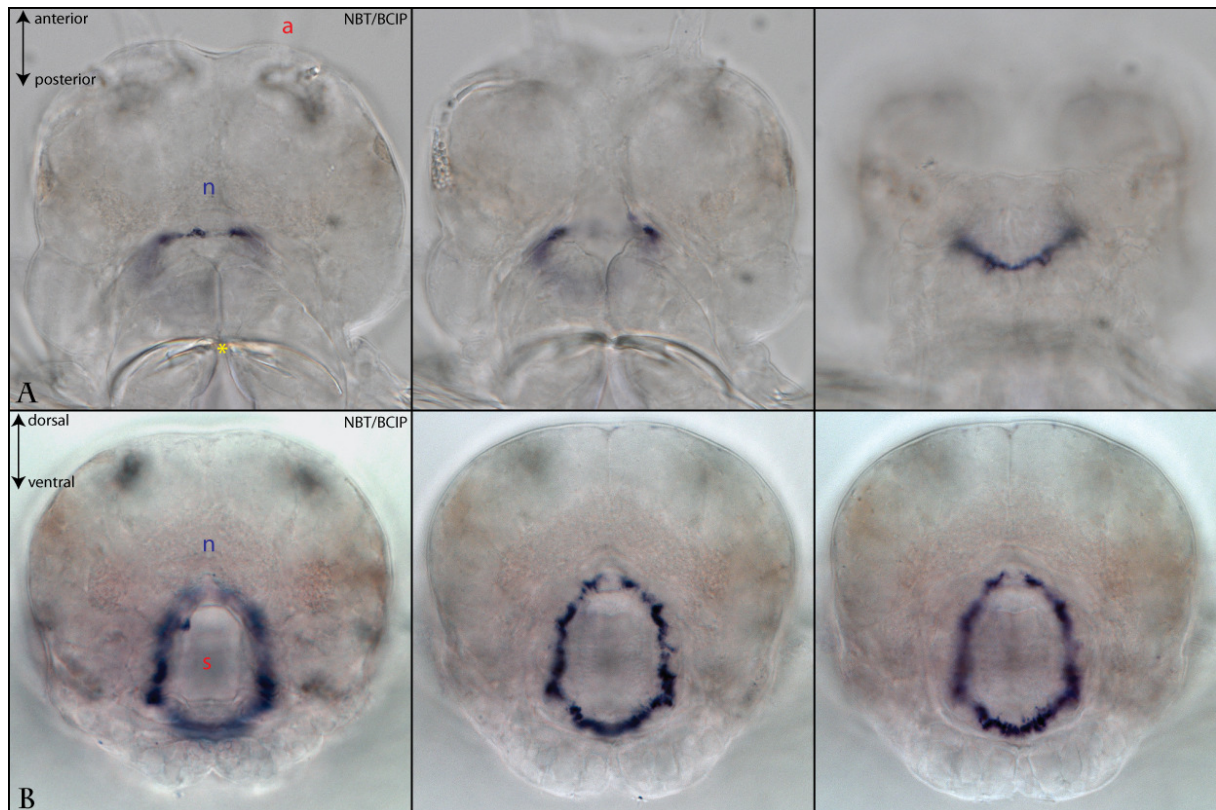


Figure 38 WMISH against hiont in 14dpf.

anterior “a”, posterior “p”, dorsal “d”, ventral “v”

A) Dorsal view on prostomium, focussing from dorsal to ventral (from left to right).

B) Apical view on prostomium, focussing from apical to posterior (from left to right).

See text for details. Yellow asterisk indicates position of the jaw. a= antenna, n= neuropil, s= stomodaeum

6.3.3 HIOMT LIKE

6.3.3.16 CLONING

Upstream primer “Pdu hiomtlikeup3” and downstream primer “Pdu hiomtlikelo2” gave an amplicon of 746bp length (assembled contig 756bp) corresponding to an N-terminal gene fragment of presumably *hiomt like*.

6.3.3.17 EXPRESSION

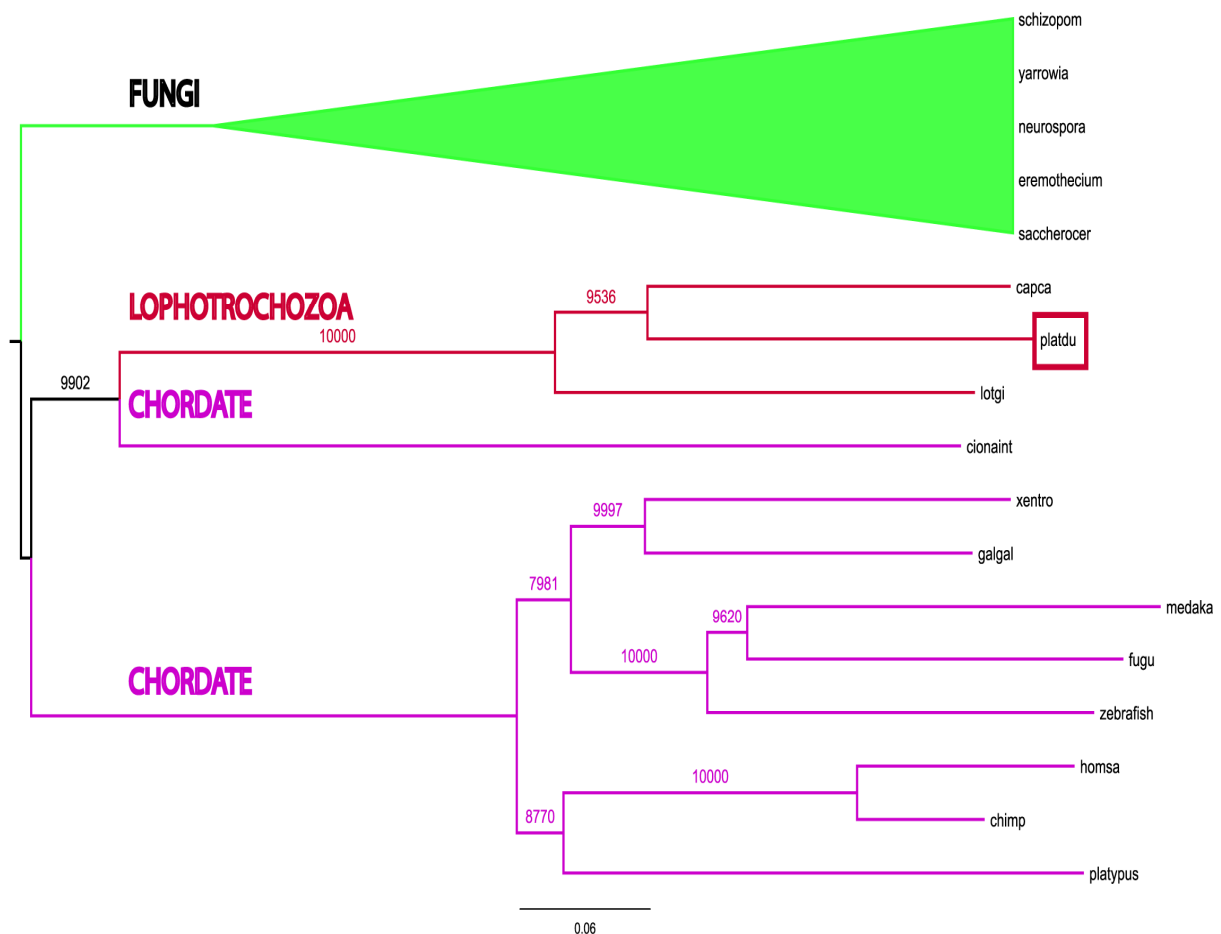


Figure 39 phylogenetic tree of hiomt like orthologues.

Numbers are bootstrap values. Alignment is based on aa sequences. Tree is generated using NJ algorithm. *Pdu* HIOMT LIKE is marked by red frame; it clusters together with orthologues of the same phylum.

I translated my cloned *hiomt like* sequences and compared representative orthologues in phylogenetic relationships with my clones (Figure 39). The *Platynereis* protein clusters together with orthologue from *Capitella capitata*, another annelid. With a gastropode HIOMTL at their base they form a lophotrochozoan branch which clusters together with an urochordate, which branch from higher chordates. Fungal HIOMTL branch apart from the other.

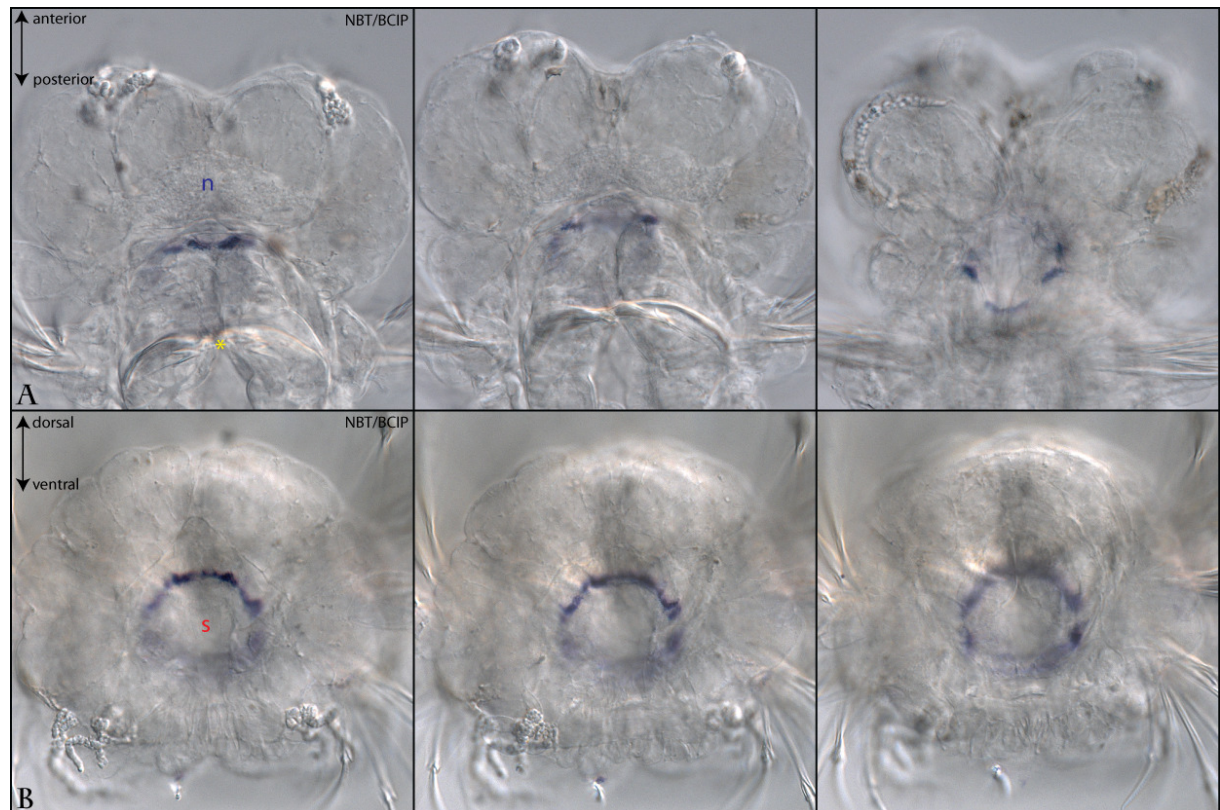


Figure 40 WMISH of *hioMt like* in 9dpf worms.

A) dorsal view on prostomium. NBT/BCIP staining specific for *hioMt like* expression in the region around the stomodaeum. Focussing from dorsal to ventral, from left to right.

B) Apical view. Focussing from anterior to posterior, from left to right.

See text for details. Yellow asterisk indicates position of the jaw. n= neuropil, s= stomodaeum.

From my amplicons I transcribed an antisense riboprobe of approximately 750bp length and had a look into expression of *hioMt like* (Figure 40). The staining in the WMISH showed a circum-oral structure in 9dpf old worms. The structure seems to be discontinuous especially at the ventral curvature. Dorsal and ventral curvatures of the structure are only weakly joined lateral from the esophagus. Peristomal, between the posterior border of the cerebral ganglion and the anterior end of the pharynx, cells are stained and form the dorsal curvature of the structure. The structure then bilaterally projects around the stomodaeum to the ventral curvature, which is more posterior. The structure's ventral curvature ends next to the ventral bow of the stomodaeum.

6.3.4 PDF

6.3.4.18 CLONING

Upstream primers “Pdu pdfup1” and “Pdu pdfup2” with downstream primer “Pdu pdflo2” gave a gene fragment of 567bps or 540bps length respectively.

6.3.4.19 EXPRESSION

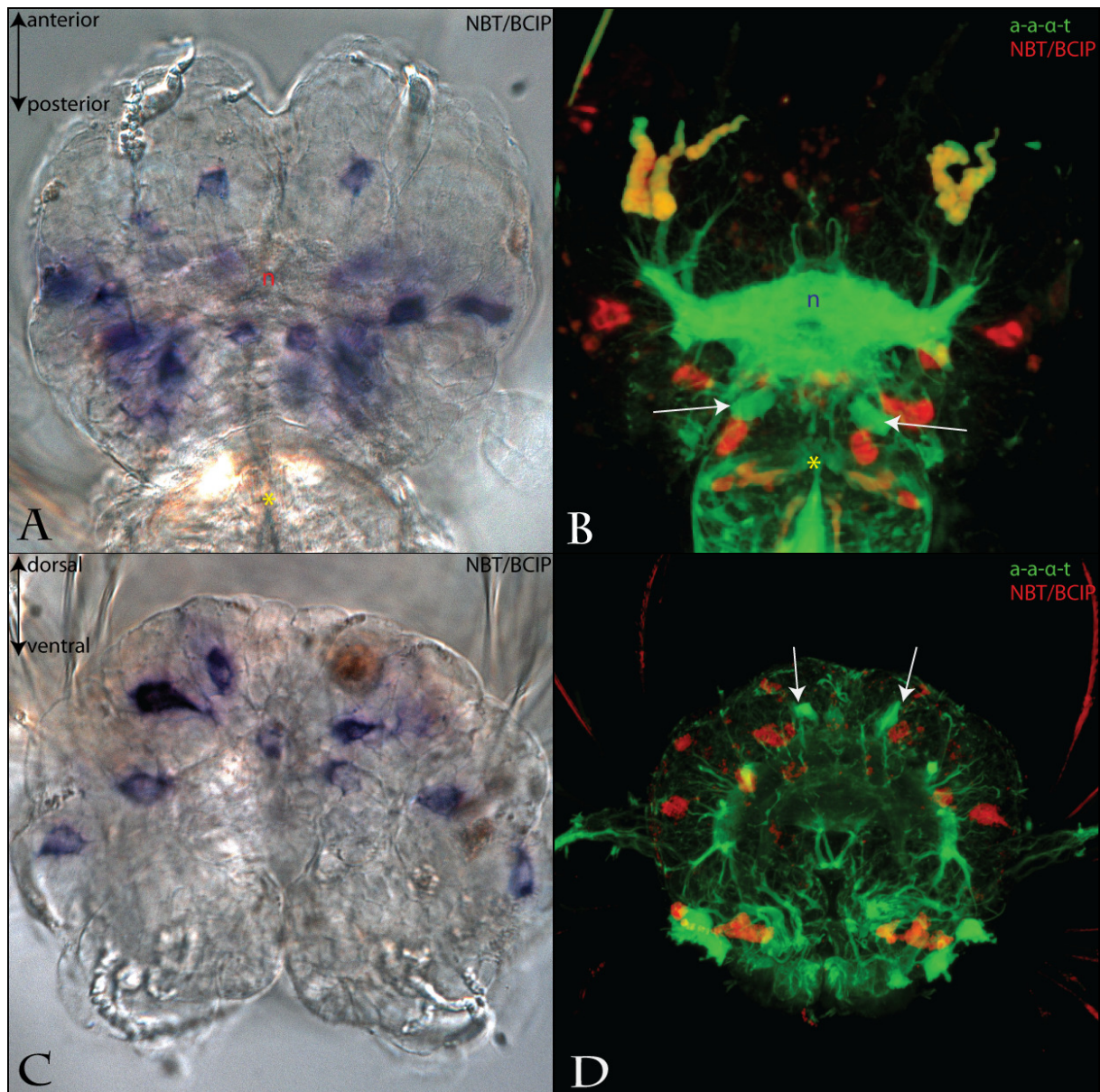


Figure 41 WMISH with *pdf* in 14dpf worms.

anterior “a”, posterior “p”, dorsal “d”, ventral “v”

A) Apical view on worms shows bilateral symmetrical expression of *pdf*.

B) Dorsal View on worm shows the paired *pdf*-expressing cluster in the prostomium .

C,D) z-projections of confocal scans. Green colour corresponds to anti-acetylated α -tubulin marking axons. Red colour derives from either specific NBT/BCIP reflection marking location of *pdf*-transcripts, or unspecific reflections from jaws, chaetae and antennae. C) apical view on specimen D) dorsal view on specimen. White arrows point to the ciliary PRCs in the brain of *Platynereis dumerilii*. Yellow asterisk indicates position of the jaw. n= neuropil

My cloned gene fragments enabled the transcription of a antisense riboprobe of 550 bp length. Staining of *Pdu pdf* in the 14dpf old worm revealed bilateral symmetric subsets of cells in the prostomium (Figure 41). At least five *pdf* expressing paired cluster can be identified in the apical view (Figure 41 A). From a dorsal view (Figure 41 B) on the prostomium at least 6 paired subsets can be distinguished. Interestingly two pairs of *pdf* expression domains are located in the vicinity of ciliary, clock gene expressing PRCs (Figure 41 C, D). A first domain in the very anterior part of the prostomium, medial and posterior from the antennae is relatively prominent. A second peristomal domain at the posterior end of the cerebral ganglia, anterior to the pharynx and the most medial ones can be identified. Dorsolateral to this domain another one can be distinguished. Lateral from the most medial ones is a forth cluster, which in turn lies in the same coronal plane as the fifth and sixth cluster. The fifth and sixth domains are more lateral and ventral. The sixth cluster is the most lateral one.

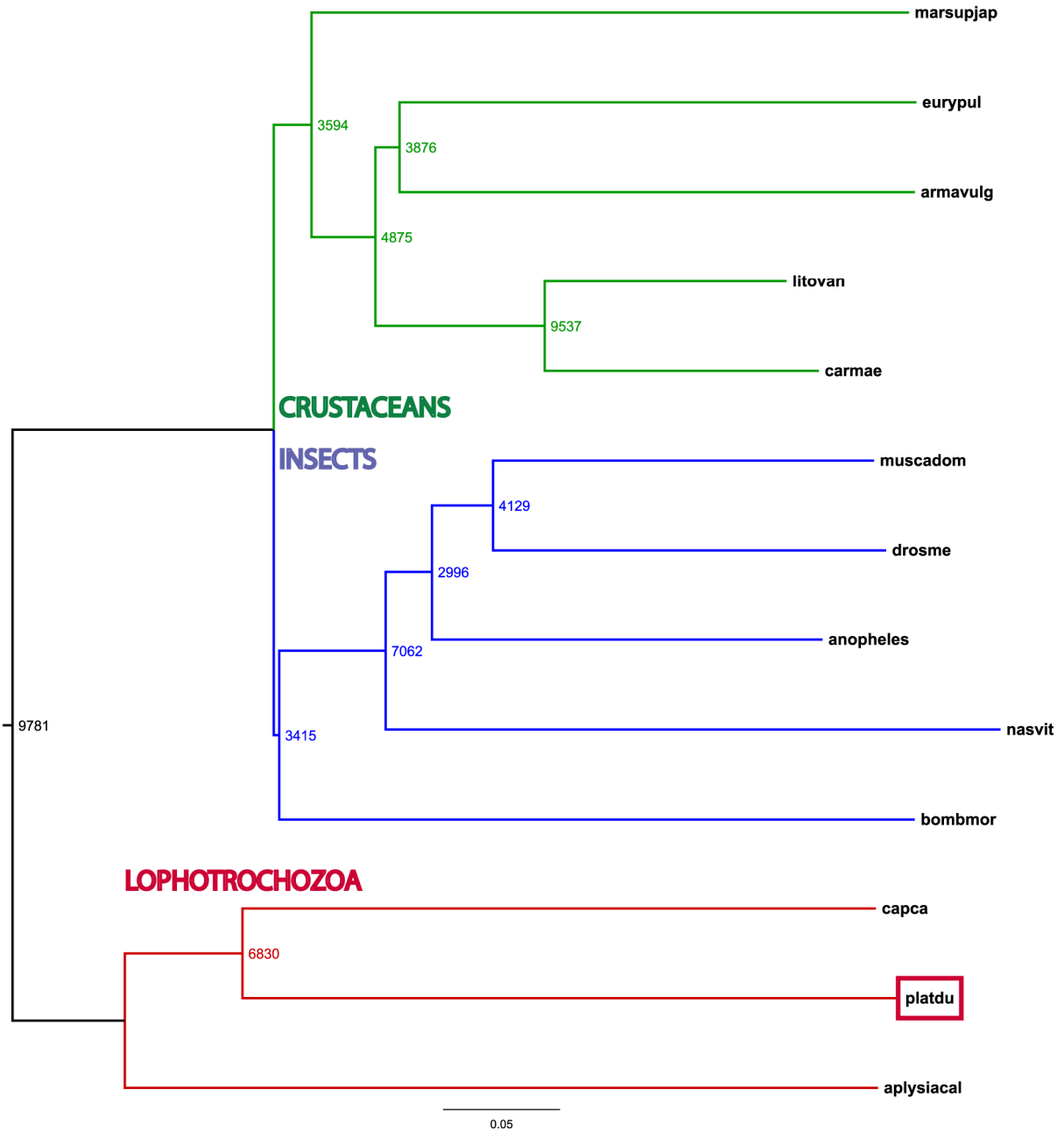


Figure 42 Phylogenetic tree of PDF orthologues

Alignment is based on the primary sequence. Tree is generated using NJ algorithm. Numbers at branches are bootstrap values. Pdu PDF is enframed with red box. See text for details.

Relationships of *Pdu* PDF with orthologues from representative species were tested with a phylogenetic tree (Figure 42). *Platynereis* PDF clusters together with an orthologue from *Capitella capitata*, another annelid. Both in turn branch together with *Aplysia californica*, a gastropod. These three belong to the lophotrochozoan group and branch apart from insects and crustaceans. Insects and crustaceans in turn cluster together but branch off.

7 Discussion

In this section I start off by revising earlier studies with my own experimental data. Then I will point out new findings I obtained during my experiment and additional considerations. Possible implications of my findings and an outlook will constitute the end. The conclusion sums up the general trend of all findings.

7.1 *Role of cerebral adult eyes in circadian rhythm*

I tried initially to uncover the adult eyes' involvement in entrainment of endogenous lunar- as well as circadian clock by up to date methods. Addressing the effect on lunar rhythm was exempted due to issues with regeneration. My inquiry about their involvement in the circadian clock of *Platynereis dumerilii* has not been carried out so far. The technique for a molecular read out and the discovery of circadian clock gene expression in *Platynereis* made this possible.

In a very general sense I affirmed the results C. Hauenschild obtained in his study [114]. Although I tested the entrainment of the circadian clock and did not cover the lunar light driven photoperiodicity. I never assessed the spawning of treated animals. The approach however was very similar – production of artificial blind animals that were tested for adaptation of their endogenous cycle to a new exogenous rhythm. Like Hauenschild I reliably targeted adult cerebral eyes. I could also shed some light on contradictory issues underlying the former and my own experiment. The fact that I made the same observations but had the advantage of up to date methodology enabled my study to have a closer look on the principles. Reported issues were the problem of regenerating eyes and the need for subsequent ablation trials, which I could affirm during my experiment. I oriented the time course of my subsequent ablations by that of Hauenschild who blinded the animals in a weekly interval starting off with 3 month old worms. A striking difference is the very high mortality rate in my experiments (70% after two ablations), which was kept increasing by each additional ablation trial. In the mentioned study animals are reported to be effectively ablated up to 28 times (mortality probability 38%). Comparing the setup I propose that in my experiment damage was delivered more precisely and more reproducible to the target. The cause for this discrepancy in mortality is something that is still open to question. But incubation in filtered artificial sea water and omitting contamination by algae administration improved survival. While comparison of short and long duration of the ablation procedure itself showed that duration time had no impact on mortality, but the number of trials did have an effect. An improvement in survival might be achievable by adding antibiotics to the water containing treated worms to prevent bacterial infection. An usual measure in *Platynereis dumerilii* transplantation experiments[131]. In contrast to the earlier study I could show that I really targeted the adult rhabdomeric eyes by means of WMISH. Thereby I also could show that actually two ablation trials are sufficient to abolish *Pdu r-opsin* expression in 14 dpf to 1 month old worms. This condition is then at least stable for one week after the surgery in 1 month old specimens.

This difference is a major issue in the experiment of Hauenschild. He lacked a tool to verify the absence of the cerebral rhabdomeric eyes. Although the challenge posed by regeneration of what is maybe the eye was recognized, he had no control besides the bare eye whether or not the tested subject possesses functional eyes. Also regenerating ectopic pigment spots or eyes are hard to spot under the light microscope. After all phototransduction is a signalling cascade using second messengers (rhabdomeric PRCs: DAG) that greatly amplify the initial signal [15]. The enlarged membrane surface of PRCs employs vast amounts of photopigment. Therefore it is essential for the experiment that not a single light sensory cell persists in test

subjects. With regard to these limits the results from the early study are doubtful in their result and interpretation. I made some improvements in the experimental setup and the quality control concerning the results in that I used WMISH to look for residual *Pdu r-opsin*. Yet I could not demonstrate that absence of rhabdomic PRCs at the adult eyes' location is valid for 3 month old worms as it is valid for 1 month old. The data from 3 month old worms though was even more critical for the experiment as they comprise the actual test group. This means I was able to establish the procedure for the 3 month old worms but could not verify the success. After all a WMISH for *Pdu r-opsin* that should generate the needed data failed due to technical issues. The important result would be the *Pdu r-opsin* loss specifically at the site of the adult eyes. On the other hand I could show the transient loss in photopigment in 14dpf to 1 month old worms specifically at the adult cerebral eyes by means of UV ablation. I assessed the question of *Pdu r-opsin* loss by measuring relative transcription levels of *Pdu r-opsin* in the heads of the test group and control via qPCR. It has to be noted that the measured *Pdu r-opsin* levels showed no obvious trend or oscillation throughout 24 hours, which is due to the fact that eyes amongst animals are of different size. Especially when comparing the three individual biological replicates (Figure 30) for *Pdu r-opsin* expression no resemblance or common pattern/oscillation can be obtained. Eyes are growing mitotically throughout the atoke life of *Platynereis dumerilii*, and although from the same age they can show developmental dispersion. Consequently the *Pdu r-opsin* levels resulting from the anterior ocelli contribute to the total amount. It also has to be pointed out that these two different methods – qPCR and WMISH – are also differently sensitive and vary in the limit for detection. Theoretical LOD in qPCR [132] is 8-12 transcript copies at a CT value of 38. The reported LOD for hybridisation with DIG labelled probe is one single copy among 1µg DNA in southern blot. The sensitivity of a DIG labelled RNA probe in a WMISH can only be measured empirically. It is largely depending on the length of the probe influencing the amount of incorporated DIG labelled nucleic acids, the applied WMISH procedure and duration of the enzymatically driven staining reaction. Therefore the LOD of WMISH in *Platynereis dumerilii* can only be estimated and might be less sensitive than qPCR. Therefore the comparability between my two tested stages is impaired by using different approaches. To really have comparable data qPCR measurements with identical thresholds have to be carried out. In the case of a WMISH identical conditions are difficult to obtain. But given the fact that both are very sensitive methods the data remains trustworthy.

Also the general trend of my data rather supports the notion that adult cerebral eyes are not necessary for entrainment of rhythmicity, but circadian in contrast to lunar. Despite given the fact that circadian clock genes might not be elements of the lunar clock (Zantke, J., unpublished) there may be need for crosstalk between both systems e.g. without the circadian oscillations no anticipation of the night would be possible, although the leaving of tubes and swimming behaviour could be triggered by darkness and not a result of circadian anticipation. It has been shown that the relative alteration between nocturnal light and nocturnal darkness is the key component to lunar photoperiodicity, not the overall duration of light exposure [112]. In constant light (LL) *Platynereis dumerilii* spawning becomes random, the same is true for constant darkness (DD). Therefore both systems have to measure the duration of light and dark phases during the day. This task is functional identically and could be conveyed by the very same sensory organ. Although the functional segregation of both systems may also affect their input stage, being the photoreceptive cells. Thus both would receive their signals via independent sensory cells. Neither of the two possibilities can be ruled out by my results. Or it can be like in *Drosophila* or mice in the case of the circadian clock, where other photosensors can complement the loss of another. In mammals rods, cones and ipRGCs all contribute to entrainment of the central pacemaker. The results of my circadian phase inversion first of all demonstrated that 72 hours entrainment is a long enough

stimulus to entrain the endogenous circadian clock of *Platynereis dumerilii*. Considering the fact that 12 hour delay/advance in a 24 hour cycle is the maximal period for a shift. Every hour more or less means a shorter shift period. Not surprisingly the light was the only needed factor to reset the endogenous circadian clock. All groups, positive, test and negative (data not shown) could adapt to the new cycle. Determination of clock genes' expression is ideal for a molecular read out of endogenous circadian rhythm, because there are no masking effects that can occur when tracking activity. In all biological replicates a robust and similar trend could be observed. Second with strong reservation the experiment revealed that adult eyes might be not required in the entrainment of the circadian clock. This is due to the simple fact that the test group could adapt to an inverted cycle. The expression profiles of *bmal* and *period* resembled the positive control, which in turn indicates alignment with the inverted circadian cycle. This means exogenous light stimulus is received via sensory components other than adult cerebral eyes. Yet this hypothesis can not be backed up by the measurement of residual rhabdomeric PRCs of the test group. The fact of *Pdu r-opsin* persistence in treated specimens has to be taken into account upon interpretation of the adult eyes' impact on circadian clock. And it is certainly a flaw in the argument disavowing involvement of cerebral adult eyes in circadian clock. The same underlying flaw is true for the earlier study with blinded worms. No control of actual blindness leaves the result from C. Hauenschild with reasonable doubt and the existence of anterior ocelli in *Platynereis dumerilii* was also not known back then.

7.2 Adult cerebral eyes are not the only rhabdomeric sensory cells in the prostomium

Although the abundance of *Pdu r-opsin* in ablated specimens 3 days after the procedure is significantly decreased compared to untreated, decreased *Pdu r-opsin* level is still contradictory to my hypothesis. But there is reason that aids my argumentation. The decreased *Pdu r-opsin* amounts in treated worms might result from residual sensory cells that I failed to destroy in the course of the two ablation trials. It has to be mentioned that the eyes of *Platynereis* are growing their complete lifespan.[14] . This obviously affects the amount of tissue to be destroyed as well as the regeneration capabilities. But it might be as well the regenerative capacity that achieves to regrow destroyed tissue within the 72 hours to a certain extent. Or it might be the fact that I failed to reliably target anterior ocelli and hence did not destroy them. As I was not able to proof their location and subsequent destruction in 3 month old worms this later fact seems very likely. If this is the case and all detected *Pdu r-opsin* originates from the mentioned rhabdomeric ocelli, it would mean adult cerebral eyes were depleted in PRCs and non-functional. Summarized the residual *Pdu r-opsin* levels in ablated specimens result either from remainders of adult eyes, from regeneration, or from AOs. If only the anterior ocelli comprised residual *Pdu r-opsin*, as consequence my hypothesis would be rendered valid, stating adult eyes are not required for the entrainment of the circadian clock. Complete non-involvement can not be assayed by this experimental setup, because it also might be that other PRCs have a compensatory action.

By means of qPCR data no evidence towards any of the suggested explanations can be gathered. This is why a subsequent WMISH against *Pdu r-opsin* would a necessity. When comparing relative expression levels of *Pdu r-opsin* transcripts in heads the abundance is really high and surpasses housekeeping genes like *cdc5*, marking mitotic cells. Even in the twice ablated population *Pdu r-opsin* levels were high. High amounts are not surprising after all OPSINS are incorporated in the enlarged apical cell surface, providing a large area for OPSIN distribution. As I used the Δ CT method to compare initial mRNA levels the character to describe the amount is the uncalibrated CT value, which derives from the cycle number the

fluorescent signal crosses the threshold. The threshold has to be well above baseline and ensure the reaction is in the exponential phase. If set wrongly it won't measure the abundance correctly. For different initial template amounts different thresholds have to be set, which in my experiments were determined automatically. However average difference in CT values between ablated and non ablated is 4,2 cycles, corresponding to a $(2^{4,2})$ theoretical 18,4 fold difference in abundance over the whole 24h. Comparing constantly expressed *cdc5* to *Pdu r-opsin* levels in non ablated worms, *cdc5* is coming up approximately 6,3 cycles later, corresponding to 78.8 fold difference. *Pdu r-opsin* levels in ablated specimens reach the threshold about 2,9 cycles earlier than *cdc5* levels, corresponding to roughly 8 fold higher amount of *Pdu r-opsin* in initial abundance. Thus it can be stated that *Pdu r-opsin* is highly abundant in the prostomium of *Platynereis dumerilii* in relation, but total or absolute quantification is not possible with the data. Obtaining absolute numbers anyway would not be of help because it is meaningless to relate them to. There is no way to discern whether reduction corresponds to total loss of PRCs in adult eyes but not in anterior ocelli or to remaining *Pdu r-opsin* in adult eyes. Another observation is that *Pdu r-opsin* expression pattern in the course of 24 hours does not exhibit a deducible rhythmicity. But this is mainly due to the fact that sampled animals are not exactly of equal size and stage.

But to proof some of these assumptions a *Pdu r-opsin* WMISH for twice ablated 3 month old worms would deliver the appropriate data. As denoted my experiment failed but anyways there were issues regarding this procedure that have to be circumvented in order to achieve a trustworthy read-out. A technical problem comprises the bleaching of the adult eyes. The presence of pigment in the eyes is masking staining from NBT/BCIP making it hard to see a signal. Either there is a method to bleach the eyes or one has to use the RE-strain instead and accept the higher mortality. Or use a light emitting chromophor like a fluorescent signal. Otherwise only a readout concerning the larval can be achieved because they have less incorporated pigment.

Regarding the anterior ocelli information on their exact location and successful destruction in 3 month old worms would be an essential information. As I only could proof their existence and location in 21 and 28 dpf old larvae, where they are easy to find. After subsequent growth of the worm tracking the anterior ocelli is hindered by appearance of pigment cells. Their earliest appearance during development, the tissue giving rise to them and their persistence in the maturing worm are issues to be assessed. After all the observed ocelli could be remainders of the larval eyes, which are supposed to disappear during development (between 3-6dpf) and rather move medially (96hpf). If these detected ocelli are indeed the larval eye remainders it would be contradicting the assumption of their disappearance during development. Another aspect that could be covered is their functionality. For larval eyes their role in the trochophora is elucidated, but may be open for revision in older stages be it that they persist in adult animals. In older stages the benthic lifestyle and movement via parapodia would render the control of ciliated cells for movement obsolete. Thus the reason for their persistence would be interesting to know. As pointed out in the introduction the larval eyes would correlate more closely to the retinal ganglion cells in vertebrates, which are responsible for circadian clock entrainment. Thus circadian entrainment might be the corresponding function if these anterior ocelli originate from the larval eyes. If those cells are different from the larval eyes again their function remains elusive as well. Thus the reason for their existence and their origin would be interesting to know. As the apical prostomial area is known to harbour chemo- and mechanosensory cells [14], the additional competence for light sensing would make the apical prostomium a multi-sensory unit. In the end it might be very simple and adult cerebral eyes as well as anterior ocelli have their role in vision. Due to the limited field of sight of adult cerebral eyes additional ocelli extend the visual field of *Platynereis*. The next question is if they really are employed for vision or rather luminance detectors for circadian entrainment. Without analysing WMISH data for various genes (TFs known for eye development, *Pdu ath*,

Pdu pax6, *Pdu six1/2* and of course *Pdu r-opsin*) from stages older than five days and assaying their innervation, no reliable conclusions can be made. Also inquiries into their morphology and supporting cells could provide useful information.

However without clear results if those detected *Pdu r-opsin* levels stem from the anterior ocelli or not, my data is not solid enough to fully back up the notion of non-involvement of adult eyes in rhythmicity of the circadian clock. Again a WMISH for *Pdu r-opsin* in 3 month old, treated specimens would cover two issues, the *Pdu r-opsin* loss at the adult eyes' location and the location of the anterior ocelli.

7.3 Regeneration

Concerning the possible regrowth of rhabdomeric PRC there are two issues. One issue is the characterization of the appearing pigmented structures and the other issue is the evaluation of the actual regeneration process of the adult cerebral eye. My experiments could show that pigmented structures were appearing at the place of ablated adult eyes, sometimes grew in displaced locations and multiple structures could be observed. A WMISH against *Pdu r-opsin* from ablated specimen showed no signal from PRCs after one week of regeneration, although pigmented structures could be spotted as an after-effect of ablation. Worms exhibiting such regenerative behaviour could mature normally and did not show unusual spawning. Annelids are reported to vary in their regenerative capabilities [133, 134]. The regeneration needs parts of the nervous system in Polychaetes [135]. At least the observed structures clearly develop due to regenerative action as it is a reaction of ablation. To test whether the regenerated structures are functional and indeed rhabdomeric PRCs follow-up experiments have to be carried out. Also the time lapse of the process is vital information. To have a look into the regeneration process the next step would be to carry out a WMISH against *Pdu r-opsin* with ablated worms that surpassed >7 days of regeneration. This regeneration study would provide information about the need for further ablations and determine if pigmented structures are principal rhabdomeric PRCs. Also the time window for *Pdu r-opsin* absence or re-appearance respectively can be narrowed down. To actually monitor off-target damage on subjacent neural tissue inflicted by the ablation procedure, immunostaining of acetylated tubulin of specimen directly after the procedure might be carried out. In my inquiry it does not look like neurons are impaired, but this can be due to regenerative action because my specimens had 7 days to regenerate their tissue.

For really monitoring neuronal regeneration this method is not appropriate because individuals have to be sacrificed. A look into the process intravitaly yields more reliable data than to look at different worms at different times after ablation.

7.4 Enzymes of the Melatonin Pathway

As shown in the results section my work on the melatonin pathway consisted of cloning orthologues for *aanat*, *hiomt* and look into their expression by means of WMISH. I also cloned an N-terminal gene fragment from presumable *Pdu hiomt like*. Due to its incompleteness it can not be verified to be an orthologue of *hiomt like*. A 3'RACE to the C-terminus would clarify the gene's identity. Except for *hiomt like* their classical biological role

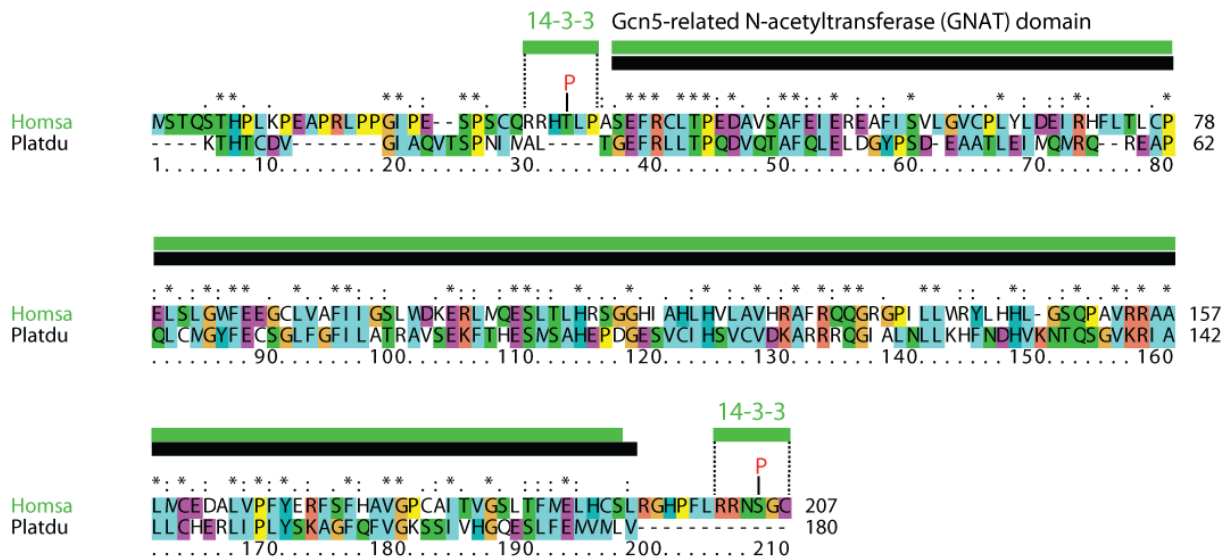


Figure 43 Alignment of primary sequences of vertebrate and *Pdu* AANAT

Green coloured bars indicated domains of human *aanat*. Black coloured bar indicates *Pdu* *aanat* domains. No flanking regulatory domains are found on *Pdu* AANAT. Whereas in vertebrate *aanat* N- and C-terminal PKA-dependent phosphorylation sites are found which control binding to 14-3-3 proteins and ultimately activation and stability. Red "P" marks phosphorylated aa, "14-3-3" marks binding motif for 14-3-3 protein(s)

is the production of melatonin, which coordinates systemic nocturnal rhythm by being endocrine secreted. In vertebrates the cells it is being synthesized and released from are pinealocytes. A feature of pinealocytes is therefore the co-expression of *aanat* and *hiomt* and the release of melatonin. I could confirm co-expression of these two genes in *Platynereis dumerilii* (Figure 34, Figure 38).

For the cloned *Pdu aanat* orthologue no regulatory regions flanking the catalytic core on N- or C- terminus could be found (Figure 43). In vertebrate orthologues 14-3-3 proteins are reported to bind to the AANAT regulatory sequences upon cAMP dependent phosphorylation [136] thereby regulating protein activity. This argues against posttranscriptional control of *Pdu* AANAT. It also argues for *Pdu aanat* to be a member of the non-vertebrate *aanats*, which of course is not surprising. It also lacks the proline containing tripeptide in the catalytic domain seen in vertebrates. The phylogenetic tree (Figure 33) supports this view and *Pdu* AANAT rather resembles AANATs from the lower chordate, *Branchiostoma*, and of course the annelid, *Capitella capitata*. Also the results of quantification experiments carried out by Juliane Zantke favour transcriptional control of final enzyme activity. It is not proven that *aanat/hiomt* expression directly precedes melatonin synthesis, but there is evidence that AANAT and HIOMT abundance is regulated on the transcriptional level (Zantke, J., unpublished). The actual involvement of *Pdu aanat* and *hiomt* in the melatonin pathway has not been demonstrated yet. Substrate affinity for serotonin is something that has to be proven as well by biochemical tests. Yet *Pdu* AANAT is a member of the Gcn5-related N-acetyltransferases, but if it is a true serotonin-N-acetyltransferase is not yet clear. If *Pdu* AANAT does not perform the common melatonin synthesizing function of pineal and retinal

AANATs, it might serve detoxification of arylamines by acetylating them [85]. Especially in photoreceptive cells formation of toxic bis-retinylalkylamines, metabolites of the photopigment, is prevented by action of AANAT and it is argued that HIOMT enhances this reaction [85]. Light dependent activity of 2 amphioxus AANATs could also be demonstrated, but no free running oscillations were observed [89]. Which means activity of both amphioxus AANATs is not driven by the circadian clock because the light/dark rhythm immediately decays in LL and DD. Yet it is regulated by light. In the case of amphioxus non-classical roles for AANATs are suggested that could also be valid for *Platynereis dumerilii*. As mentioned detoxification reactions of arylalkylamines is an important function as they can react non-enzymatically with aldehyde groups, and deplete vital biogenic aldehydes (e.g. retinaldehyde) and result in toxic products. These arylalkylamines might be xenobiotics and taken up as part of the diet. The circum oral expression of *aanat* would also be plausible, when considering this “digesting” function.

Pdu aanat is expressed in a very distinct circum-oral structure, seemingly coexpressed with its biochemically downstream enzyme *Pdu hiomt*. Also the presumably *Pdu hiomt like* is defined to that certain location, although its substrate affinity is not clear, neither is its involvement in melatonin production. To test if the prominent ring shaped structure might correspond to the circumoesophageal connectives or commissures, which join the dorsal brain with the ventral nerve cord an immunological co-staining against α -tubulin would be a follow-up experiment to ascertain the location. Melatonin secretion therefore does not derive from the infracerebral gland. Anyways coexpression of *Pdu aanat* and *Pdu hiomt* argues in favour for a classical role in melatonin synthesis. Characterization of the *aanat/hiomt/hiomtlike* expressing cell type is the next step. By means of comparative biology molecular markers expressed by these cells may be analysed to assess the question of their homologous cell type. If *aanat* and *hiomt* expression is considered to be a common feature of pinealocytes, so is their light receptive ability and intrinsic oscillator among sub-mammalian species, but the latter two features are lost in late evolution [137]. Pinealocytes of many chordate species are known to express *c-opsins* and therefore being light receptive. Earlier experiments identified *Pdu c-opsin* in the brain cells of 48 hpf old *Platynereis* larvae [17] and assessed their intrinsic circadian clock function and vicinity to *bmal* expressing cells. The obvious question is if those ciliary PRCs or other PRCs (anterior ocelli?) innervate the *aanat/hiomt/hiomt like* expressing cells or if they themselves are expressing any kind of clock gene. Clock gene co-expression would also argue for the classical role of *Pdu aanat* and *hiomt*. An experiment to show possible light influence on *aanat* (and *hiomt*) expression seems appropriate. With *aanat/hiomt/hiomt like* expressing cells in a primary cell culture additional experiments are thinkable. If *aanat/hiomt* expression in cell culture is light dependent and circadian free-running in constant conditions, this might argue for an intrinsical oscillator as well as for a directly photoreceptive element of the circadian oscillator like insect CRY. Also it would be interesting to know the melatonin releasing sites, if melatonin is found in the hemolymph, or released into the gut. Although mammalian pinealocytes show diurnal fluctuations in dense core vesicles, melatonin release is not depending on this exocytosis pathway. Melatonin release happens independent from golgi apparatus derived vesicles. Absence of typical neuroendocrine releasing machinery would not disqualify cells from a melatonin releasing function. Melatonin can cross lipid bilayers passively. When looked at *Pdu hiomt* orthologues the first aspect is the mentioned co-expression with *Pdu aanat*, but another interesting piece of data is the N-terminal diversity in some of the obtained transcripts (Figure 36). As shown in the results section translated clones exhibit a histidine rich region at their N-terminus. When inquired for signal peptide no pattern was recognized. Literature on histidine rich region at the N-terminus of proteins often indicated a binding function of divalent cations /metal [138, 139] or pH dependent folding [140], at low pH the stretch will be protonated and therefore positively charged. There are reports that

HIOMT is inhibited uncompetitive (Ca^{2+} , Mg^{2+}) and competitive (Cu^{2+}) by divalent cations [141]. Thus His rich region might function as non-competitive inhibitory domain. Inhibition of *hiomt* to fine tune rhythmic activity by adjusting intracellular ion concentration seems reasonable [142]. Though another question is which of the *hiomt* isoforms actually is expressed when, under which circumstances? I cloned it from larval RNA therefore both variants are expressed already early.

The role for *hiomt like* is elusive. It is thought to be a fusion product because one domain is homologue to *hiomt* and the other homologue to *maf/orfE* genes. I could not completely verify the identity of *Pdu hiomt like* due to the fact that I only cloned an N-terminal fragment resembling the *mafE/orfE* domain and not the complete gene. The substrate affinity is unknown. On one hand it is co-expressed with *hiomt*. It might exhibit compensatory action, when also binding to and catalyze transfer of a methylgroup to N-acetyl-serotonin. On the other hand it might be catalyzing non-melatonin related reactions. Summing up my results I am able to reason that *aanat*, *hiomt* and *hiomt like* are co-expressed in the same cells. To really verify my assumption a double ISH may be the next step. As outlined determination of the cell type and proof of melatonergic character are important data to obtain in successive experiments.

To test the influence on maturation, regeneration and photoperiodicity various experiments can be considered. They can be as easy as administration of luzindole (a melatonin receptor antagonist), or labeling and subsequent destruction of the according cells. Subsequent evaluation of the absence or presence of melatonin and its impact on the mentioned biological processes. Overexpression and measuring effects. Knock out or knock down of one of those genes and doing the same evaluations.

7.4.1 The horizontal gene transfer hypothesis

As outlined in the introduction *aanat* and *hiomt* are regarded as exemplary candidates for HGT (3.3.3.5). The cloned orthologues from *Platynereis dumerilii* already contradict this hypothesis by their mere presence. The existence of *aanat* in *Platynereis* means that the orthologue from *Amphioxus* might be more likely obtained vertically because *aanat* is already present in an eukaryotic metazoan from the lophotrochozoan branch. Therefore a common ancestor at the base of bilateria seems plausible. The absence of *aanat* from other species might be explained by gene loss or more likely the low resolution of the sub-vertebrate phylogenetic relationships of AANAT. Data on AANAT, HIOMT in invertebrates is scarce. This might be responsible for the notion of HGT. The lack of introns in *Amphioxus* therefore also might be more likely explained by loss than HGT, although genomic data concerning existence of introns in the *Pdu aanat* gene is lacking.

Being non-vertebrate the *Pdu aanat* exhibits all features of the non-vertebrate orthologues. As mentioned before *Pdu AANAT* resembles its non-vertebrate orthologues from amphioxus, green algae, fungi and bacteria in common protein domain motifs and distinguishes it from vertebrate AANATs. This relation is supported by the phylogenetic tree for *Pdu AANAT* (Figure 33). Its exclusive co-expression to the same cellular structure though links it to the classical vertebrate function of melatonin synthesis. It is argued that AANAT specificity for melatonin occurred during early vertebrate evolution, after the gene was obtained by *Amphioxus*. If *Pdu AANAT* is involved in melatonin synthesis it also argues against HGT, because only vertebrate AANATs are thought to have this function.

More data on *Pdu aanat/hiomt* will provide further insights into evolution of these gene families and melatonin synthesis. The existence of *Pdu hiomt* and *Pdu aanat* already fill a gap in the former empty and maybe misleading space between bacteria, fungi, algae and the chordates.

7.5 Pigment dispersing factor

A *pdf* orthologue was found in and cloned from *Platynereis dumerilii*. Therefore it is a highly conserved gene amongst protostomia, found in nematodes, crustacean and insects, in mollusca and for the first time in annelida. Thus *pdf* is present in both major protostomian groups, lophotrochozoa and ecdysozoan (see the phylogenetic tree for PDF, Figure 42). In all these species it might likely act as a circadian clock output. Considering the fact that it is highly conserved it might originate from a common ancestor at the very base of the protostomia. *Pdf* has not been found in deuterostomia. A neuropeptide with the analogous function in deuterostomia would be vasoactive intestinal peptide (VIP) [143].

My expression analysis of PDF in 14dpf old worms yielded several PDF expressing neurons in the brain of platynereis, which seem to be organized in bilateral symmetric clusters. In larval 72hpf stage diurnal variations in PDF expression between 9am and 9pm (data not shown) could be assessed. Some clusters seemed to constantly express PDF, whereas others seem to exhibit circadian fluctuation in PDF levels. The general trend was that at 9am expression in all PDF clusters was monitored, whereas at 9pm PDF expression was restricted to consistently expressing clusters. This data was in accordance with quantification experiments from Juliane Zantke (unpublished). This particular PDF oscillation could not be reproduced in 14dpf so far. Due to the fact that this pattern could not be reproduced in older stages or free-running specimen the observed expression might be an artefact that is not due to regulation by the endogenous circadian clock but due to direct extrinsic light regime. After all PDF was discovered in crustaceans to regulate pigment allocation according to external light conditions [92]. As PDF was shown to be expressed in pacemaker neurons in *Drosophila*, coexpression with clock genes would be interesting. Another interesting point would be the temporal release of PDF in *Platynereis*. In Insects PDF abundance is not controlled on transcriptional level but posttranslational by the timing of its release. Therefore an immunological staining might better reflect the temporal control of PDF than an ISH. The phylogenetic tree puts *Platynereis dumerilii* PDFs apart from crustaceans or insects, to two other lophotrochozoan orthologues. It is not an insect type PDF, where PDF is employed as clock output and its not resembling crustacean PDF either, where it is used as hormone to control pigment dispersion as well as for a circadian output. Thus its function in *Platynereis* remains speculative, maybe a clock output and a pigment controlling hormone?

7.6 Conclusion

Adult cerebral eyes might not be required for circadian entrainment due to outcome of my ablation experiments. The clock could be entrained to an inverted LD rhythm in blinded animals. I was also able to identify additional rhabdomeric ocelli at the anterior prostomium not described in literature so far. These in turn might be involved in circadian entrainment.

Successful cloning and finding of concerted allocation of the enzymes of the melatonin pathway to a circum-oral structure are first pieces of data to elucidate the roles of *aanat*/*hiomt* in *Platynereis dumerilii*. The existence of *Pdu aanat* and *Pdu hiomt* is contradictory to the HGT hypothesis. Their exclusive co-expression favours employment in the melatonin synthesis, although a function in detoxification is thinkable. Data from invertebrate orthologues is scarce and biological functions among species are far away from being clear.

PDF turns out to be highly conserved in prostomia due to its existence in lophotrochozoa and ecdysozoan. I also was able to have a look on the cerebral expression of *Pdu pdf*, a neuropeptide with assumed dual functions. In some cellular clusters in the brain *Pdu pdf* might be directly a circadian clock output.

Platynereis dumerilii proves to be a useful entry point to important biological questions, not only from a developmental and evolutionary perspective but also from a chronobiological point of view. Its circadian clock can be accessed now by standard methods in molecular biology. And first cues are shedding light on its lunar clock.

8 Appendix

8.1 Abbreviations

5-HT	5-Hydroxytryptamin
aa	aminoacids
AANAT	Arylalkylamine N-acetyltransferase
ath	atonal
AO	Anterior ocelli
bHLH	basic Helix Loop Helix
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like
CaMK	Ca ²⁺ /calmodulin-dependent protein kinases
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CK1δ	Casein kinase 1 delta
CK1ε	Casein kinase 1 epsilon
CLK	Circadian Locomotor Output Cycles Kaput
CRE	cAMP response elements
CREB	cAMP response element-binding
CRY	Cryptochrome
CYC	Cycle
DAG	diacylglycerol
DBT	Doubletime
DD	Dark Dark
dpf	days post fertilisation
DREAM	Downstream regulatory element antagonist modulator
ERK	Extracellular-signal-regulated kinase
EST	expressed sequence tags
EtOH	ethanol
GTP	guanosine triphosphate
HAT	Histone acetyl transferase
HGT	horizontal gene transfer
HIOMT	Hydroxyindole-O-methyltransferase
HIS	histidine
hpf	hours post fertilisation
ICER	Inducible cAMP Early Repressor
IP3	Inositol trisphosphate
ipRGC	intrinsically photosensitive retinal ganglion cells
ISH	in situ hybridisation
LD	Light Dark
LL	Light Light
LNv	ventral lateral neurons
LOD	limit of detection
LSCM	laser scanning confocal microscopy
MAPK	Mitogen-activated protein kinase
MeOH	methanol
mRGC	melanopsin-expressing retinal ganglion cells
NAS	N-Acetylserotonin
NE	Norepinephrine
NJ	Neighbor-joining
NMDA	N-methyl-D-aspartic acid
norpA	no receptor potential A

PACAP	Pituitary adenylate cyclase-activating peptide
pax	paired-box-gene
PCR	polymerase chain reaction
PDE	Phosphodiesterase
PDF	Pigment dispersing factor
PDH	Pigment dispersing hormone
Pdu	Platynereis dumerilii
PER	Period
PIP2	Phosphatidylinositol 4,5-bisphosphat
PK	Proteinase K
PKC	Protein kinase C
PLC	Phospholipase C
PNS	photoneuroendocrine system
PP2a	Protein phosphatase 2
PRC	photoreceptive cell / photoreceptor cell
PTTH	Prothoracicotropic hormone
qPCR	quantitative real time polymerase chain reaction
RACE	rapid amplification of cDNA ends
RHT	retinohypothalamic tract
RORa	Retinoic acid-related orphan receptor
RORE	ROR response elements
ROS	reactive oxygen species
SCN	suprachiasmatic nucleus
sLNv	small ventral lateral neurons
SPC	shading pigment cell
TIM	Timeless
TRP	transient receptor potential channels
TRPL	transient receptor potential like channels
VIP	Vasoactive intestinal peptide
WMISH	whole mount in situ hybridisation

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11 Curriculum vitae

Stefan Florian Keplinger

Date/Place of birth: 17.01. 1984, Linz, Austria

Marital status: not married

E-mail: s.kep@gmx.net

Education

1994-2002	BRG Fadingerstrasse, Linz
2002-2003	Military Service, Austrian Army
2003-2010	Molecular Biology, University of Vienna

Research Experience

6/2009-6/2010	Diploma Thesis Max F. Perutz Laboratories, Vienna Dr. Kristin Tessmar-Raible “Influence of the adult eyes on lunar and circadian rhythms in <i>Platynereis dumerilii</i> ”
11/2008-12/2008	Training Center for Brain Research, Vienna Prof. Michael Kiebler “Overexpression of candidate miRNAs in primary embryonic hippocampal neurons”
12/2008-1/2009	Training Center for Brain Research, Vienna Prof. Michael Kiebler “Construction of an artificial GFP sensor”
2/2008-4/2008	Training Max F. Perutz Laboratories Prof. Rudolf Schweyen “Screen for mitophagy-deficient mutants in yeast”